

Postreplication Labeling of E-Leaflet Molecules: Membrane Immunoglobulins Localized in Sectioned, Labeled Replicas Examined by TEM and HVEM

JOSEPH E. DINCHUK, TIMOTHY J.A. JOHNSON, AND JOHN E. RASH
*Department of Anatomy, Colorado State University,
Fort Collins, Colorado 80523*

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ABSTRACT Conventional freeze-fracture techniques were combined with immunogold labeling and with plastic embedding and sectioning to analyze the distribution of membrane immunoglobulins (mIgs) and their associated intramembrane particles (IMPs) in E-face replicas of murine B-lymphocyte plasma membranes. Immunogold labels were applied to cells after the process of freeze-fracture and replication. Conventional stereoscopic transmission electron microscopic examination of sectioned, labeled replicas (SLRs) revealed that the gold-labeled mIgs were bound to and localized on the outer leaflets of split and replicated membranes. The gold labels were attached to the external determinants of the mIg molecules, which were retained beneath and contiguous with the replicated E-faces. The mIgs were also localized on the external surface of unreplicated microvilli. In addition, thick sections examined by high-voltage transmission electron microscopy (HVEM) revealed large expanses of replica with well-resolved IMPs. mIgs colocalized with small-diameter (<60 Å) IMPs in E-face replicas of B-lymphocytes whose mIgs were patched by anti-immunoglobulin. Thus, postreplication E-surface labeling of split and replicated membranes is a high-resolution technique that is suitable for the study of membrane protein distribution in E-face replicas and contiguous nonreplicated tissue.

INTRODUCTION

High-resolution labeling of individual cell membrane molecules would clarify how alterations in membrane protein distribution and number may be correlated with changes in biological activity. Freeze-fracture and freeze-etch electron microscopy facilitate study of the cellular distribution of membrane proteins because they allow high-resolution, three-dimensional examination of large areas of both replicated cell surfaces (freeze-etch) and split membrane faces (freeze-fracture). Previous attempts to localize membrane receptors in replicas have relied on either (1) labeling cell suspensions before replication of the intact or split membrane surface (prereplication labeling, label-fracture, or label-etch, e.g., Abbas et al., 1975; Aguas and Pinto da Silva, 1983, 1984, 1985; Harding et al., 1983; Karnovsky and Una-

nue, 1973; Pinto da Silva and Kan, 1984; Smith and Revel, 1972; Unanue et al., 1973) or (2) labeling split membranes after shadowing with platinum but not carbon coating (which might also be called shadow-label or postshadow labeling; Rash, 1979; Rash et al., 1978, 1980a,b). In this report, we will consider a sample that has been shadowed with platinum and subsequently coated with carbon to be replicated. This study concentrates on the postfracture, postreplication labeling (replica-label) of membrane immunoglobulins (mIg) with colloidal gold. We are able to label mIg molecules on the external leaflet surface (E-surface) which lies immediately beneath the replicated E-face. We

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Address reprint requests to Joseph E. Dinchuk, Department of Pathology, Colorado State University, Fort Collins, CO 80523.

thus demonstrate that the antigenicity of mIg is maintained through the processes of fixation, freeze-fracture, and labeling.

Splenic lymphocytes were selected as a model system because they are easily obtained and are isolated with a mixed population of easily recognized cell types that serve as internal controls for the specificity of labeling. B-lymphocytes have large numbers of immunoglobulin molecules inserted into their membranes at certain stages of their development (Rabellino et al., 1971). The two most common classes are mIgD and mIgM (Owen et al., 1974; Vitella et al., 1975). Unlike serum immunoglobulins, mIgs have an additional hydrophobic α -helix which is likely the transmembrane portion of these molecules (Hood et al., 1984). Initial attempts to define the distribution of mIg in replicas of murine B-lymphocytes utilized deep etching of prelabeled cell suspensions. Anti-Ig antibodies were coupled to ferritin and/or hemocyanin. After deep etching and shadowing, these attached labels appeared as noticeable bumps in the replicas (Abbas et al., 1975; Karnovsky and Unanue, 1973; Karnovsky et al., 1972; Unanue et al., 1973). Those labels were deemed unsuitable for high-resolution labeling of replicas because either the label (hemocyanin) is too large to permit molecular localization or the label (ferritin) is difficult to recognize on the platinum replica (Carter and Staehelin, 1979; Schmidt and Buchheim, 1982).

In this study, colloidal gold was chosen as the electron-dense label. In contrast to other electron-dense macromolecules such as ferritin (Rash, 1979; Rash et al., 1978, 1980a,b), colloidal gold is easily visualized on replicas (Harding et al., 1983; Mannweiler et al., 1982) and is also easily prepared in many sizes. To label mIgs in fractured and replicated B-lymphocytes, we utilized a new version of a post-fracture, postreplication labeling scheme. This technique preserves recognizable IMPs and retains the biological molecules of interest. The fracture-label method was unsuitable for this study because that technique does not preserve IMPs as in conventional replicas (Aguas and Pinto da Silva, 1983, 1984, 1985; Pinto da Silva et al., 1981a-c). The label-fracture technique (Pinto da Silva and Kan, 1984) apparently results in the collapse of cell projections onto the nearby replica. This obscures label because of the electron density of undigested cytoplasm (Pinto da Silva and Kan, 1984). Because B-cells were often quite villous and because the villi on these cells were heavily labeled, we decided

to use a different approach to correlate the distribution of labeled mIg with IMPs.

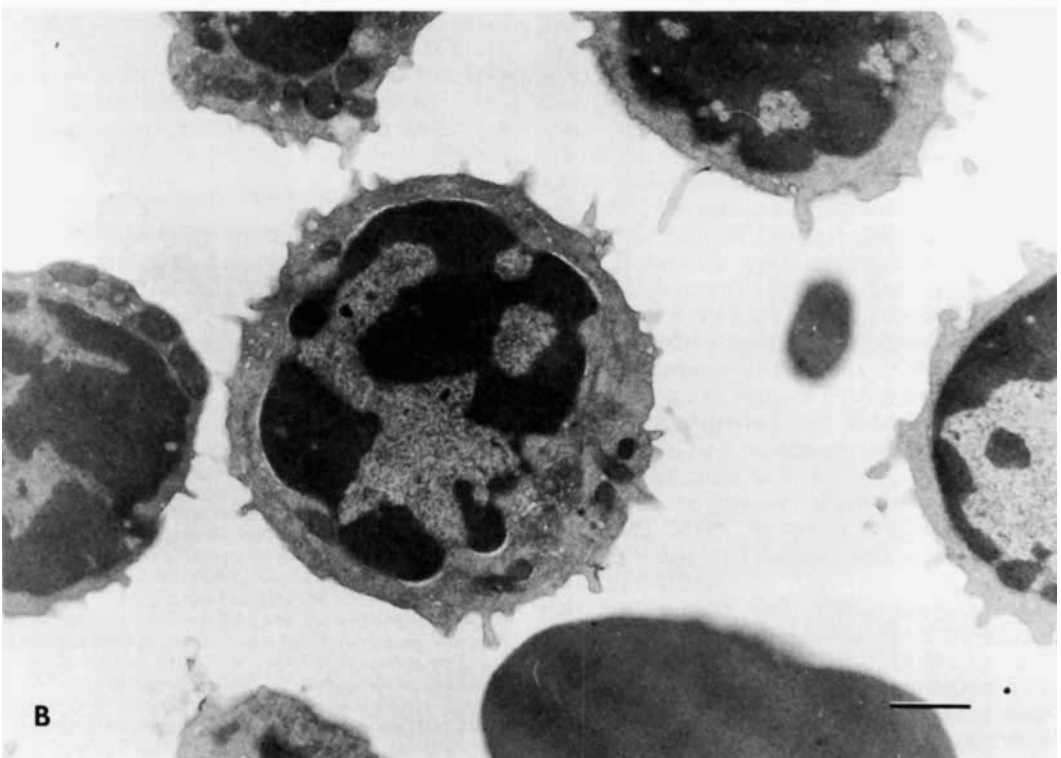
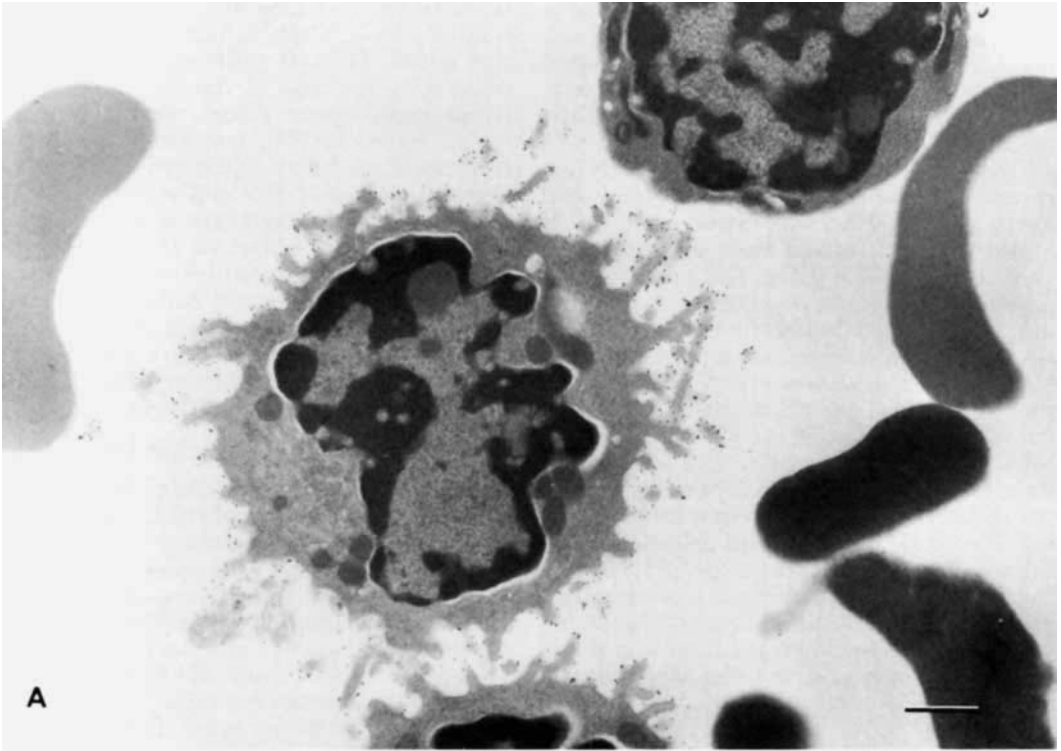
We observed in preliminary label-fracture deep-etched preparations that colloidal gold labels directed against mIg were located on E-surfaces but not on P-faces (unpublished observations, also see Karnovsky et al., 1972). We then found in shadow-label experiments that E-leaflets were labeled but P-faces were not labeled (unpublished observations). These experiments suggested that either mIg partitions with the E-leaflet and/or that since the P-faces were platinum shadowed, available binding sites were masked by the platinum shadow (Dinchuk et al., 1986). We also observed that without carbon stabilization, the E-leaflets were highly twisted and poorly suited for morphological evaluation. Therefore, to localize the mIg in freeze-fracture replicas of large aggregates of cells, a labeling technique was devised which retains normal (replicated) IMP morphology yet allows simultaneous labeling of the external determinants of the same molecule. This version of the SLR technique was designed to label externally exposed macromolecules that have their transmembrane components visualized as IMPs. In addition, the replica-label scheme used in this work does not alter the partitioning of mIg due to previous attachment of antibodies and colloidal gold to unfractured cells. This labeling technique has allowed us to determine with improved resolution (100–200 Å) the distribution of mIg in murine splenic lymphocytes and to identify their associated IMPs.

