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Molecular cytochemistry of CD3 and CD4 antigens in human lymphocytes as studied by label-fracture and by fracture-label

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Label-fracture and fracture-label membrane immunocytochemistry are used to analyze the surface distribution, dynamics and partition on fracture of CD3 and CD4 antigens of human T lymphocytes. Redistribution of the antigens, induced by treatment at 37°C with specific monoclonal antibodies, results in patching and capping of the labeling as observed in label-fractured specimens. Examination of platinum/carbon replicas of freeze-fractured plasma membranes of antibody-treated cells does not reveal recognizable domains of intramembrane particles. However, in cells where the aggregation of intramembrane particles is induced by incubation with glycerol, colloidal gold-labeled CD3 and CD4 molecules are seen confined to particulate domains of the membrane. Therefore, the lack of visible aggregation of intramembrane particles in patched or capped regions of the membrane implies that migration of CD3 and CD4 antigens with concentration in domains of the membrane is achieved contemporaneously with export of other non-capped integral membrane proteins from the same regions, in a process of diffusional equilibrium. Examination of fracture-labeled specimens shows that CD4 molecules partition on fracture with the inner protoplasmic face of the plasma membrane. This partition illustrates the transmembrane attitude of the antigen molecule and is a probable consequence of interaction of the protein with other components of the membrane or with the cytoskeleton.

Introduction

The CD3 and CD4 antigens expressed on the surface of human T lymphocytes are transmembrane glycoproteins, whose sequence and peptide chain topology is now established [1,2]. The CD3 antigen (a group of non-polymorphic proteins; [1]) is associated to the T cell receptor molecule and is, therefore, expressed on all T lymphocytes. The CD4 antigen (a single peptide chain) [2,3] characterizes a subset of T cells and its glycoprotein does not appear to be associated to other receptor proteins. Functionally, the CD3 antigen appears to be responsible for the transduction of the activation signal [1], the CD4 antigen appears to function as a recognition site for class II HLA antigens and to be part of the receptor for immunodeficiency virus (HIV) [4].

Recognition of antigens and signal transduction are dynamic membrane phenomena that are likely to require conformational changes, lateral displacements and the cross-linking of receptor complexes [5]. At the ultrastructural level, these processes will be best studied by freeze-fracture, where splitting of biomembranes along their bilayer continuum reveals the position of structures – the intramembrane particles – generally taken to represent the sites of integral membrane proteins (see Refs. 6 and 7 for reviews). At present, the only ultrastructural approaches that can relate directly the distribution of an antigen or receptor to a specific class of structural component within the plane of the membrane involve the combination of freeze-fracture and immunocytochemistry. The first of these approaches – 'label-fracture' (see Fig. 1) – provides coincident images of the distribution of a cytochemical marker and a platinum cast of the fractured membrane [8]. To this end, isolated cells are labeled, freeze-fractured, and the fractured faces are replicated by evaporation of

Abbreviation: PBS, phosphate-buffered saline.

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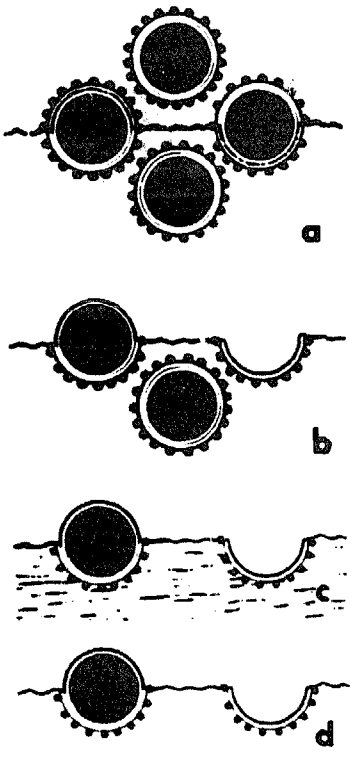

Fig. 1. Label-fracture. (a) Cells in suspension are labeled and frozen; (b) Freeze-fracture splits the plasma membranes of labeled cells into exoplasmic halves (with attached surface label; right) and protoplasmic halves (which remain attached to the cell body; left). Pt/C evaporation produces a high-resolution cast of the fractured cells (only label at the interface of fracture is exposed and shadowed); (c) Fractured, shadowed specimens are thawed and washed with distilled water which removes unfractured cells. Exoplasmic membrane halves remain attached to the replica. Coincident images of the Pt/C replica of the E face and the surface label are produced. Cells with fractured P faces (left) remain attached to the replica (here, electron density of the cell body prevents observation of the P face); (d) The replicas are then mounted on electron microscope grids, dried, and observed (from Ref. 8).

platinum. On thawing, unfractured cells are washed away, but the outer halves of the labeled and fractured plasma membranes remain attached to the replica. Thus, in label-fracture specimens it is possible to compare in a single, coincident image, the distribution of a surface antigen (judged by colloidal gold labeling) with that of the intramembrane particles revealed by freeze-fracture. In the other freeze-fracture cytochemical approach that we use here – 'fracture-label' – specimens are first freeze-fractured and thawed and then split membrane halves are labeled. With the fracture-label method, we showed that it is possible to study the partition of transmembrane proteins in freeze-fractured cells (for reviews, see Refs. 9–12).

Here, we use label-fracture as a molecular cytochemical approach to study the surface distribution and dynamics of CD3 and CD4 transmembrane glycoproteins. In addition, we use the fracture-label approach to analyze the partition during fracture of CD4 glycoproteins.

