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# THE REDISTRIBUTION OF MEMBRANE SURFACE IMMUNOGLOBULIN INDUCES THE REARRANGEMENT OF SOME MEMBRANE INTEGRAL PROTEINS

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## Summary

We investigated whether the redistribution of surface membrane receptors is associated with rearrangement of integral membrane proteins. Using a newly developed process, which combines histochemical analysis with an immunofluorescence or immuno-electron microscopy-staining technique, we studied the redistribution of two membrane-bound enzymes, 5′-nucleotidase and ATPase, on mouse splenic lymphocytes and B lymphoma cells induced by anti-mouse immunoglobulin antibodies. Labeling and capping of the membrane surface immunoglobulin induced a similar rearrangement of both 5′-nucleotidase and ATPase from uniform distribution at 4°C into 'patches' and caps at 37°C.

The modulation and redistribution of cell membrane components following interaction with multivalent ligands such as antibodies or lectins (capping) have been used as tools to further our understanding of the topographical distribution and lateral mobility of receptors in the membrane plane and possibly of the mechanism of signal transduction across membranes. Capping of surface macromolecules is the end result of an energy-dependent chain reaction. This process begins with the binding of a ligand to an appropriate surface receptor, followed by its aggregation into 'clusters' and 'patches' which are finally collected into one pole of the cell [1,2].

Although the capping phenomenon has been studied extensively, its exact mechanisms are unclear and controversial [1—8]. One specific question, which remains unanswered, deals with the effects of the redistribution of one membrane component on other adjacent membrane components. Most attention has been focused on the interaction of membrane proteins with the cytoskeletal elements collected at the cytoplasmic phase of the cell membrane [9—12]. Considerably less effort has been given to the aspect of redistribution and signal (information) transduction within the membrane plane. Therefore, we wished to determine whether the redistribution of surface immunoglobulin (Ig) molecules brought about by the binding of specific antibodies could affect the organization of two unrelated membrane integral proteins, i.e., 5′-nucleotidase and $Mg^{2+}$-ATPase. Surface Ig is a protein which is strongly associated with the plasma membrane surface and functions in recognition of antigen [13].