MATERIALS AND METHODS

Preparation of colloidal gold conjugates

Colloidal gold (≈ 180 Å) was prepared according to the methods of Frens (1973) and

Fig. 1. Control preparations. A: Thin section from a region below the plane of the replica in a postreplication labeled spleen cell pellet. (Although all of the cells in a fixed cell pellet are in contact with adjacent cells at some point, the section plane gives the false impression of a suspension.) Two labeled B-lymphocytes are visible among unlabeled lymphocytes and unlabeled red blood cells. Although the most heavily labeled cells are usually villous, no distinctive morphology separates labeled from unlabeled lymphocytes. In preparations like these, approximately 10–15% of lymphocytes show a similar high labeling density (> 500 gold labels in this ~ 0.1 - μm section of the B-lymphocyte (center) suggests $> 30,000$ gold labels/cell). B: This control preparation was processed as in A but was incubated with a nonimmune goat primary antibody instead of goat antimouse immunoglobulin antibody. No labeling is evident on any cell type. Membrane blisters are commonly seen in these preparations. Bar, 1.0 μm .



Horisberger and Rosset (1977) and titrated to pH 9.0 with 0.1 M K_2CO_3 . Smaller gold colloid (100 Å) was prepared by the method of Slot and Geuze (1985). Antibody-gold complexes were prepared by a modification of the methods of Geoghegan and Ackerman (1977). Affinity-purified, lyophilized, and salt-free rabbit antigoat antibody (Kirkegaard and Perry Laboratories) was dissolved in 0.1 M acetic acid and mixed with an equivalent volume of 0.1 M K_2CO_3 . The antibody was diluted to a concentration of 0.1 mg/ml in 5 mM glycyglycine buffer (final pH 8.2). To 25 ml of a stirred gold colloid suspension, 2 ml of antibody solution was added. After 30 minutes at 20–25°C, the pH was decreased to 7.0 by the slow addition of 0.1 M acetic acid and incubated overnight at 4°C. The colloid/antibody mixture was washed repeatedly by centrifugation (De Mey, 1983) in a solution of 5 mM Tris (pH 7.5). The final gold pellet was diluted in a 5 mM Tris buffer (pH 7.5) containing 0.04% sodium azide as a preservative and stored without the addition of stabilizing agents. The IgG-gold preparation was stable for approximately 3 months when stored at 4°C.

Preparation of spleen cell pellets

Four spleens were removed from adult Balb/C mice and rapidly minced in phosphate buffer containing glucose and salts (PGS), pH 7.0 (140 mM NaCl, 5 mM KCl, 10 mM Na_2HPO_4 , 5 mM NaH_2PO_4 , 0.2% glucose). This solution was maintained at 37–39°C to minimize phase separation of membrane lipids and displacement of IMPs (Unanue et al., 1973; but see Wunderlich et al., 1974). All subsequent steps through fixation were carried out at 39°C. Tissue cubes were vortexed at maximum speed on a Vortex Genie for 60 seconds and large pieces of tissue were allowed to settle. The supernatant was removed and centrifuged at 500g for 5 minutes. The cell pellet was resuspended in fresh PGS buffer. The suspension was incubated in fresh PGS buffer for 2–3 minutes between centrifugation steps to remove Fc-bound mouse IgG (Kumagai et al., 1975). The washing process was repeated five times. To the final suspension (10 ml), 400 μ l of 50% glutaraldehyde was added. This preparation was incubated at 39°C for 5 minutes, whereupon 1 ml of 1.0 M glycine was added to quench excess glutaraldehyde (Johnson, 1986) and to bind cells into a loose pellet. Immediately after glycine addition, the cell suspension was centrifuged at 500g for 5 minutes. The

supernatant was removed, and a fresh solution of 2.0% glutaraldehyde in PGS buffer was added. The cell pellet was gently separated from the wall of the centrifuge tube, sliced into several pieces with a bamboo splint, cooled to 4°C, and incubated in the same solution for an additional 50 minutes. Fresh solutions of 100 mM glycine in PGS buffer were introduced over a 4-hour period until no evidence of further glutaraldehyde/amine colored products (Johnson, 1986) was present. Fixed spleen cell pellets were incubated overnight in 25% glycerol in PGS buffer, frozen on 3-mm Balzers stubs by immersion in freezing dichlorodifluoromethane, and stored in liquid nitrogen.

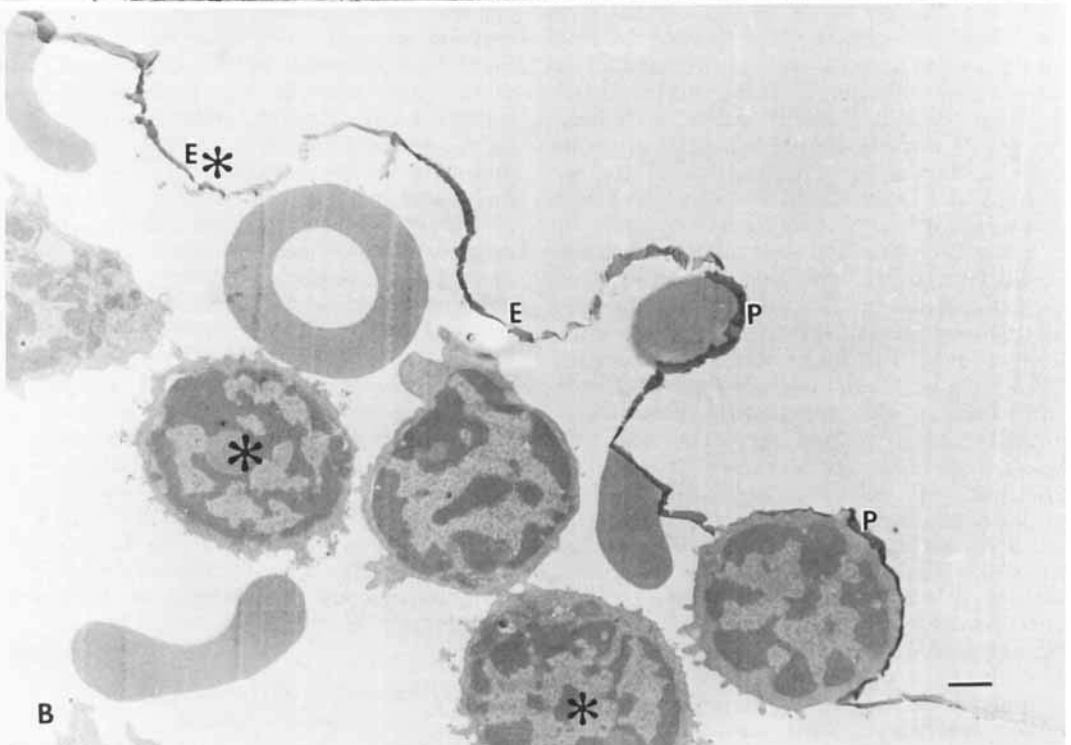
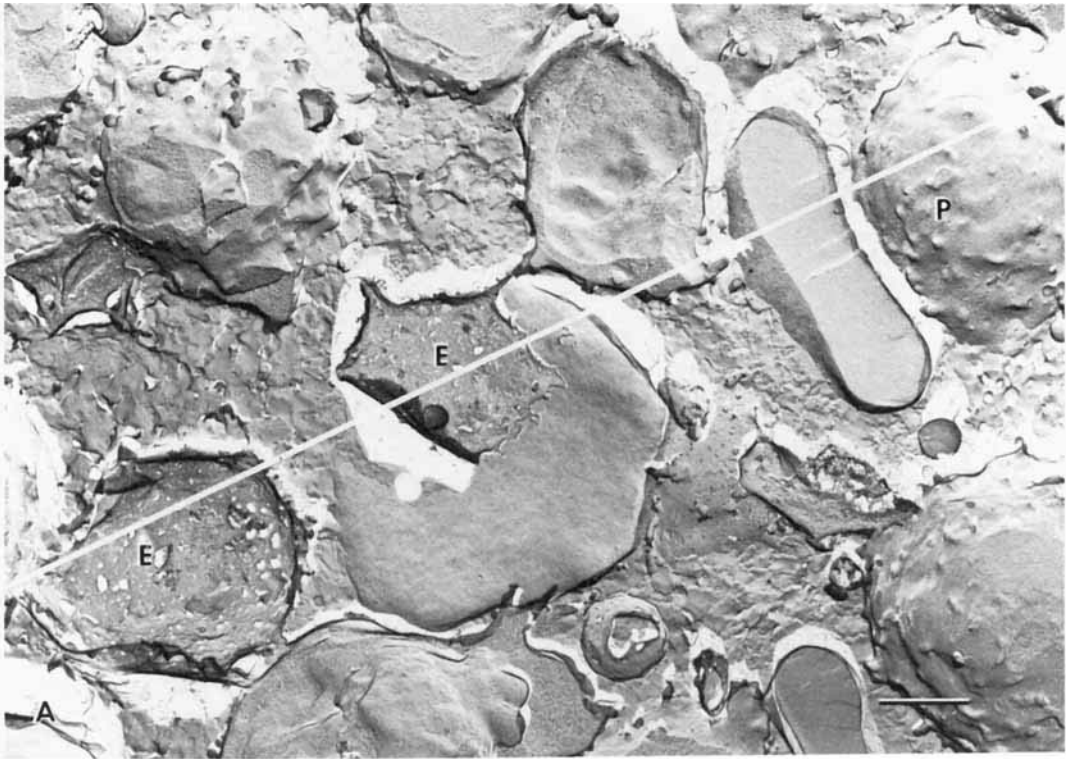
Preparation of patched spleen cell pellets