Materials and Methods

Cells

Human peripheral lymphocytes were isolated from fresh heparinized blood of healthy donors by Ficoll/Hypaque density-gradient centrifugation and washed three times in phosphate-buffered saline (PBS). The native distribution of CD3 and CD4 antigens was assessed by treating lymphocytes unfixed or chemically fixed (1% glutaraldehyde in PBS, 1 h, 25°C) in OKT3 or OKT4 monoclonal antibodies (50 μg/ml, 5 μl/10^6 cells, 1 h, 4°C) (Orthodiagnostic, Raritan, NJ) [13]. Clustering and capping of CD3 and CD4 antigens was induced using two experimental protocols: (a) lymphocytes were incubated in OKT3 or OKT4 solutions for 1 h at 4°C, followed by incubation with anti-mouse IgG-FITC (1 : 100 in PBS) (Sigma, St. Louis, MO) for 1 h at 4°C and, after washing, by an additional 30 min at 37°C [14]; (b) redistribution of surface antigens was induced in a single incubation step in OKT3 and OKT4 for 1 h at 25°C. Redistribution of antigens was followed by immunofluorescence microscopy. Unfixed lymphocytes treated only with OKT3 and OKT4 were fixed in glutaraldehyde before addition of fluorescent anti-mouse IgG. To induce the clustering of intramembrane particles, unfixed lymphocytes were impregnated in 30% glyceroi in PBS for 1 h at 37°C [15,16].

Label-fracture

For the label-fracture procedure, all cells were labeled with colloidal gold-protein A as above, re-fixed in 1% glutaraldehyde in PBS (1 h, 25°C), impregnated with 30% glycerol, frozen, freeze-fractured in a freeze-fracture device (−105°C, 10^{-6} mmHg) and replicated by evaporation from a platinum/carbon gun. The replicas were floated into distilled water, washed repeatedly in distilled water, picked by adhesion to formvar-coated grids and observed with a transmission electron microscope.

Fracture-label

All cells (treated or untreated with the monoclonal antibody OKT4 as above) were fixed in 1% glutaraldehyde in PBS (1 h, 25°C), centrifuged and embedded in 30% bovine serum albumin (30% in distilled water) cross-linked with glutaraldehyde (added at 50% (w/w) to a final concentration of 1%). The resulting 'gels' were sliced into small pieces, impregnated in 30% glycerol in PBS and frozen in Freon 22 cooled by liquid nitrogen. Frozen gels were fractured in liquid nitrogen by repeated crushing with a glass pestle, thawed in 1% glutaraldehyde, 30% glycerol in PBS, gradually de-

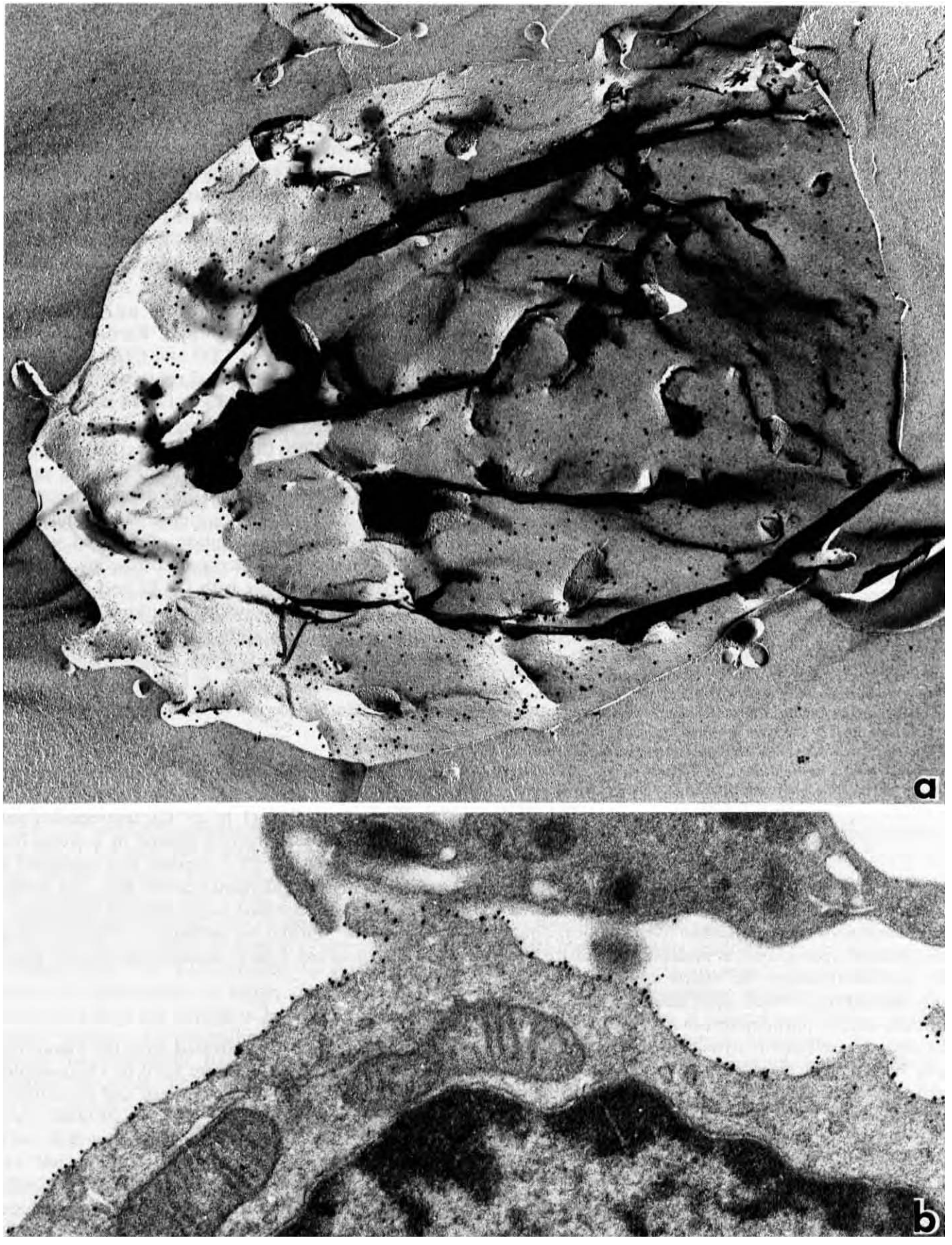

Fig. 2. Native distribution of CD4 antigens on human lymphocytes: the distribution of the immunolabeling is uniform over the cell surfaces as shown in both label-fracture images (a) and conventional thin section (b). In (b) an unlabeled platelet near the CD4 positive cell is evident. Magnification factors: (a) ×34359 (original ×33000); (b) ×34359 (original×33000).

glycerinated and washed two times in PBS (see Refs. 9–11 for an explanation of fracture-label). The gel fragments were incubated in the OKT4 monoclonal antibody 50 μg/ml (1 h at 4°C), labeled with colloidal gold (prepared by the citrate method) coated with protein A [17] (Pharmacia Fine Chemicals, Uppsala, Sweden) and processed for thin-section electron microscopy (fixed in 1% osmium tetroxide in veronal acetate buffer, stained with uranyl acetate, dehydrated in acetone and embedded in Epon 812).