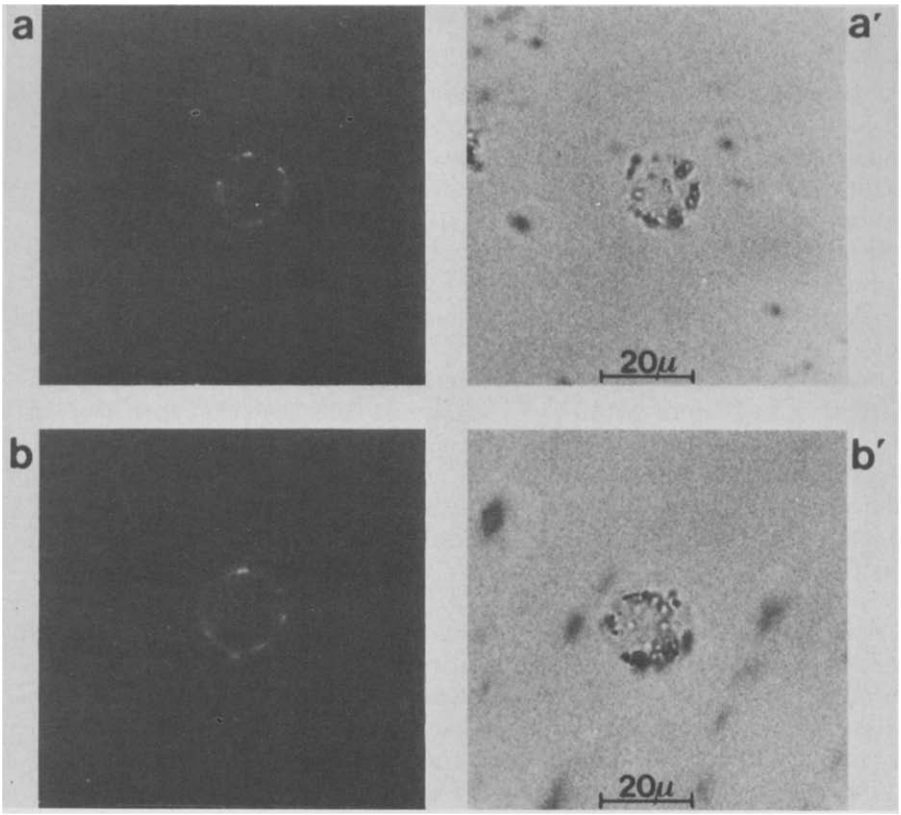
Spleen lymphocytes were harvested from BALB/c strain mice and purified by Ficoll-Hypaque centrifugation. 100 μg/ml of either fluorescein- or ferritin-conjugated rabbit anti-mouse Ig antibodies (Miles, Yeda, Rehovot, Israel) were added to $5 \cdot 10^6$ cells/ml in Hank's balanced salt solution for 30 min at 4°C. After being washed three times, the cells were reincubated for 5 min at either 37 or 4°C: washing and fixation with 1% formaldehyde in 155 mM NaCl for 5 min at 4°C terminated the reaction. After the washes, the cells were incubated for 20 min at 37°C with freshly-filtered histochemical media according to the method of Gomori [14]. Histochemical media contained either (a) 80 mM Tris-malate (pH 7.2), 10 mM $MgCl_2$, 1 mM ATP and 3.6 mM $Pb(NO_3)_2$ (for ATPase visualization) or (b) 100 mM Tris-malate (pH 8.5), 10 mM 5′-AMP and 3.6 mM $Pb(NO_3)_2$ (for 5′-nucleotidase visualization). The cells were then thoroughly washed in saline to remove traces of free phosphate and processed for either fluorescence microscopy or for transmission electron microscopy. For fluorescence visualization, the cells were exposed to 1% ammonium sulfide for 1 min (24°C) and washed with saline. The cells were examined and photographed with a Zeiss microscope equipped with epifluorescent illumination and filter setting for both bright field and fluorescein fluorescence. For transmission electron microscopy visualization, the cells were refixed with 2.5% glutaraldehyde in 0.1 M sodiumcacodylate buffer, pH 7.4 (60 min, 4°C) and post-fixed with 1% $OsO_4$ in the same buffer (60 min, 4°C). Cells were stained en bloc with uranyl acetate, dehydrated with a graded series of ethanol, infiltrated and embedded in Spurr's low-viscosity medium. Thin sections were examined without further counterstaining in a Hitachi-HU-12A electron microscope.

Incubation of spleen lymphocytes with fluorescein-anti-Ig at 4°C, followed by fixation and histochemical staining, produced an intense ring-like or slightly patchy distribution of the fluorescein (Fig. 1a and b) as well as the lead sulfide deposits (Fig. 1a′ and b′). The formaldehyde fixation was sufficient for immobilization of the Ig molecules, since no difference could be observed in the fluorescence distribution when fixed cells reincubated at 37°C (during the histochemical reaction) were compared to those incubated

under the same conditions at 4°C, the temperature at which enzyme activities are inhibited. Temperature elevation to 37°C prior to fixation resulted in a redistribution of the fluorescent staining into a single cap in 89% of the stained cells (Fig. 1c and d) at the same time the enzyme products were located in a distinct zone on the cell surface (Fig. 1c′ and d′). Examination of control cells, which were incubated under the same conditions (37°C) but without the antibody, did not reveal any alteration in the enzyme distribution (histochemical staining) as compared to cells incubated at 4°C. This finding rules out the possibility that temperature elevation (37°C) per se was responsible for the localization or rearrangement of the two membrane-associated enzymes. The localization pattern of the membrane enzyme product could be easily distinguished from the pattern of the formed Ig cap (Fig. 1c and 1c′). In approx. 50% of the capped cells the two types of stain coincided at the same pole. Formation of two distinct lead sulfide precipitates was observed in approx. 17% of the cells, one in the fluorescein Ig cap zone and the other in the opposite pole. The remainder of the cells (33%) exhibited an ill-defined zone of lead sulfide deposits relative to Ig fluorescence cap.