To compare the distribution of colloidal gold labels and of IMPs in replicas of lymphocytes that had their mlg patched prior to fixation and labeling, spleen cells were isolated and washed as above. After five washes in PGS, the cells were resuspended in PGS to which affinity-purified goat antimouse IgG+IgM (heavy and light chains, H+L) (Kirkegaard and Perry Laboratories, KPL) was added to a concentration of 20 μ g/ml. Patching without capping was carried out at 39°C in the presence of 30 mM sodium azide (Taylor et al., 1971). After a 15-minute incubation period, the cells were washed five times in fresh PGS (39°C), fixed with glutaraldehyde into the form of a pellet, frozen and stored as described above.

Preparation of red blood cell pellets

Red blood cells were selected as controls for labeling of replicas and labeling of non-

Fig. 2. Comparison of a deep-etch replica and sectioned, labeled replica (SLR) of a spleen cell pellet. A: Low-magnification view of a deep-etched spleen cell pellet. This image reveals the tight packing and cell-cell contacts in the pellet. The unsupported E-face images (E) are susceptible to etching damage (as indicated by the curling and tearing of these portions of the replica) while P-face, E-surface, and cytoplasmic fracture materials are resistant to etching damage. B: SLR (postreplication labeled). The narrow strip of replica in B is comparable to the section of replica indicated by the line in A and contains E-face (E), P-face (P), and cytoplasmic fracture material. Comparison with Figure 1 shows that microvilli are sheared away by the fracture process but retained as unreplicated components with E-face replicas. The presence of intact microvilli in the subjacent thin section complements the information provided in the replicated portion of cell membrane. Two labeled intact cells and one labeled E-face image (asterisks) contrast with the other unlabeled portions of this preparation. Bar, 1.0 μ m.



quenched, glutaraldehyde-fixed cells because these cells possess no membrane immunoglobulin and should therefore exhibit only nonspecific labeling when subjected to the same labeling steps as spleen cell pellets. Blood (0.5 ml) was removed from a Balb/C mouse by cardiac puncture with a heparinized syringe. The red blood cells were washed by successive pelleting and resuspended in warm PGS buffer (containing 2 mM EDTA for red cell washes). The washing process was repeated five times. The red blood cells were fixed into a pellet and quenched with glycine as for the spleen cells. Fixed red blood cell pellets were incubated in distilled H₂O for 2 days (five changes) and placed in 40% ethanol for 20 minutes prior to freezing on 3-mm Balzers stubs by immersion in freezing dichlorodifluoromethane. Frozen pellets were stored in liquid nitrogen.

Postreplication labeling and embedding of spleen cell pellets

Cell pellets of both the untreated and patched lymphocyte preparations were fractured at -150°C in a Balzers BAF 301 freeze-fracture device and immediately coated with Pt/C at a 45° angle followed by rotary evaporation of carbon at 15° to the normal. The replicated cell pellets were thawed in PGS buffer (three changes) and equilibrated for 30 minutes in labeling/blocking buffer (LBB, consisting of 0.1 M MOPS buffer, 0.1% gelatin, 0.05% Tween 20, pH 7.4; LBB prevents nonspecific labeling of replicated surfaces; see Results and Discussion). Samples were then placed in LBB containing approximately 20 $\mu\text{g}/\text{ml}$ affinity-purified goat antimouse IgG+IgM (H+L)(KPL). All labeling steps were carried out at 4°C . The pellet was incubated in this solution for approximately 10 hours, washed in LBB 12 times, and then incubated for 24 hours in LBB containing rabbit anti-goat immunogold. In a control preparation, an intermediate step of incubation with rabbit anti-goat IgG (for 10 hours at 20 $\mu\text{g}/\text{ml}$) preceded gold labeling. In another control preparation, the initial labeling was carried out with nonimmune goat IgG (20 $\mu\text{g}/\text{ml}$ in LBB) for 12 hours. The sample was then washed in LBB before incubation in rabbit anti-goat immunogold (affinity-purified rabbit anti-goat IgG (H+L)(KPL)) for 24 hours. The pellets were labeled at 4°C in the immunogold suspension for approximately 24 hours, washed in LBB, and then washed three times in PGS buffer. The pellets were fixed in 2.0% glutaraldehyde in PGS buffer for 1 hour, postfixed in 1.0% osmium tetroxide, and stained en bloc in 0.4% aqueous ura-

nyl acetate. After conventional washing and dehydration, cell pellets were infiltrated with Spurr's resin (Polysciences) and oriented replica-side down in flat embedding molds.

Postreplication labeling and embedding of red blood cell pellets

Red blood cell pellets were fractured at -150°C in a Balzers BAF 301 freeze-fracture device and immediately coated with Pt/C at a 45° angle followed by rotary evaporation of carbon at 15° to the normal. The replicated pellets were thawed in PGS buffer and labeled as for spleen cell pellets except that PGS buffer was substituted for LBB in all labeling steps. This procedure allowed us to demonstrate the role of LBB. Samples were washed as before except that PGS was substituted for LBB. The red blood cell pellets were then postfixed and embedded as for the spleen cell pellets.

Sectioning the plastic-embedded, labeled replicas for TEM and HVEM

Pellets were cut away from polymerized blocks with a jeweler's saw and reoriented, with fresh cyanoacrylate resin, so that the replica was at the sectioning surface. Thick sections were obtained along the plane of the block face, transferred to a microscope slide with a platinum wire loop, and stained with toluidine blue. Sections were examined in a light microscope until cells and replica were observed in the section. Typically, ten or fewer sections 1 μm thick were necessary to reveal both cells and replica in sections. Subsequent sections were taken for electron microscopic examination. Thin sections (pale gold) were stained for 5 minutes in Sato's Pb citrate (1967) at $20-25^{\circ}\text{C}$ and examined at 80–120 kV in a Philips 400T electron microscope equipped with a $\pm 60^{\circ}$ tilt stage. Thick (1–5 μm) sections were picked up on 3-mm copper grids and stained for 2 hours at 20°C in Sato's Pb citrate. Thick sections were then carbon coated on both sides before examination at 750 kV on a Jeol 1000 electron microscope equipped with a $\pm 45^{\circ}$ goniometer stage (Laboratory for High-Voltage Electron Microscopy, University of Colorado, Boulder, CO 80302).