Results

Label-fracture

In label-fractured specimens, images of the outer, exoplasmic fracture faces of plasma membranes are observed superimposed on those of the distribution of the surface antigens and receptors as revealed by colloidal gold cytochemistry (see Fig. 1 for interpretation).

Label-fracture images of the native distribution of CD3 and CD4 antigens, as observed in cells that were chemically fixed before the addition of antibodies, showed that both CD3 (data not shown) and CD4 antigens (Fig. 2a) were uniformly distributed over the entire cell body. The same pattern of distribution was also observed in thin sections of unfixed and glutaraldehyde-fixed lymphocytes immunolabeled at 4°C (Fig. 2b). In these samples, fixation with glutaraldehyde did not result in substantial reduction of labeling. Density of the surface immunolabeling shown in label-fracture images was comparable to that observed in thin sections (average thickness 100 nm) of cells from the same experiments, as assessed by counting the gold particles for μm of plasma membranes.

Incubation of unfixed lymphocytes with OKT3 or OKT4 monoclonal antibodies at 4°C, followed by anti-mouse IgG at 37°C resulted in patching of the colloidal gold-protein A label into clearly demarcated domains (Fig. 3a, b). Examination of stereo pairs (Fig. 3a, b) showed that the colloidal gold label and, therefore, the antigens remained at the surface of the membrane i.e., without noticeable extracellular extrusion.

Close inspection of the ultrastructure of replicas of exoplasmic fracture faces failed to show any parallel codistribution of intramembrane peptides, i.e., no specific population of particles was discernable. The density of intramembrane particles in the areas of CD3/CD4 clustering was indistinguishable from that observed in unlabeled areas. Similar results could be observed even when capping of the antigens to one pole of the cell was observed, as shown in Fig. 4 for CD4 antigens.

Examination of the ultrastructure of exoplasmic and protoplasmic faces was performed in replicas of untreated (Fig. 5a, b) or antibody-treated (Fig. 5c), unlabeled specimens, after conventional cleaning of the replicas with 5% sodium hypoclorite and washing. On close observation of regions that were shadowed from a high angle, a low density of intramembrane particles is revealed (Fig. 5b). However, as the angle of shadow decreases along the curved replica of a membrane fracture face, a higher density of smaller, lower particles becomes apparent (Fig. 5b). With this in mind, we re-observed label-fractured specimens; again, we failed to observe the existence of any recognizable domains on the exoplasmic fracture faces of cells where patching and capping of CD3/CD4 antigens was induced. In the previous set of experiments we induced the patching of a surface antigen and attempted to discover whether this event was accompanied by a parallel accumulation of intramembrane particles. Next, we attempted the reverse, i.e., to see whether the aggregation of intramembrane particles would lead to parallel re-positioning of surface antigens. To this end, we pre-incubated human lymphocytes in PBS containing 30% glycerol (v/v) (Fig. 6a), a treatment that has been shown to induce co-aggregation of all the intramembrane particles (regardless of diameter or height) into randomly contoured aggregates [6,7,15,18]. Examination of glycerol-treated, label-fractured cells showed that most gold spheres were associated with the co-aggregates of intramembrane particles (Fig. 6b). These experiments established that the transmembrane glycoproteins bearing CD3/CD4 antigens correspond to components that, on treatment with glycerol, are translationally displaced into the intramembrane particle co-aggregates.

Control experiments in which we omitted the monoclonal antibodies from the immunolabeling procedure showed more than 90% reduction of the labeling over all surfaces.

Fracture-label

In fracture-label experiments, thin sections of both protoplasmic and exoplasmic halves of plasma membranes revealed the typical aspect of interrupted unit membrane segments. This appears to be due to reorganization of the fracture monolayers into interrupted bilayered structures as discussed elsewhere [19–21].

To analyze the partition of CD4 antigens during fracture with the exoplasmic and protoplasmic halves of split plasma membranes, we immunolabeled freeze-fractured lymphocytes with monoclonal antibody OKT4 and colloidal gold-protein A. We observed that, whereas labeling over protoplasmic halves was moderate and uniformly distributed, exoplasmic halves remained virtually unlabeled (Fig. 7a, b). In these fracture-labeled specimens, the density of immunolabeling over the protoplasmic faces was lower than that observed on the unfractured surfaces, as expected because of reorganization events of the inner protoplasmic half of the membrane occurring during thawing of freeze-fractured