In order to rule out the possibility of cross-reaction between the anti-mouse Ig and the membrane enzyme, the antisera were absorbed with mouse red blood cells twice. No fluorescent erythrocyte could be detected in either absorption cycle. The absorbed and unabsorbed sera yielded identical results.

The conversion of the enzymatic product, lead phosphate, produced large aggregates of lead sulfide that can be visualized in bright field microscopy. Therefore, it was possible that the rearrangement of this enzyme product stain in Ig-capped cells could have been due to an experimental artifact. In order to rule out this possibility and to examine more directly the relative arrangement of 5′-nucleotidase and ATPase to the ferritin-labeled anti-Ig on the cell surface, we next examined the cells by immunoelectron microscopy. In cells incubated at 4°C (20 min) the ferritin label was distributed uniformly over the entire cell surface either as single particles or as small clusters (Fig. 2a). In the same cell samples which were treated for enzyme visualization, a broad continuous layer of the ATPase product was visualized on the external cell surface (Fig. 2b). The lead phosphate precipitate masked completely the ferritin molecules due to similarity in the electron density of the lead and the ferritin. Incubation of cells with ferritin-anti-Ig at 4°C (20 min) followed by a 5-min incubation at 37°C induced morphological alteration of the labeled cells (Fig. 2c). A polar orientation of the cytoplasm was apparent with an accumulation of the ferritin label over this pole. The rest of the cell surface was unlabeled. Fig. 2d—f represents examples of cells incubated as above followed by ATPase histochemical staining. The ferritin label cannot be visualized due to masking by lead phosphate precipitate: however, ferritin-labeled endocytosed vesicles were observed in the cap zone (Fig. 2c and d). In Fig. 2d, a cell is shown with a co-capping of the enzyme with the Ig molecules. Few, if any, enzyme product deposits are seen elsewhere. In another cell sample (Fig. 2e) one large cluster of lead phosphate can be seen in the cap zone accompanied by smaller but separate




clusters over the entire surface of the cells. Fig. 2f shows a cell with two separate large clusters of the enzyme product. Lead phosphate deposits are often seen in the cells' mitochondria. Because of formaldehyde fixation, the cells become permeable to the substrate, which is then also available for enzymatic degradation by mitochondrial ATPase. No alteration in the localization of the enzyme product could be observed in Ig-negative cells.

To further correlate the movement of surface immunoglobulins and the membrane enzymes, we studied the phenomenon in a homogeneous population of B cells, B-lymphoma W-279 (obtained from Dr. N.L. Warner) The results clearly demonstrated that, following 10 min of incubation at 4°C, both the fluorescence and the ATPase product were distributed in a ring-like manner over the entire cell surface. Temperature elevation to 37°C for 10 min induced redistribution of fluorescent staining into a single cap in approx. 49% of the cells which was accompanied by the rearrangement of the lead sulfide precipitate (data not shown).