Preparation of etched spleen cell pellet replicas

For preparing freeze-etch replicas, spleen cells were isolated, fixed, and quenched with glycine. The pellets were then washed in several changes of distilled water, incubated in

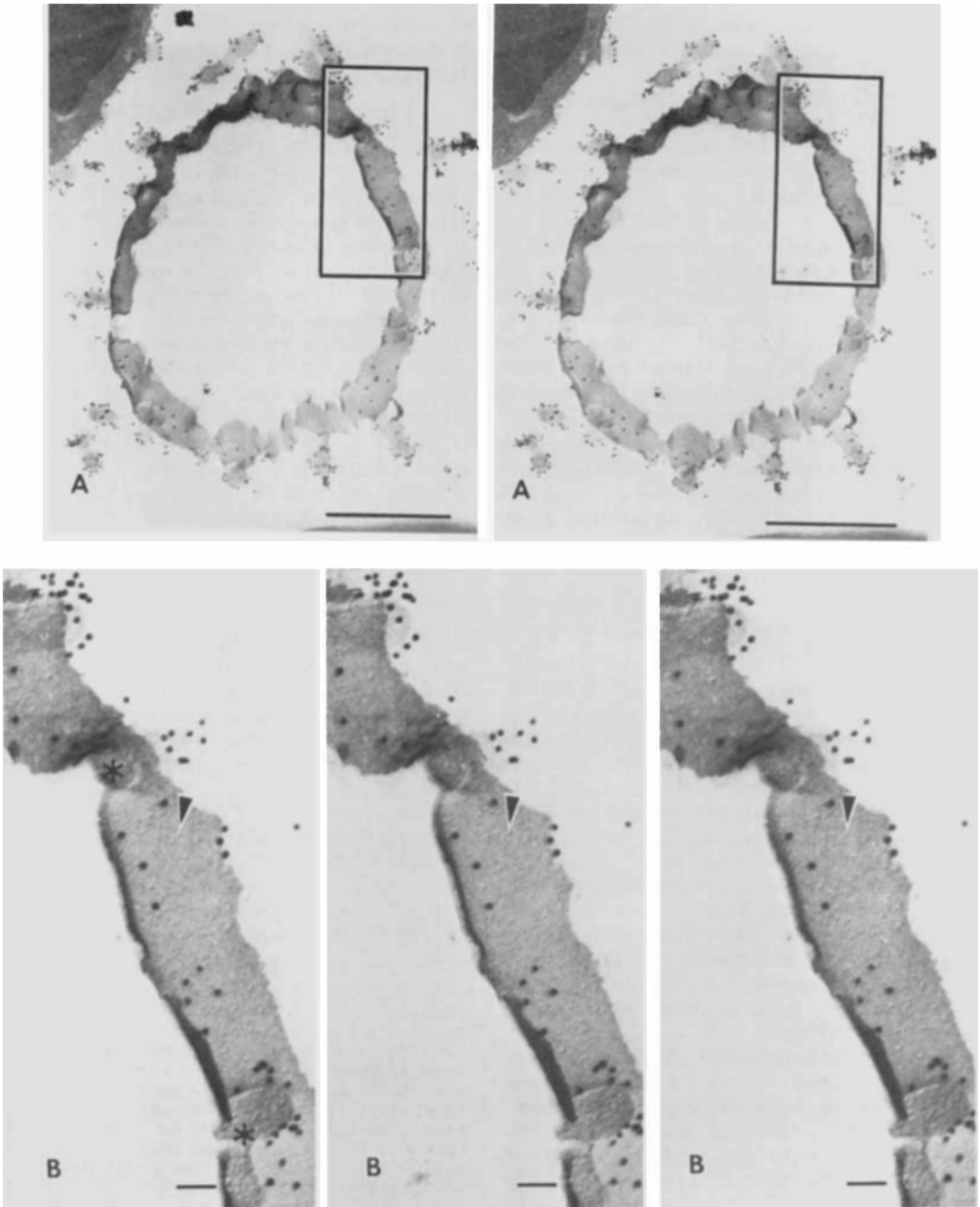


Fig. 3. Semithick section (ca. $0.25 \mu\text{m}$) examined at 120 kV contrasts with the thin (ca. $0.1 \mu\text{m}$) section (80 kV) image in Figure 2B. A: The postreplication labeled E-face of a B-lymphocyte can be contrasted with a labeled but unreplicated intact B-lymphocyte and a nearby unlabeled red blood cell. The distinct sidedness of label and contour of the E-face replica can be discerned and IMPs are barely detectable at this magnification. As in Figure 1A, the gold ligand labels the microvilli at a higher density than on contiguous, non-microvillous (replicated) membrane. At higher magnification (B), the correct stereo perspective (pair of photos on the left) of

the area outlined in A reveals that virtually all of the colloidal gold label is located below the plane of the replica (i.e., on the intact external membrane leaflet or E-leaflet). Viewing the image in reverse stereo perspective (right-side pair) helps clarify the sidedness of label. Gold labels (180 \AA) are rarely seen adjacent to large-diameter ($\sim 100 \text{ \AA}$) IMPs (arrowhead) but are commonly seen underlying small-diameter ($30\text{--}60 \text{ \AA}$) particles. High densities of gold label are evident in regions where the cytoplasm of several microvilli is cross-fractured (asterisks). Bar, $1.0 \mu\text{m}$ (A), $0.1 \mu\text{m}$ (B).

40% ethanol (an etchable cryoprotectant, Schiller and Taugner, 1980) for approximately 30 minutes, and frozen on conventional Balzers stubs in a pool of freezing dichlorodifluoromethane. Samples were stored in liquid nitrogen. After they were fractured at -108°C , the samples were etched for 8 minutes at a vacuum of 2×10^{-7} mBar. The specimens were then replicated with Pt/C at an angle of 45° followed by rotary shadowing with carbon at an angle of 15° . Thawed samples were placed into a solution of 2.5% sodium hypochlorite in 0.1% sodium dodecyl sulfate (Tom Stewart, personal communication) for 5–10 minutes. This solution was prepared immediately before use because the SDS is rapidly decomposed by sodium hypochlorite. These cleaning steps were repeated two times before placing the replicas in a solution of 2.5% chlorox for an additional 5 minutes. The cleaned replicas were slowly equilibrated with distilled H_2O , picked up on copper grids, and photographed on a Philips 400T electron microscope.

RESULTS

Labeling specificity

A pellet of spleen cells contains a mixed population of leukocytes and erythrocytes (Fig. 1). In all sections examined, only cells that had the typical morphology of lymphocytes were well labeled. Other identified cell types (e.g., RBCs) were unlabeled (Figs. 1–3). Eosinophils and granulocytes (not shown) were also unlabeled. Since B-cells at certain stages of development have a high density of immunoglobulin determinants in their plasma membranes (Rabellino et al., 1971), and since other identifiable cell types were essentially unlabeled, we concluded that the heavily labeled cells in these preparations are B-lymphocytes.

In all of these experiments, Fc-bound mouse IgG was removed during the washing steps (Kumagai et al., 1975). However, the possibility existed that the goat and/or rabbit immunoglobulins were nonspecifically adsorbed to mouse Fc receptor during the antibody incubation steps (Winchester et al., 1975). In a control study for Fc binding, no labeling was observed in specimens that were incubated in nonimmune goat IgG prior to incubation in rabbit anti-goat immunogold (Fig. 1B). In additional experiments, specimens that were incubated in goat antimouse IgG+M and "blocked" with rabbit anti-goat antibody prior to incubation in rabbit anti-

goat gold showed no labeling of any cell type (second control, not shown). These results confirmed that the immunogold bound only to mIg in these preparations.

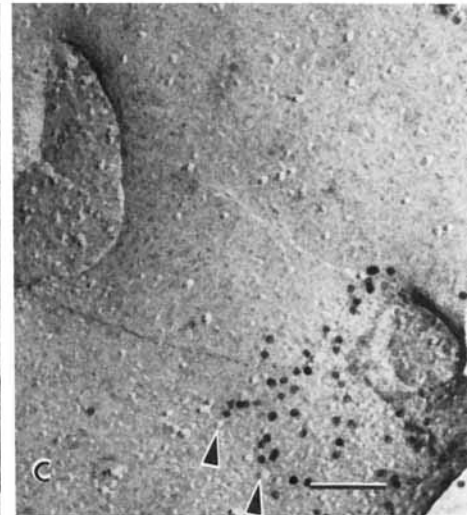
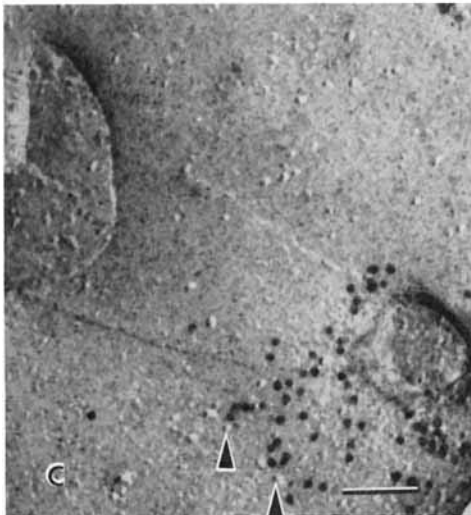
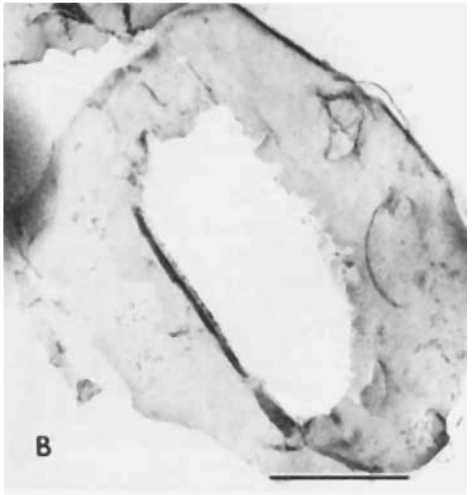
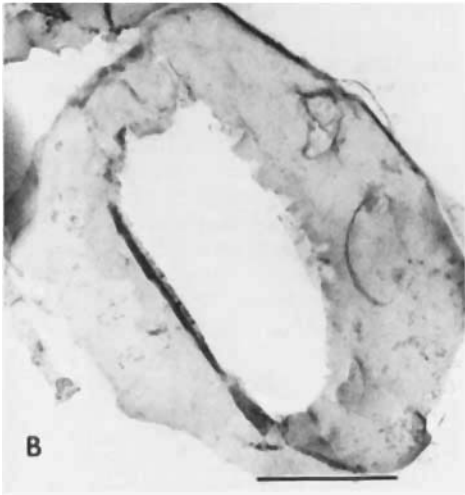
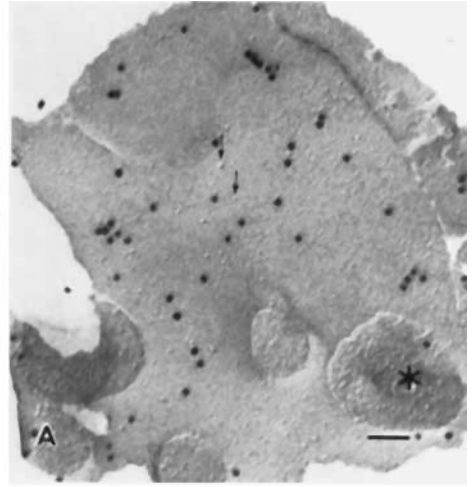
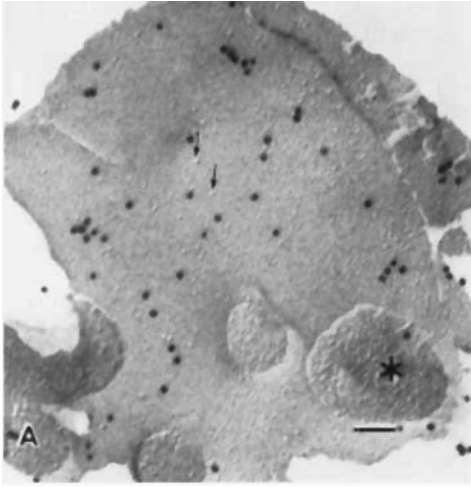
Sectioned replicas of spleen cells