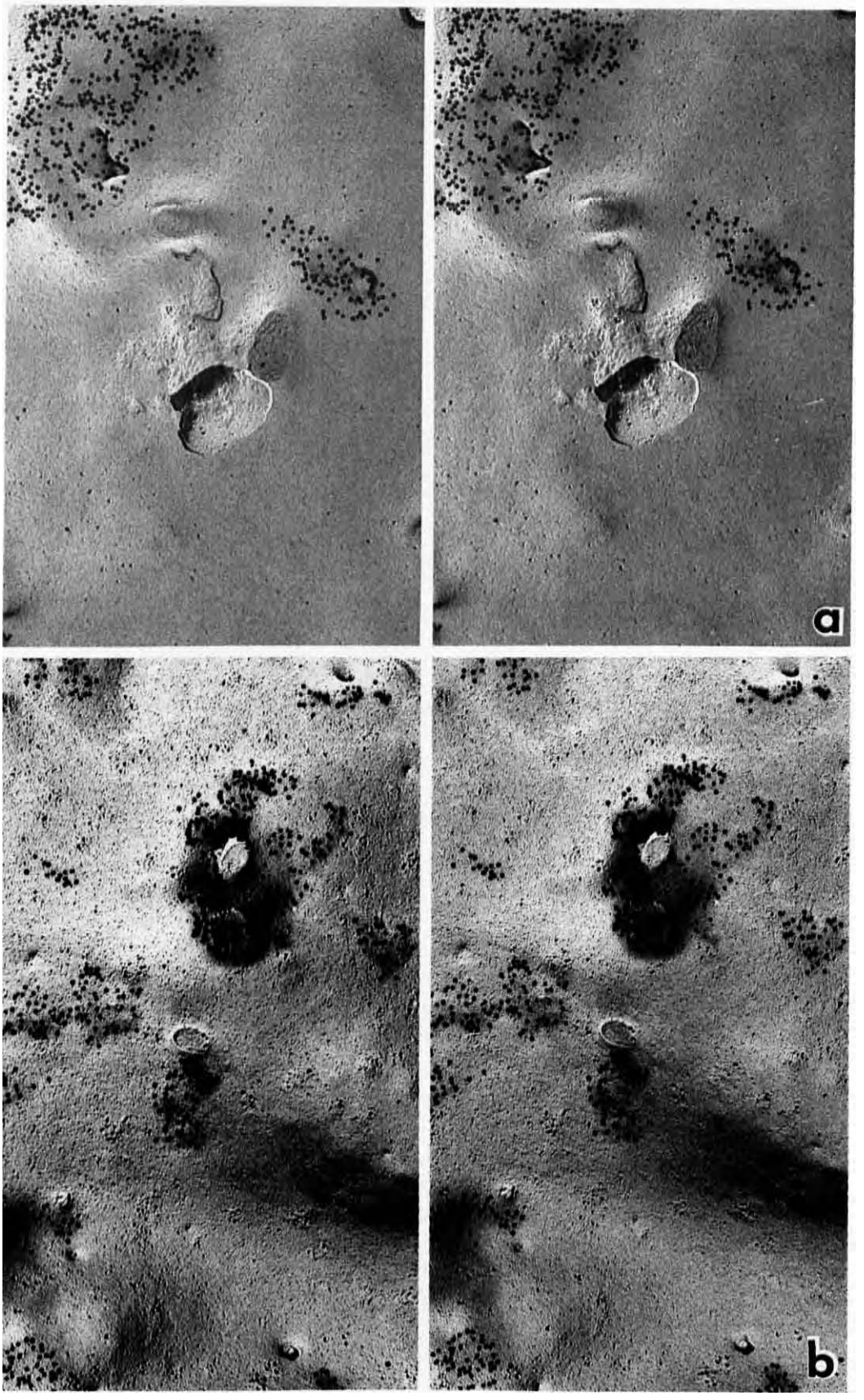

Fig. 3. Patching of CD4 and CD3 antigens induced by incubation with antibodies at 37°C: the surface immunolabeling is concentrated in domains, whereas no parallel codistribution of intramembrane particles is evident on the exoplasmic faces. Stereo pairs of label-fractured lymphocytes treated with OKT4 (a) and OKT3 (b) monoclonal antibodies. Magnification factors: (a) ×54046 (original×40000); (b) ×51342 (original×38000).