The staining technique described here enabled us to follow the effect

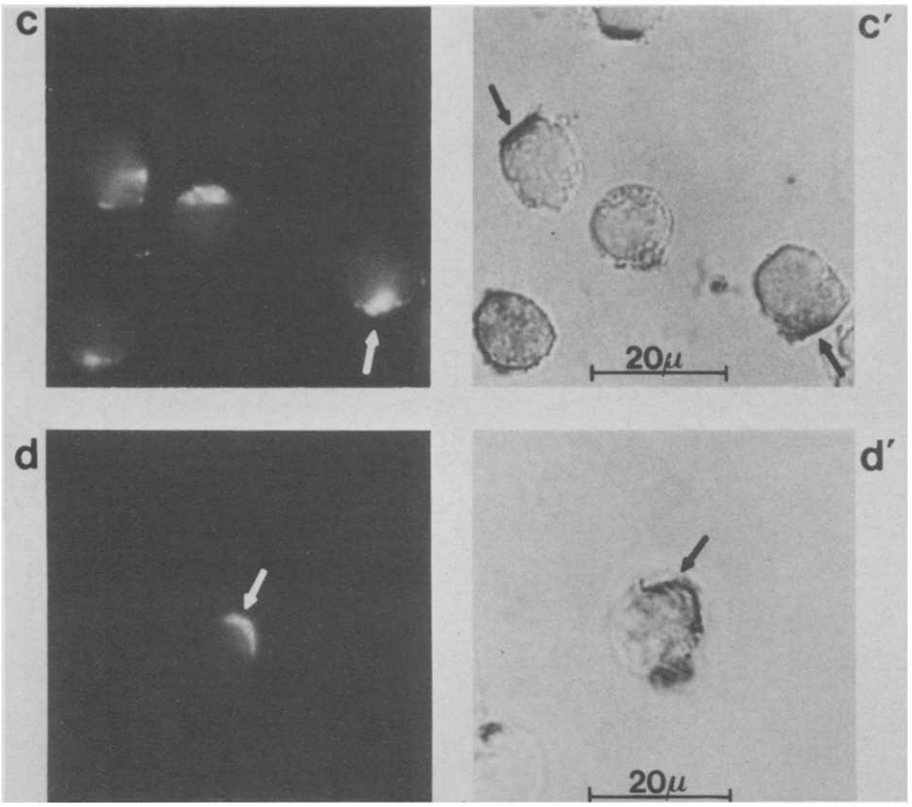
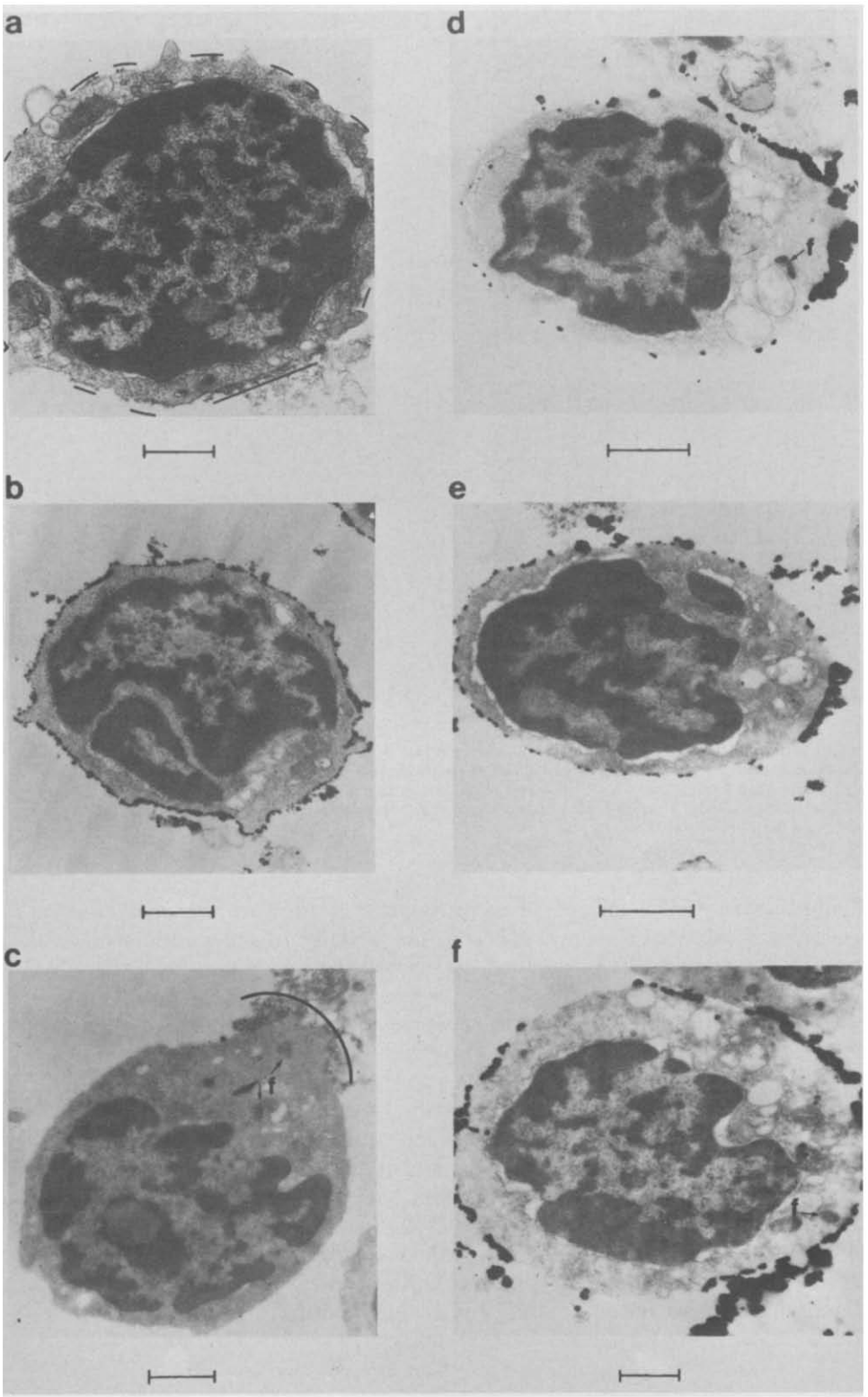