Spleen cell pellets were fractured and deep-etched prior to replication and tissue digestion. Among the various cell types in splenic tissue are the easily recognized RBCs and lymphocytes (Fig. 2A). The replica in Figure 2A is of an etched preparation, whereas the sectioned, labeled replica preparations were not etched prior to replication. By imagining that the replica viewed in Figure 2A is still associated with the replicated cellular material beneath, one can visualize how a section passing through the replica (indicated by the line in Fig. 2A) would generate the image seen in Figure 2B. The cytoplasmic markers retained in the sectioned replica simplify the identification of replicated cells. In addition, the differentiation of E- from P-faces becomes trivial, even to the neophyte.

Postreplication labeled, sectioned replicas

Spleen cell pellets were frozen, fractured, immediately shadowed with Pt, and then coated with carbon. By this process the split membranes are stabilized and the IMPs are preserved and replicated as in conventional freeze-fracture replicas. Replicated samples were thawed, labeled, postfixed, embedded in plastic, and sectioned parallel to the replica surface. Since the replicated surface of fractured biological material is not flat, a section cut parallel to the replica surface reflects that inherent contour (compare Figs. 2 and 3). The P- and E-face replicas seen in Figure

Fig. 4. Sectioned, labeled replica of B-cells with unpatched (A) and patched (B,C) mIg. A: Thin-sectioned replica of a B-cell labeled with 180 Å colloidal gold directed against mIg. Colloidal gold labels are most often seen near small-diameter (30–60 Å) and not large-diameter (>60 Å) IMPs (arrows). Other tilt-angle stereo pairs (not shown) can reveal IMPs obscured by gold label. The asterisk marks the cytoplasm of one of four cross-fractured microvilli. B: High-voltage (750 kV) thick-section image of the replicated outer membrane leaflet of a B-cell (about one-third of the cell membrane is seen in this section) that had its mIg patched with goat antimouse Ig prior to labeling with rabbit anti-goat Ig bound to 100-Å colloidal gold. Clusters of gold label are seen scattered underneath the E-face replica of this cell. C: A high-magnification view of the region located above the magnification bar in B. A cluster of 100-Å gold labels underlies a patch of small-diameter E-face IMPs. A few larger-diameter IMPs (arrowheads) are observed in this patch as well. Labels are concentrated at the base of a cross-fractured microvillus. Bar, 0.1 μm (A), 1.0 μm (B), and 0.1 μm (C).



2 are easily discerned at the margin of the tissue analog (Fig. 2B). Normal cell shapes are maintained in replica slices and conform well to the cell outlines in unreplicated cells (Figs. 1, 3A). In addition, HVEM of thick sections (Figs. 4B, 5A) permits study of the distribution of label in areas of replica that may be almost as large as those prepared by conventional replica methods.

In the surface of E-face replicas, microvilli typically remain intact in the subjacent thin section, lying in direct continuity with their cross-fractured bases in E-face replicas. Thus, identifiable and antigenically recognizable cellular material (cytoplasm and membranes), remains associated with both E-face and P-face replicas and helps to identify replicated cells.

The external membrane leaflets (located beneath replicated E-faces in split membranes) of B-cells is often well labeled (Figs. 3-5). Replica image quality remains high throughout processing, even when viewed through several microns of epoxy resin (Figs. 4B,C, 5A,B). E-face replicas typically contain an abundance of distinct IMPs of different sizes (30-100 Å; Figs. 3-5). In high-magnification images of "unpatched" B-lymphocytes, colloidal gold labels are rarely seen underlying large-diameter (>60 Å) E-face IMPs (Figs. 3B, 4A) yet are often close to small diameter (~30-60 Å) IMPs. For a closer correlation of IMPs or pits associated with the monodisperse colloidal gold, mIgs were patched before colloidal gold labeling to determine if clusters of IMPs (or pits) of a discrete size class would be formed and if, after labeling, these would be uniquely associated with clustered gold labels. Thick-section images of B-lymphocytes (Figs. 4B,C, 5) reveal patches of small-diameter (30-60 Å) E-face IMPs overlying clusters of 100-Å colloidal gold. Occasionally, larger-diameter IMPs are seen at the margins of a patch (Fig. 4C), but more often only small-diameter (<60 Å) IMPs are localized in a patch (Fig. 5B).

Uneven distribution of mIg on B-cell surface

The density of labeling was not uniform over the entire surface of unpatched label cells. As seen in postreplication labeled E-face replicas (Fig. 3) and in thin sections of conventionally labeled cell pellets (Fig. 1A),

microvilli were more heavily labeled than contiguous, nonmicrovillous membranes. This was confirmed in numerous thin-section and deep-etch-labeled replica preparations (data not shown) and agrees with the observations of De Petris (1978). Labeling of replicated and contiguous nonreplicated membrane surfaces (Fig. 3) indicates that the antigenicity of mIg in replicated regions is retained in the normal distribution in the subjacent external half-membrane leaflets after fixing, freezing, fracturing, replicating, and thawing.

Sidedness of label

Stereoscopic analysis of thick and semi-thick sections of postreplication labeled specimens reveals that label is preferentially bound to the positively stained leaflet beneath the replicated E-face (Figs. 3-5). Reverse stereo ("intaglio") images help clarify the sidedness of label (Fig. 3B). These micrographs reveal that split membrane leaflets are stabilized by the platinum coat, that antigenicity is maintained in the externally exposed (nonreplicated) portions of the transmembrane proteins (mIgs), and that a portion of each mIg is embedded within and replicated as an E-face IMP in the platinum-stabilized E-leaflet. In addition, these images reveal that nonspecific binding to the platinum surface is minimized by appropriate blocking techniques (Figs. 2-5). The replicas attached to erythrocytes and unlabeled lymphocytes (Fig. 2b) are as devoid of gold label as the areas outside of the clustered label in Figures 4C and 5B. The patching experiments show that the gold label is not displaced laterally by washing, postfixation, embedding, or sectioning steps and that label does not appear on replicated solvent or other unexpected areas.

LBB prevents nonspecific binding of colloidal gold labels to replicas

A pellet of red blood cells was fractured, replicated, thawed, and labeled for mIg in the absence of labeling/blocking buffer (LBB). Since there were virtually no lymphocytes in this preparation, the labeling evident in this preparation is necessarily nonspecific. The absence of LBB allowed RBCs and the replicated intercellular medium to appear heavily labeled (Fig. 6). These images, in contrast to the images in Figures 2B-5, indicate that in the absence of LBB, one or more components of the labeling reagents are preferentially