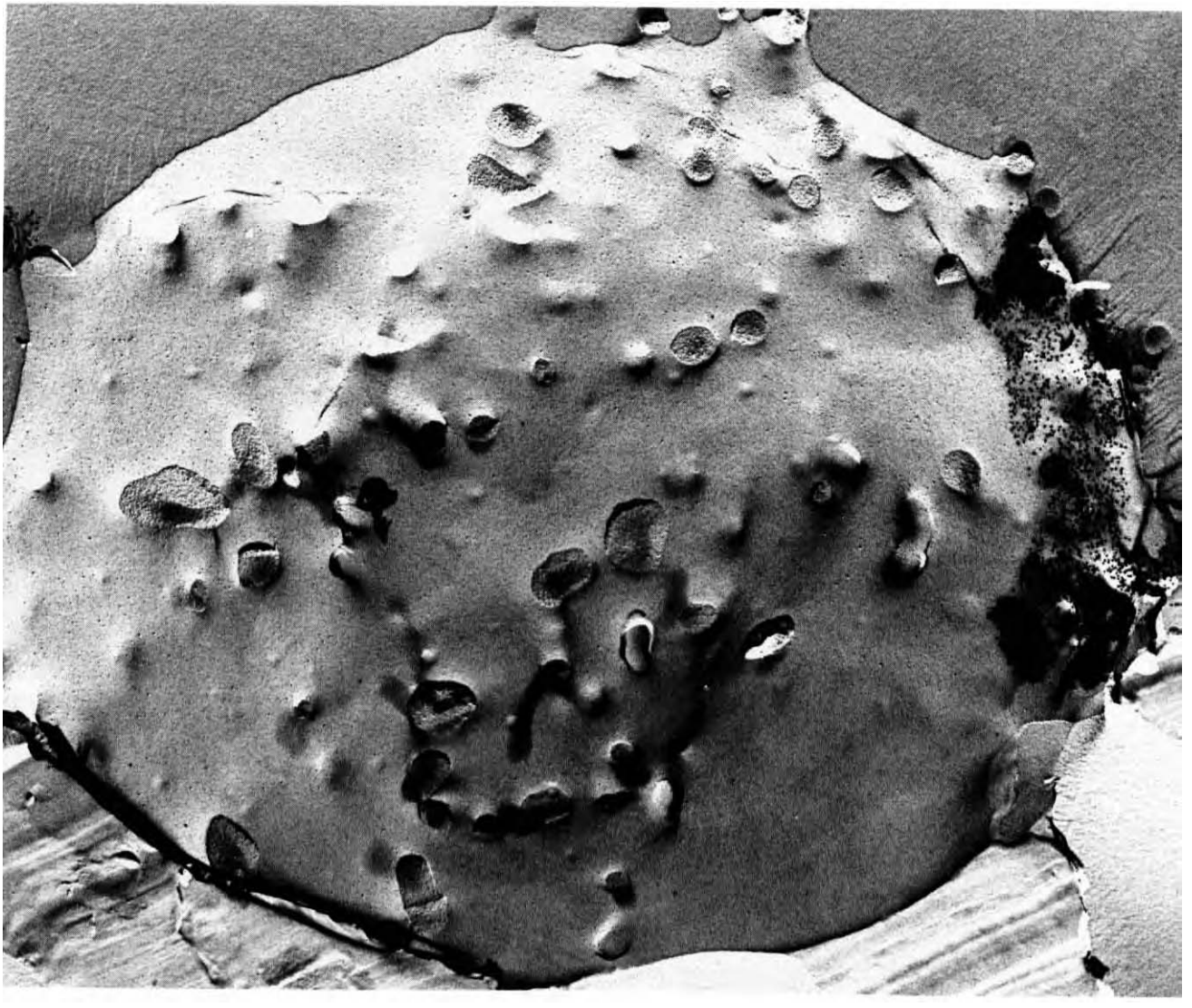

Fig. 4. Capping of CD4 antigens induced by incubation with antibodies at 37 °C: the immunolabeling is concentrated at one pole of the cell surface in a label-fractured CD4-positive lymphocyte. Magnification: ×26 982 (original ×25 000).

specimens [6,7,9]. Next, we analyzed the partition on fracture of CD4 antigens in cells in which we had induced the clustering of the antigens before fixation and fracture. In these specimens, CD4 antigens were again confined to the protoplasmic halves of plasma membranes, but the colloidal gold was clustered (Fig. 8a, b), often associated to cell projections (data not shown). In control experiments, we omitted the monoclonal antibodies from the immunolabeling procedure. These specimens showed virtually no labeling over protoplasmic or exoplasmic membrane halves.

Surprisingly, we were unable to observe the partition of CD3 antigens during fracture: no immunolabeling on both protoplasmic and exoplasmic faces was seen in all experiments, despite the clear surface labeling observed in unfractured cells.

Discussion

We used label-fracture to relate the patterns of distribution of CD3 and CD4 antigens on the surface of human T lymphocytes, before and after redistribution with monoclonal antibodies, to that of the intramembrane particles revealed by freeze-fracture. Label-fracture (and also thin-section electron microscopy) showed that, in resting human lymphocytes, the two antigens – both expressed on transmembrane proteins [1–3] – are uniformly distributed along the plane of the membrane. As in these experimentally undisturbed membranes the distribution of intramembrane particles is also uniform, it was not possible to relate the intramembrane particles revealed by fracture to the surface antigens. It was therefore necessary to cause experimentally the displacement of either of the antigens (through antibody mediated patching/capping) or of the intramembrane particles (through glycerol-induced co-aggregation).

Redistribution of CD3 and CD4 antigens, as induced by labeling with specific monoclonal antibodies at 37 °C, caused the migration of the CD3 and CD4 glycoproteins into clearly demarcated domains. These domains