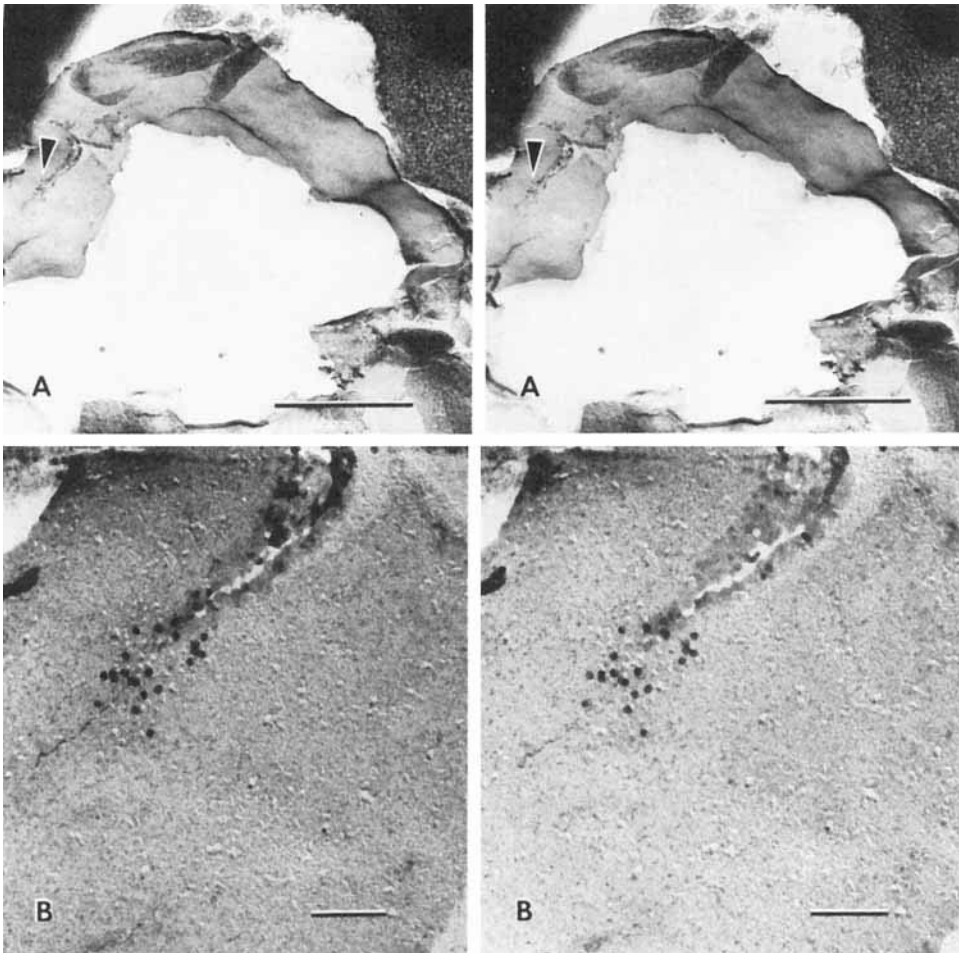


Fig. 5. Thick-sectioned labeled replica of a B-cell that had its mIg patched before replication and labeling with 100-Å colloidal gold. A: A distinct cluster of colloidal

gold (arrowhead) is shown at higher magnification in B. Particle size ranges from 30 to 60 Å. Larger particles are not found in this patch. Bar, 1.0 μm (A), 0.1 μm (B).

bound to the finely divided platinum and/or carbon layers of the replica. Thus, LBB (or equivalent) is required to prevent nonspecific binding to the replica.

DISCUSSION

Maintenance of antigenicity and replica quality

We have used a new version of a high-resolution labeling technique (Rash et al., 1978) to study the distribution of membrane immunoglobulins in the outer "half" membranes of fractured and replicated B-lympho-

cytes. This labeling occurs because the tissue analog (without basement membrane and extracellular barriers) allows the label to gain access to E-surfaces by diffusing between the cells of the pellet (Fig. 7). The outer membrane leaflet is well stabilized by the replication process, and antigenicity is maintained in the externally exposed moieties throughout fixation, cryoprotection, freezing, fracturing, and multiple labeling and washing steps which may last up to 48 hours. This application of the SLR technique demonstrates high specificity of labeling while maintaining high replica quality (i.e., excellent morphology, in-

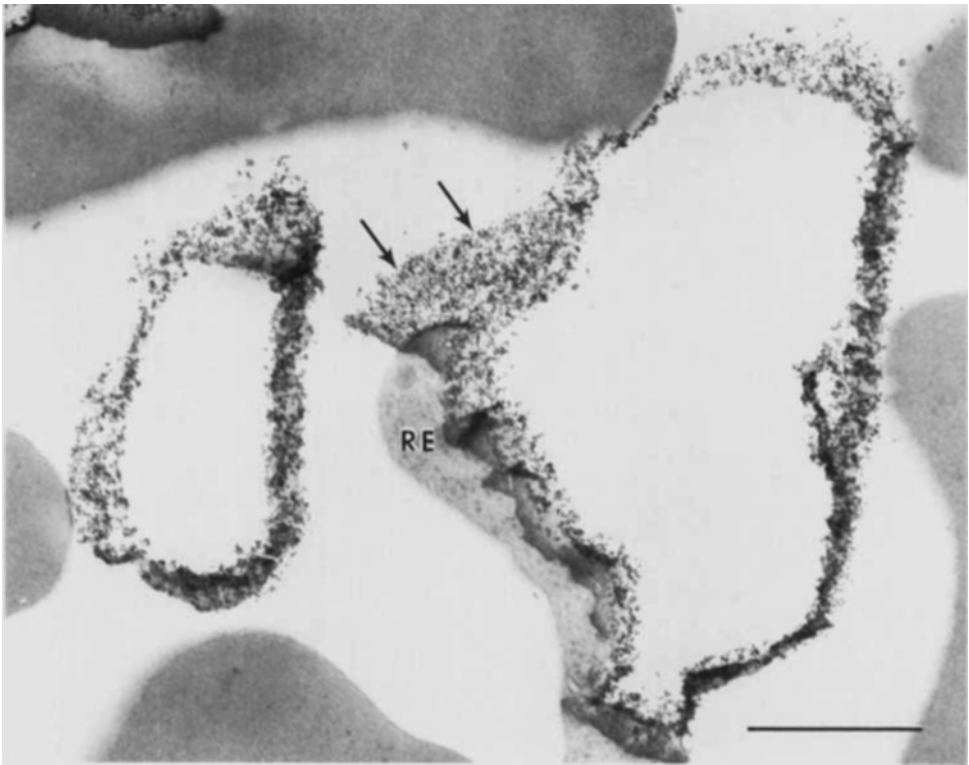


Fig. 6. Control preparation of red blood cells that was labeled with anti-immunoglobulin colloidal gold reagent in the absence of labeling/blocking buffer (LBB). Examination of replicated cytoplasm and replicated intercellular medium (arrows) confirms the high level of nonspecific "labeling" that is associated with the absence of LBB during the labeling procedure. Stereoscopic

examination of such preparations (not shown) reveals that most gold is attached to the platinum/carbon side of E-face replicas (i.e., the true surface of the intact half-membrane leaflet is not markedly labeled) and to both sides of replicated intercellular medium. Reticulocyte, RE. Bar, 1.0 μm .

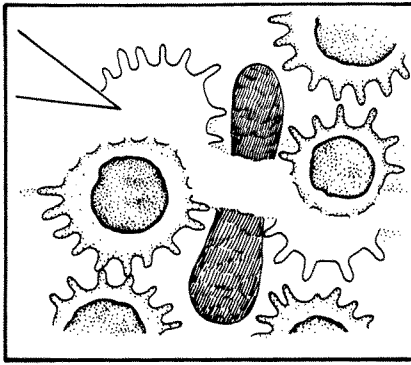
cluding the presence of well-defined IMPs and pits, with diameters ranging from 30 to 100 Å). Once the reagents are prepared, this technique takes little more time than a standard colloidal gold labeling experiment and is a simple, reproducible procedure (Fig. 7).

With this technique, replicas remain connected with intact portions of cell membrane and cytoplasmic material that did not cleave away during the fracture process. The association of identifiable cellular material with the replica is of considerable value because it helps to identify replicated cells within a heterogeneous population. This association also allows the direct comparison of labeling density in flat areas of cell membrane vs. areas of rough contour (e.g., microvilli) which otherwise are likely to be sheared away and therefore not be incorporated into the fracture plane in conventional replicas.

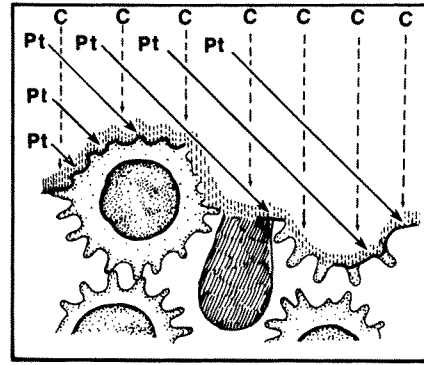
Because E-leaflets are stabilized by the addition of a carbon coat after platinum shadowing, there is no need for a supporting gelatin or agarose matrix (e.g., Aguas and Pinto da Silva, 1983, 1984, 1985; Pinto da Silva et al., 1981a-c; Rash, 1979; Rash et al., 1978, 1980a,b), which limits access of label to those areas that are not in contact with the matrix. This tissue analog method allows E-surface labeling of tightly apposed cells that are linked together with glutaraldehyde. In contrast, replicas of loose cell suspensions (Pinto da Silva and Kan, 1984) disaggregate upon thawing, thereby limiting the ability to directly compare many cells at one time.

Criteria for establishing specificity of replica labeling

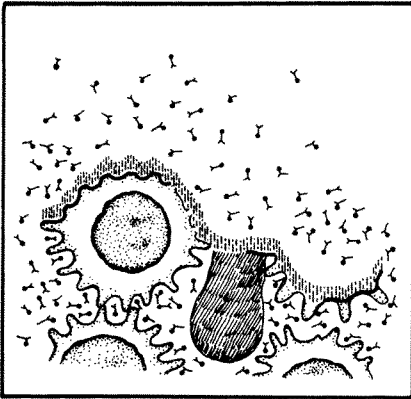
The blocking buffer control (Fig. 6) demonstrates that in order to achieve specific label-



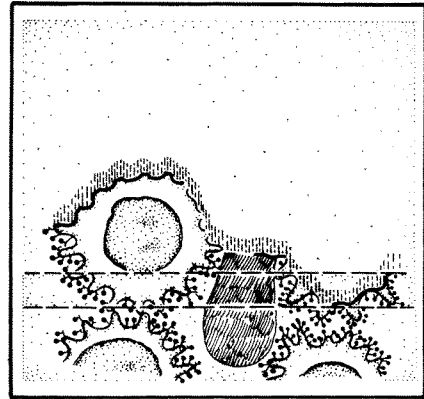
CLEAVE



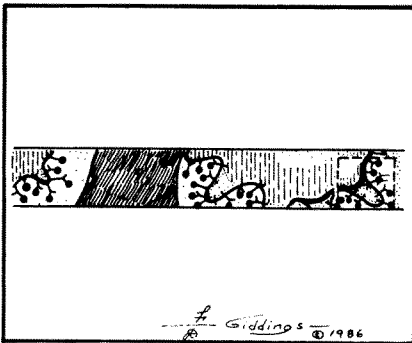
Replicate : Platinum & Carbon



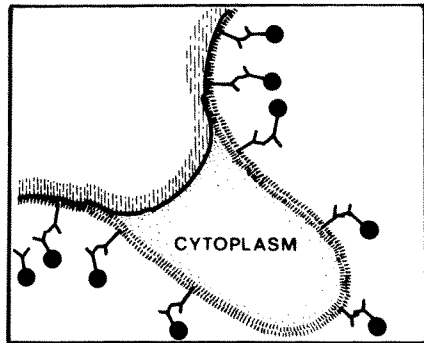
THAW & LABEL



WASH, POSTFIX & EMBED



SECTION



LABELING DETAIL

Fig. 7. Postreplication labeling. Cells are linked to each other at their extremities by fixation. After freezing and fracturing, the sample is shadowed with platinum at a 45° angle. The sample is then stabilized by addition of a carbon coat from above. Fragile E-leaflet replicas are well preserved by the deposition of carbon. If carbon is not used, the E-leaflets become highly contorted during the labeling process (unpublished observations). After deposition of carbon, the sample is thawed in blocking buffer and labeled by a double-antibody sandwich technique. Label gains access deep in the pellet by diffusing between the cells. Excess gold ligand is removed by washing in blocking buffer, and the sample

is then prepared for conventional transmission electron microscopy. Embedding in plastic preserves the quality of the replica while allowing study of associated labels and cellular material. Conventional digestion and mounting of the postreplication labeled replica is not possible with this particular methodology. Digestion of the organic links between the gold labels and the platinum replica would result in displacement of the gold and subsequent nonspecific binding of gold colloid to the platinum replica. Sections taken along the plane of the replica reveal gold ligand bound to membrane immunoglobulin sites on replicated and unreplicated portions of the sample.

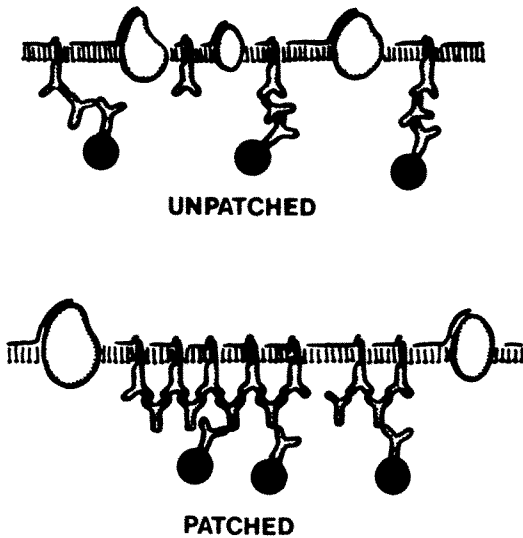


Fig. 8. Detail of E-leaflet, postreplication labeling of membrane immunoglobulin (mIg) in membranes with patched and unpatched mIg. Membranes are fixed with glutaraldehyde before adding primary and secondary (colloidal gold) antibodies in unpatched membrane and fixed before addition of secondary (colloidal gold) antibody in patched membrane. Not drawn to scale.

ing of cell receptors in replicated preparations, it is important to use an adequate blocking agent during the labeling steps. Criteria for specific labeling of components within freeze-fractured membranes should include (1) association of label with identifiable classes of cells among a population of cells, (2) lack of significant "labeling" in areas of replica that are known to be devoid of the antigen, and (3) demonstration of proper "sidedness" of label, presumably by stereoscopic examination. [In addition to these criteria, assignment of label to a specific particle class can be demonstrated only when both particles and pits are reliably and reproducibly replicated without evidence for changes in particle diameter due to water vapor contamination (Rash et al., 1979).] Finally, when appropriate, it is useful to show that labeling changes from a diffuse pattern to a clustered pattern under conditions that are known to cause patching or reorganization of the membrane component of interest.

Distribution of membrane immunoglobulin

In untreated cells, we demonstrate that membrane immunoglobulin is preferentially sequestered on the numerous microvilli that characterize heavily labeled cells. A comparison of the labeling density on replicated planar membranes and unreplicated micro-

villi readily demonstrates this point. These results have been confirmed in prefracture labeled deep-etched and thick-sectioned labeled preparations (unpublished observations).

Partitioning of membrane immunoglobulin

Previously, radioisotopes had been used to determine to which leaflet a particular membrane protein partitions after prefracture labeling (Fisher, 1982a,b; Fisher and Yanagimoto, 1986). The technique we have described addresses a similar question in a postfracture, postreplication labeled preparation. This report demonstrates that, in fixed membranes, mIg antigenic sites partition with the external membrane leaflet. Recent work by Fisher and Yanagimoto (1986) demonstrates that the covalent polypeptide backbone of any erythrocyte membrane protein is likely preserved intact after the process of membrane splitting. If this finding is applicable to other cell types such as lymphocytes, then postreplication, E-leaflet labeling offers a high-resolution method to determine which membrane proteins partition with the E-leaflet.

Distribution of IMPs/resolution of labeling techniques

It will be significant to establish, with a minimum radius of uncertainty, a direct association between an electron-dense label and a single class of IMP that partitions with the E-face. In 1982, De Groot et al. utilized conventional freeze-fracture preparations to study the distribution of E-face IMPs in the vicinity of patched cell-surface label. The high percentage of anti-Ig labeled spleen cells (70%) and the use of Ig aggregates in their labeling schemes suggests that other receptors (perhaps Fc receptors) in addition to mIg sites were clustered (Kumagai et al., 1975; Winchester et al., 1975). Our micrographs (Figs. 3B, 4A,C, 5B) show that anti-mIg label is associated with small-diameter IMPs (i.e., 30-60 Å) and not large-diameter IMPs (>60 Å). A series of varying tilt-angle stereo pairs will allow a refined definition of the IMPs mapping within 200 Å of the attached gold labels. Thus, this postreplication labeling technique may apply broadly to studies of the distribution of IMPs that partition with the E-leaflet.

Sectioned labeled replicas and HVEM

The combination of sectioned labeled replicas and HVEM presents exciting possibilities. The thick sections which can be viewed

by HVEM permit rapid examination of large areas of replica (Figs. 4B, 5A). Thick sections can be obtained in which the entire replica of a cell is included. The platinum replica and gold labels provide good markers for locating areas of interest and for focusing in the HVEM. A drawback for many, of course, is the limited availability of HVEM.

SUMMARY

We have shown (1) that the antigenicity of membrane immunoglobulins in B lymphocytes remains intact after the processes of fixation, freeze-fracture, replication, and thawing, (2) that mIg labels on and thus is shown to partition with the E-leaflet in split, fixed membranes, and (3) that mIg is associated with small-diameter (30–60 Å) IMPs in E-face replicas and not with large-diameter (>60 Å) IMPs. Thus, postreplication labeling schemes demonstrate the ability of sectioned-replica techniques to maintain biological markers with high-quality replicas, thereby allowing correlation of IMP distribution with localized membrane proteins.