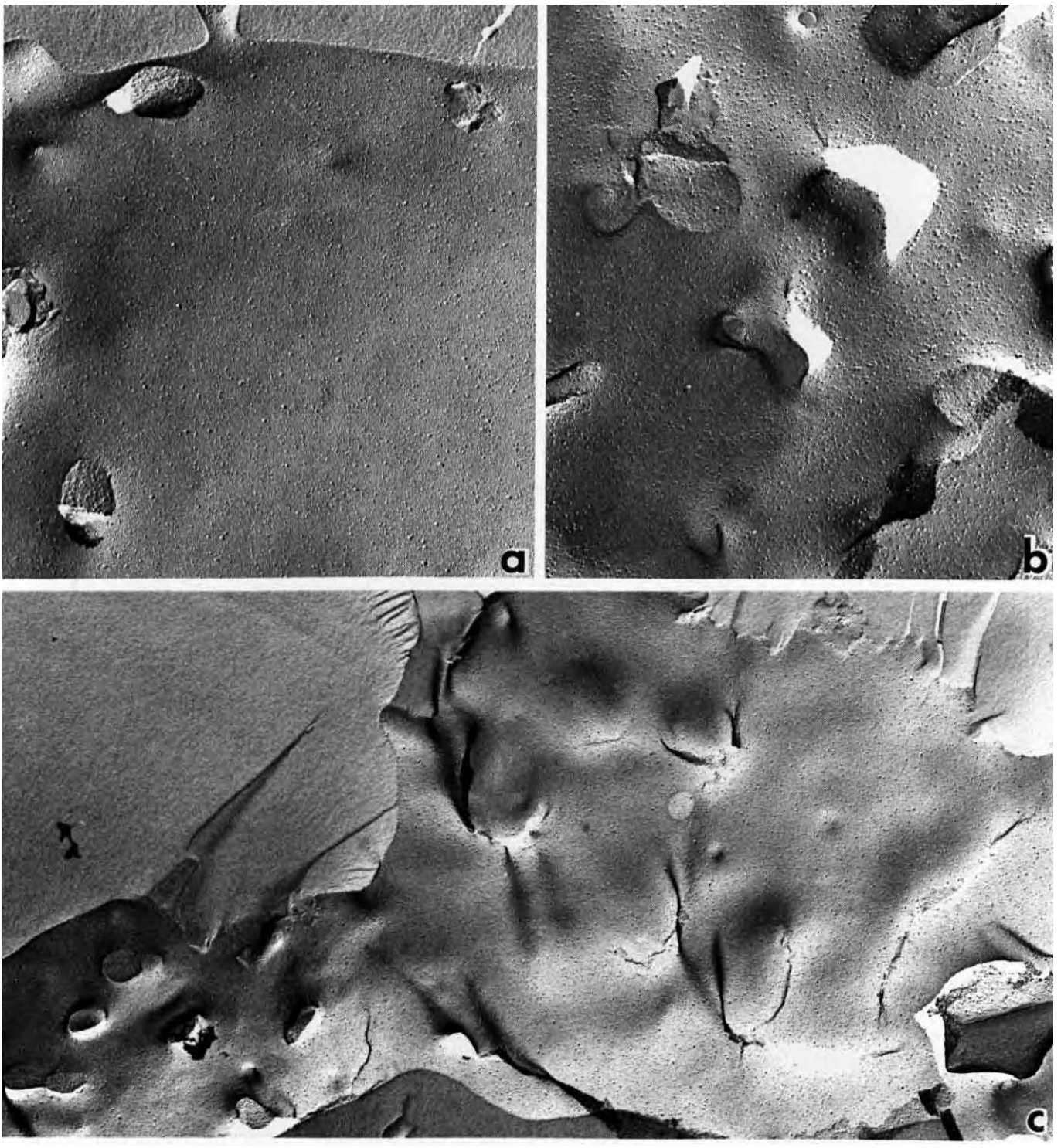

Fig. 5. Ultrastructure of E (a) and P (b) fracture faces of lymphocyte plasma membrane in conventional freeze-fracture images: no recognizable domains of intramembrane particles are apparent in either untreated (a, b) or OKT3-antibody-treated (c) lymphocytes. Magnification factors: (a) ×40960 (original×40000); (b) ×45776 (original×45000); (c) ×27465 (original×27000).

could not be related to intramembrane particles (regardless of their size or height). However, such relation became possible when, instead of altering the distribution of antigens, we altered that of the intramembrane particles by inducing their co-aggregation in PBS containing 30% glycerol (v/v). In these experiments, most CD3 and CD4 antigens were found over particulate domains of the membrane. Therefore, it is plausible that the absence of accumulation of intramembrane particles over patched or capped areas indicates that immigration of the antigen into the new domains is carried out contemporaneously with the emigration of in-

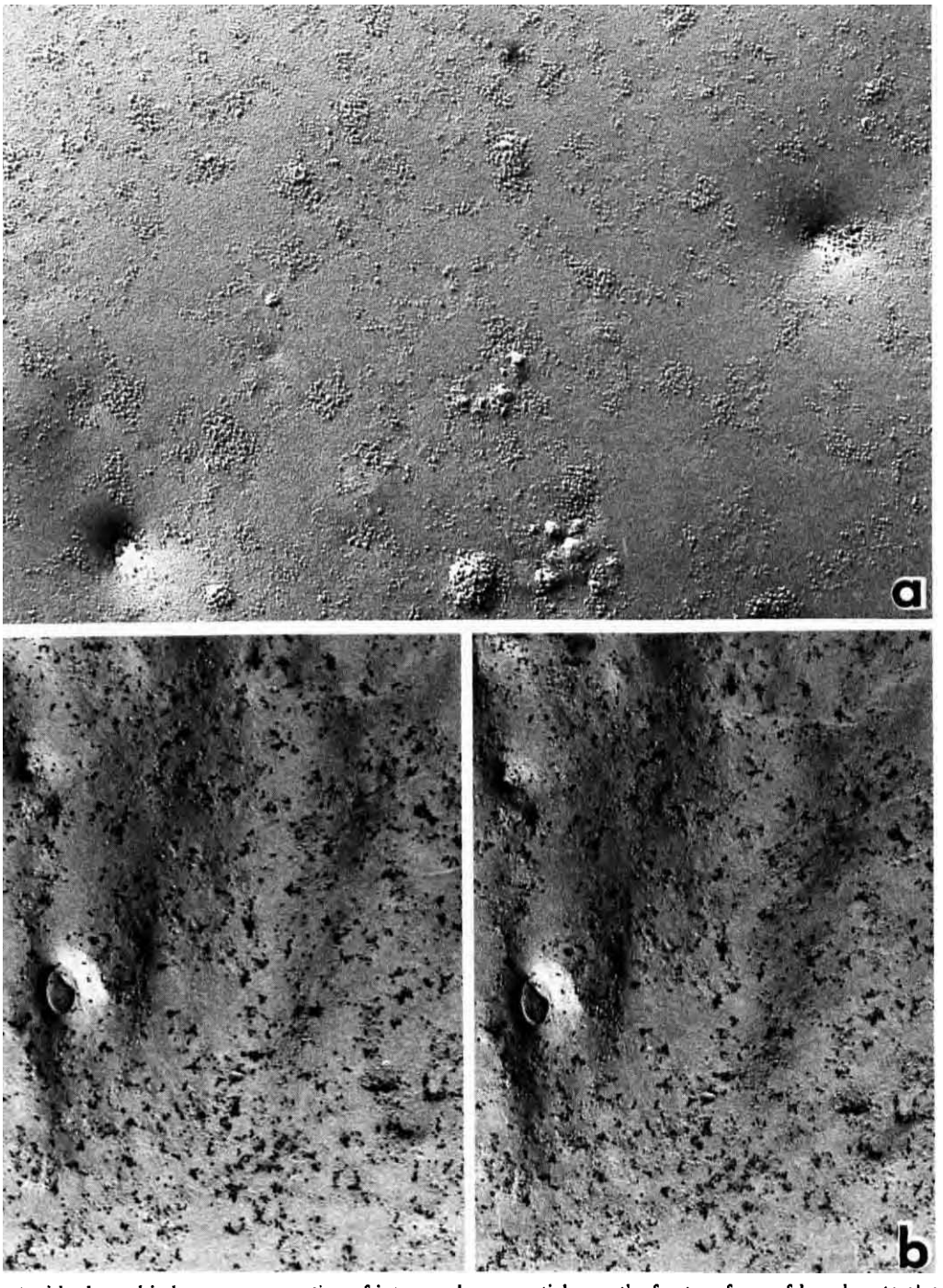

Fig. 6. (a) Treatment with glycerol induces co-aggregation of intramembrane particles on the fracture faces of lymphocyte plasma membranes. (b) Immunolabeling of CD4 antigens in a glycerol-treated cell reveals co-distribution of colloidal gold granules and intramembrane particles. Magnification factors: (a) ×63214 (original×45000); (b) ×42143 (original×30000).

tramembrane particles (other integral membrane proteins) away from the same areas. This indicates that even upon massive migration of one component into a restricted domain, the overall concentration of integral proteins along the plane of the membrane (as indicated by the density of intramembrane particles) does not appear to change so drastically that it leads to a noticeable alteration of the fracture face. In other words, the influx of the antigens into a domain appears to be contemporaneous with (and may even imply) efflux of the non-CD3/CD4 integral membrane proteins away from the CD3/CD4 cluster area, an expected outcome of planar diffusional equilibrium along the membrane continuum. A similar process is known to occur during budding of an enveloped virus from the infected cell: the viral envelope derives from the host-cell plasma membrane by interaction of the nucleocapsid with increasing amounts of viral proteins and contempora-

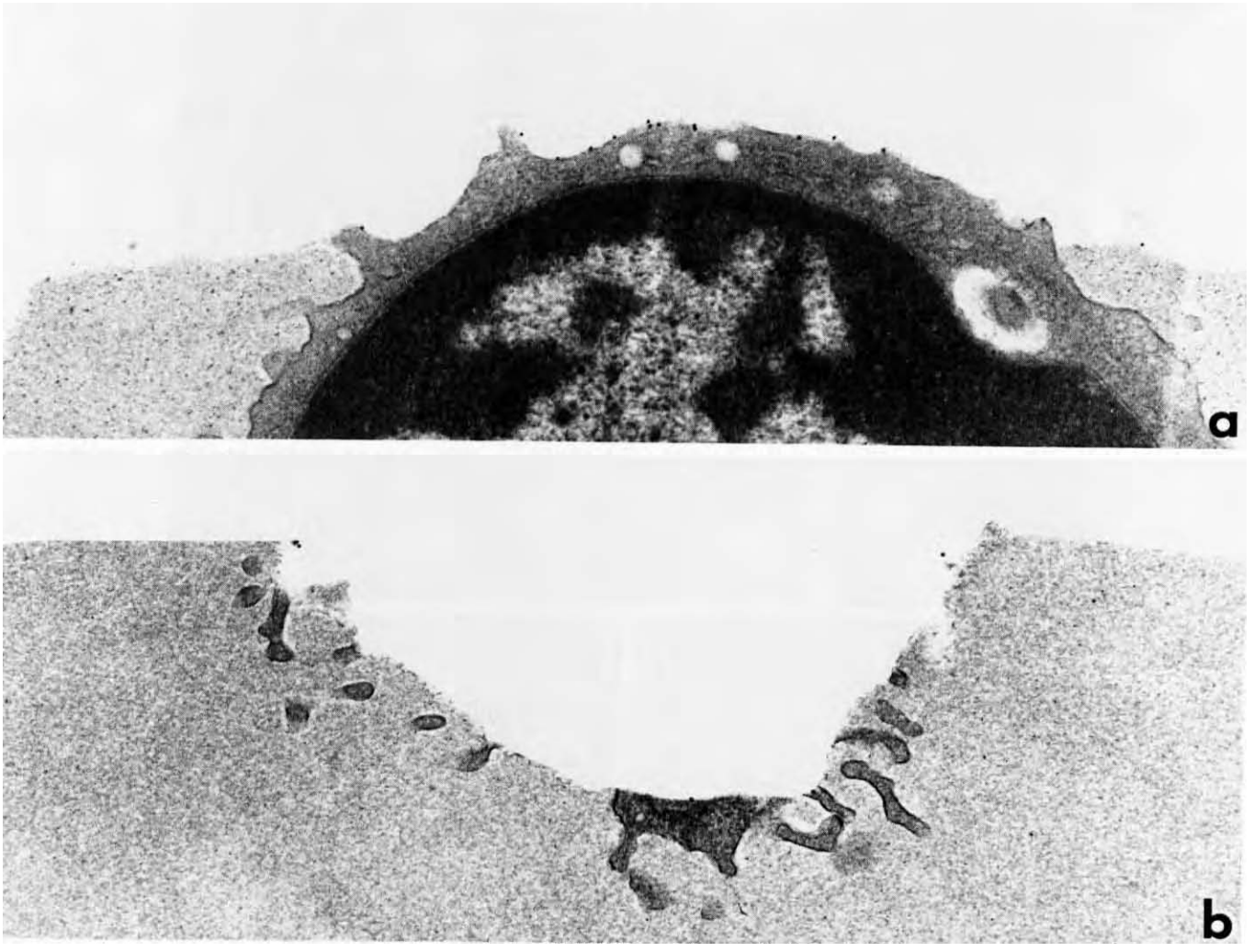

Fig. 7. Partition during fracture of CD4 antigens with the split membrane halves of plasma membranes in CD4-positive untreated lymphocytes: the immunolabeling is sparse over the protoplasmic half (a), whereas the exoplasmic half is virtually unlabeled (b). Magnification factors: (a) ×20701 (original×20000); (b) ×24842 (original×24000).

neous exclusion of the host-cell proteins [24,25]. Analogously, a concentration of surface receptors in coated pits during receptor-mediated endocytosis implies exclusion of other membrane proteins from the pits [22,23].

We used the fracture-label method to analyze the partition during fracture of CD3 and CD4 antigens with the two plasma membrane halves of human T lymphocytes. In fracture-label studies, where labeling is performed only after fracture-induced splitting of biomembranes, the transmembrane proteins can partition, as in conventional freeze-fracture, with protoplasmic and/or exoplasmic faces. However, we previously showed that surface labeling with cationized ferritin, performed before the freeze-fracture, modifies the partition of transmembrane proteins, anchoring them to the outer leaflet [21]. The preferential partition of CD4 antigens with the protoplasmic face in untreated, unlabeled prefixed CD4-positive cells indicates that the transmembrane protein expressing the CD4 antigen, despite its relatively short cytosolic tail [3], may interact with other membrane components or with cytoskeleton elements at the inner leaflet of the plasma membrane (see Ref. 9 for a discussion). Most of the transmembrane proteins studied by the fracture-label approach have been shown to partition with the outer leaflet of the plasma membrane in different cell systems, in particular, when the protein is composed of a large extracellular portion and a short cytosolic tail. In erythrocytes, for example, glycophorin partitions preferentially with the outer exoplasmic leaflet [20]. In contrast, band 3, which is strongly associated with the erythrocyte cytoskeleton, partitions preferentially with the inner, protoplasmic half of the erythrocyte membrane [19]. In Sindbis-virus-infected cells, the viral glycoproteins acquire protoplasmic partition only during the budding process of the virus, presumably because of the interaction with the nucleocapsid [26]. These findings demonstrate how interactions, more than structural