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REFERENCES

- Abbas, A.K., Ault, K.A., Karnovsky, M.J., and Unanue, E.R. (1975) Non-random distribution of surface immunoglobulin on murine B lymphocytes. *J. Immunol.*, 114:1197–1203.
- Agas, A.P., and Pinto da Silva, P. (1983) Regionalization of transmembrane glycoproteins in the plasma membrane of boar sperm head is revealed by fracture-label. *J. Cell Biol.*, 97:1356–1364.
- Agas, A.P., and Pinto da Silva, P. (1984) High density of transmembrane glycoproteins on the flagellar surface of boar sperm cells. *J. Cell Biol.*, 99:655–660.
- Agas, A.P., and Pinto da Silva, P. (1985) The acrosomal membrane of boar sperm: A Golgi derived membrane poor in glycoconjugates. *J. Cell Biol.*, 100:528–534.
- Carter, D.P., and Staehelin, L.A. (1979) Evaluation of IgG molecules, Fab' fragments and IgG-horseradish peroxidase conjugates as surface labels for freeze-etched membranes. *J. Microsc.*, 117:363–373.
- Costello, M.J., Fetter, R., and Corless, J.M. (1984) Optimum conditions for the plunge freezing of sandwiched samples. In: *Science of Biological Specimen Preparation Proceedings of the 2nd Pfefferkorn Conference*. Chicago: SEM Inc. AMF O'Hare, pp.105–115.
- Costello, M.J., and Frey, T.G. (1982) Membranous cytochrome c oxidase: A freeze-fracture electron microscopic analysis. *J. Mol. Biol.*, 162:131–156.
- DeGroot, C., Kapsenberg, M.L., and Leene, W. (1982) Observations on transmembrane structures of surface immunoglobulin in the plasma membrane of B lymphocytes. *Biochim. Biophys. Acta*, 689:275–282.
- De Mey, J. (1983) Colloidal gold probes in immunocytochemistry. In J.M. Polak and S. Van Noorden (eds.): *Immunocytochemistry: Practical Applications in Pathology and Biology*, Wright, P.S.G., Bristol, pp. 82–112.
- De Petris, S. (1978) Preferential distribution of surface immunoglobulins on microvilli. *Nature*, 272:66–68.
- Dinchuk, J.E., Johnson, T.J.A., and Rash, J.E. (1986) HVEM and conventional TEM studies of post-shadow labeled surface molecules in freeze-etch replicas. *EMSA Proc.*, 44:890–893.
- Fisher, K.A. (1982a) Spectroscopic assays for measuring quantities of erythrocyte membrane "halves" *J. Cell Biol.*, 92:44–52.
- Fisher, K.A. (1982b) Monolayer freeze-fracture autoradiography: Quantitative analysis of the transmembrane distribution of radioiodinated concanavalin A. *J. Cell Biol.*, 93:155–163.
- Fisher, K.A., and Yanagimoto, K.C. (1986) Effect of membrane splitting on transmembrane polypeptides. *J. Cell Biol.*, 102:551–559.
- Frens, G. (1973) Controlled nucleation for the regulation of particle size in monodisperse gold solutions. *Nature*, 241:20–22.
- Geoghegan, W.D., and Ackerman, G.A. (1977) Adsorption of horseradish peroxidase, ovomucoid and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-human immunoglobulin G on cell surfaces at the electron microscope level: A new method, theory and application. *J. Histochem. Cytochem.*, 25:1187–1200.
- Harding, C., Heuser, J., and Stahl, P. (1983) Receptor mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J. Cell Biol.*, 97:329–339.
- Hay, E.D., and Hastly, D.L. (1979) Extrusion of Particle-Free Membrane Blisters During Glutaraldehyde Fixation. In J.E. Rash and C.S. Hudson (eds): *Freeze-Fracture: Methods, Artifacts, and Interpretations*. New York: Raven Press, pp. 59–66.
- Hong, K., and Hubbel, W.L. (1972) Preparation and properties of phospholipid bilayers containing rhodopsin. *Proc. Natl. Acad. Sci. USA*, 69:2617–2621.
- Hood, L.E., Weissman, I.L., Wood, W.B., and Wilson, J.H. (1984) *Immunology*. 2nd ed. Menlo Park: Benjamin/Cummings Publishing Co., Inc., pp. 38–89.
- Horisberger, M., and Rosset, J. (1977) Colloidal gold, a useful marker for transmission and scanning electron microscopy. *J. Histochem. Cytochem.*, 25:295–305.
- Johnson, T.J.A. (1986) Glutaraldehyde fixation chemistry: A scheme for rapid crosslinking and evidence for rapid oxygen consumption. In M. Müller, R.P. Becker, A. Boyde, and J. Woloszewicz (eds.): *The Science of Biological Specimen Preparation for Microscopy and Microanalysis, Proceedings of the 4th Pfefferkorn Conference*. Chicago: SEM Inc., AMF O'Hare, pp. 51–62.
- Karnovsky, M.J., and Unanue, E.R. (1973) Mapping and migration of lymphocyte surface macromolecules. *Fed. Proc.*, 32:55–59.
- Karnovsky, M.J., Unanue, E.R., and Leventhal, M. (1972) Ligand-induced movement of lymphocyte membrane macromolecules II. Mapping of surface moieties. *J. Exp. Med.*, 136:907–930.
- Kumagai, K., Abo, T., Sekizawa, T., and Sasaki, M. (1975) Studies of surface immunoglobulins on human B lymphocytes I. Dissociation of cell-bound immunoglobulins with acid pH or at 37°C. *J. Immunol.*, 115(4):982–987.

- Mannweiler, K., Hohenberg, H., Bohn, W., and Rutter, G. (1982) Protein-A gold particles as markers in replica immunocytochemistry: High resolution electron microscope investigations of plasma membrane surfaces. *J. Microsc.*, 126:145-149.
- Owne, J.J.T., Cooper, M.D., and Raff, M.C. (1974) *In vitro* generation of B lymphocytes in mouse foetal liver, a mammalian "Bursal equivalent". *Nature*, 249:361-363.
- Pinto da Silva, P., Kachar, B., Torrisi, M.R., Brown, C., and Parkinson, C. (1981) Freeze-fracture cytochemistry: Replicas of critical point-dried cells and tissues after fracture-label. *Science*, 213:230-233.
- Pinto da Silva, P., and Kan, F.K. (1984) Label-fracture: A method for high resolution labelling of cell surfaces. *J. Cell Biol.*, 99:1156-1161.
- Pinto da Silva, P., Parkinson, C., and Dwyer, N. (1981) Fracture-label: Cytochemistry of freeze-fracture faces in the erythrocyte membrane. *Proc. Natl. Acad. Sci. USA*, 78:343-347.
- Pinto da Silva, P., Parkinson, C., and Dwyer, N. (1981) Freeze-fracture cytochemistry: Thin sections of cells and tissues after labeling of fracture faces. *J. Histochem. Cytochem.*, 29:917-928.
- Pinto da Silva, P., and Torrisi, M.R. (1982) Freeze-fracture cytochemistry: Partition of glycophorin in freeze-fractured human erythrocyte membranes. *J. Cell Biol.*, 93:463-469.
- Rabellino, E., Colon, S., Grey, H.M., and Unanue, E.R. (1971) Immunoglobulins on the surface of lymphocytes. I. Distribution and quantitation. *J. Exp. Med.*, 133:156-167.
- Rash, J.E. (1979) The sectioned-replica technique: Direct correlation of freeze-fracture replicas and conventional thin section images. In J.E. Rash and C.S. Hudson (eds.): *Freeze-Fracture: Methods, Artifacts, and Interpretations*. New York: Raven Press, pp.153-160.
- Rash, J.E., Graham, G.W., and Hudson, C.S. (1979) Sources and rates of contamination in a conventional balzers freeze-etch device. In J.E. Rash and C.S. Hudson (eds): *Freeze-Fracture: Methods, Artifacts, and Interpretations*. New York: Raven Press, pp. 111-112.
- Rash, J.E., Hudson, C.S., and Ellisman, M.H. (1978) Ultrastructure of acetylcholine receptors at the mammalian neuromuscular junction. In R.W. Straub and L. Bolis (eds.): *Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach*. New York: Raven Press, pp. 47-68.
- Rash, J.E., Johnson, T.J.A., Hudson, C.S., Copio, D.S., Graham, W.F., Eldefrawi, M.E., and Giddings, F.D. (1980a) Identification of intramembrane particles by pre- and post-fracture labelling techniques: A progress report. 38th Annu. Proc. Electron Microsc. Soc. Am.: 692-695.
- Rash, J.E., Johnson, T.J.A., Hudson, C.S., Giddings, F.D., Graham, W.E., and Eldefrawi, M.E. (1980b) Labelled-replica techniques: Post-shadow labelling of intramembrane particles in freeze-fracture replica. *J. Microsc.*, 126:121-138.
- Sato, T. (1967) A modified method for lead staining of thin sections. *J. Electron Microsc.* (Tokyo), 16:133.
- Schiller, A., and Taugner, R. (1980) Freeze-fracturing and deep etching with the volatile cryoprotectant ethanol reveals true membrane surfaces of kidney structures. *Cell Tissue Res.*, 210:57-69.
- Schmidt, D.G., and Buchheim, W. (1982) On the size of small protein particles determined by electron microscopy of unidirectionally shadowed freeze-etched preparations. *J. Microsc.*, 126:347-451.
- Shelton, E., and Mowczko, W.E. (1979) Scanning electron microscopy of membrane blisters produced by glutaraldehyde fixation and stabilization by postfixation in osmium tetroxide. In J.E. Rash and C.S. Hudson (eds.): *Freeze-Fracture: Methods, Artifacts, and Interpretations*. New York: Raven Press, pp. 67-69.
- Slot, J.W., and Geuze, H.J. (1985) A new method of preparing gold probes for multiple labeling cytochemistry. *Eur. J. Cell Biol.*, 38:87-93.
- Smith, S.B., and Revel, J.-P. (1972) Mapping of concanavalin A binding sites on the surface of several cell types. *Dev. Biol.*, 27:434-441.
- Taylor, R.B., Duffus, P.H., Raff, M.C. and de Petris, S. (1971) Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature New Biol.*, 233:225-229.
- Unanue, E.R., Karnovsky, M.J., and Engers, H.D. (1973) Ligand-induced movement of lymphocyte membrane macromolecules III. Relationship between the formation and fate of anti-Ig-surface Ig complexes and cell metabolism. *J. Exp. Med.*, 137:675-689.
- Vitella, E.S., Melcher, U., McWilliams, M., Phillips-Quagliata, J., Lamm, M., and Uhr, J.W. (1975) Cell surface immunoglobulin. XI. The appearance of an IgD-like molecule on murine lymphoid cells during ontogeny. *J. Exp. Med.*, 141:206-215.
- Winchester, R.J., Fu, S.M., Hoffman, T., and Kunkel, H.G. (1975) IgG on lymphocyte surfaces: Technical problems and the significance of a third cell population. *J. Immunol.*, 114:1210-1212.
- Wunderlich, F., Hoelze-Wallach, D.F., Spith, V., and Fischer, H. (1974) Differential effects of temperature on the nuclear and plasma membrane of lymphoid cells: A study by freeze-etch electron microscopy. *Biochim. Biophys. Acta*, 373:34-43.