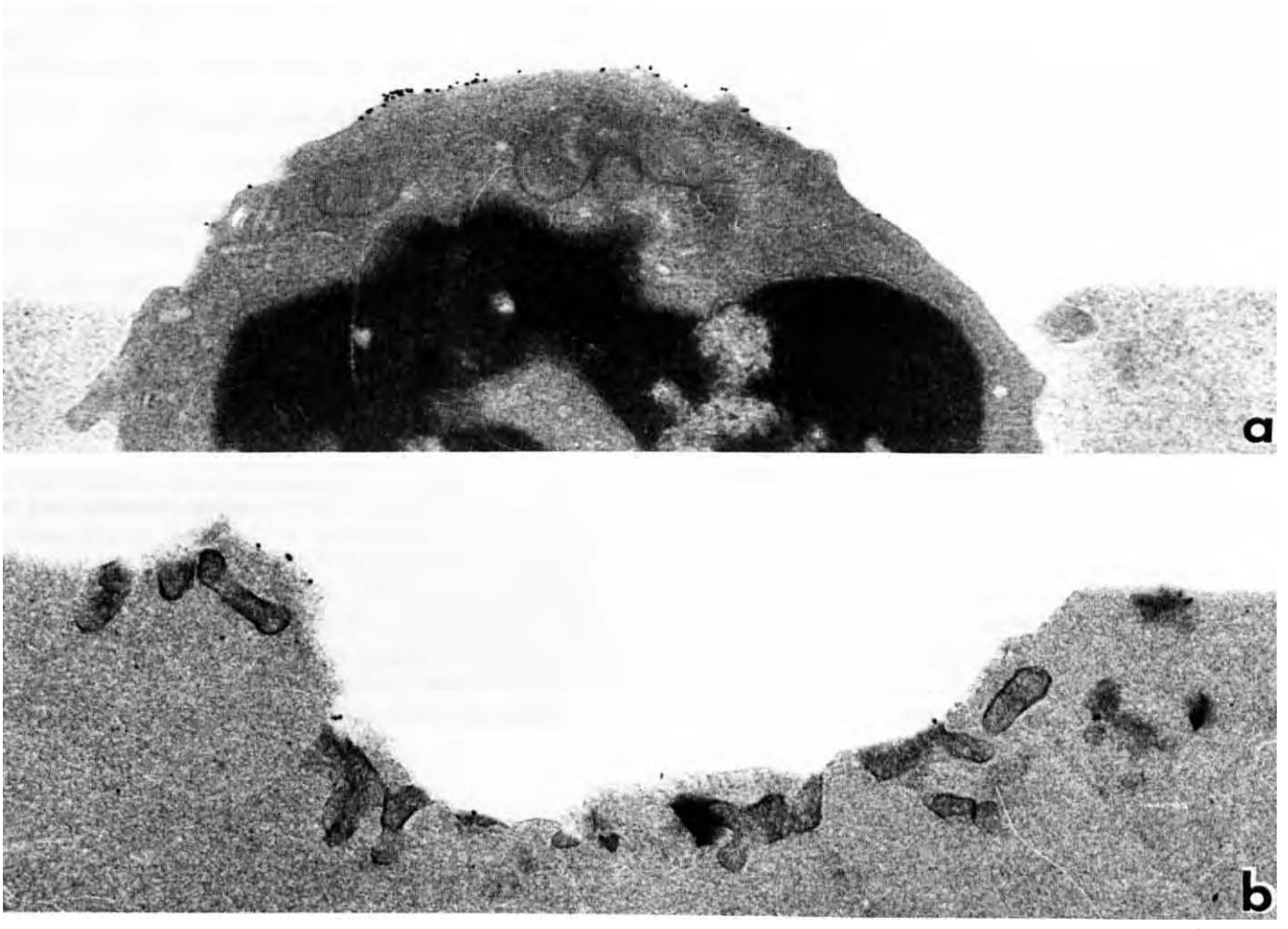

Fig. 8. Partition during fracture of CD4 antigens with the fracture faces of plasma membranes in CD4-positive cells pretreated with antibodies at 37°C to induce patching of the antigens: the immunolabeling is clustered over the protoplasmic half of the membrane (a); the exoplasmic half is virtually unlabeled (b). Magnification factors: (a) ×22640 (original×22000); (b) ×24698 (original×24000).

differences, appear to be responsible for partition, during fracture, of transmembrane proteins with the inner half of the membrane. In a cell population, such interactions may be heterogeneously expressed: in human T lymphocytes, fracture-label with the lectin wheat germ agglutinin shows heterogeneous expression of transmembrane sialoglycoproteins which partition with the protoplasmic faces of the plasma membrane [21], in agreement with the complex functions, as recognition, activation and signal transduction, operated by transmembrane proteins in these cells [5]. After clustering induced by antibodies, CD4 antigens still partition, on fracture, with the inner leaflet of the plasma membrane and appear clustered over protoplasmic faces. In this case, the preferential partition is clearer. This is interesting because clustering and capping are processes that depend upon cytoskeleton regulation [14,27,28]. We were unable to observe partition of CD3 antigens in our experiments, despite the dense immunolabeling observed over the surface of the unfractured cells, even after glutaraldehyde fixation. At present, we cannot account for this result, which might be a consequence of the complex structure of the CD3 molecule, which is not a single peptide chain, as CD4, but a group of three peptides, associated with two other chains of the T cell receptor molecule [1]. During fracture, this complex may be broken, losing its antigenicity or the epitopes recognized by the antibodies are, somehow, sterically inaccessible after fracture, or the freeze-fracture procedure may block the antibodies' ability to bind with their target antigens.

Acknowledgments

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