Fig. 1. Fluorescence (a—d) and histochemical staining (a'—d') of mouse splenic lymphocytes labeled with fluorescein-anti-Ig. The same primed and non-primed letters represent identical photographic fields. Cells were incubated at 4° C, followed by 5'-nucleotidase (a,a') and ATPase (b,b') histochemical staining: (c,c') and (d,d') are the same as (a,a') and (b,b'), respectively, followed by 5 min incubation at 37° C. Arrows mark cap zone.

of capping of specific cell receptors on the distribution of two unrelated molecules, 5'-nucleotidase and ATPase. The activity of several membrane enzymes is known to be influenced by the interaction of various ligands with membrane glycoproteins. The molecular basis for this phenomenon is still unclear. It is possible that the rearrangement of 5'-nucleotidase and ATPase following redistribution of the cross-linked peripheral proteins might contribute to the known changes in their activities [15—19].

The relative arrangement or migration of one membrane molecule to another following their cross-linking might determine their mutual physical association in the plasma membrane. Thus, $\beta_2$-microglobulin and HL-A antigen on the human lymphocyte surface are thought to be in the same 'mobile unit' [20], whereas H-2K and H-2D antigens on mouse lymphocytes are located separately [21]. However, the capping of different independent cell surface antigens has been shown to induce co-capping of the H-2 and related antigens, with no apparent physical association between the two groups of antigens [22].


a
d
b
e
c
f

Our present study clearly demonstrates that capping of specific membrane receptors can induce the rearrangement of apparently unrelated membrane molecules. The data, based on the two tested markers, imply that co-capping or simultaneous rearrangement of membrane macromolecules cannot always serve as a reliable marker for a single defined mobile unit. Moreover, the rearrangement of such membrane enzymes suggests that during capping of membrane receptors an overall change in the membrane organization occurs which may be essential for signal transduction in and across cell membranes.

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Fig. 2. Electron micrographs of spleen lymphocytes treated with ferritin-anti-Ig. (a) Cells incubated at 4°C; black lines around the cells indicate areas of ferritin molecules. (b) The same as (a), followed by ATPase staining; the ferritin molecules cannot be identified due to lead phosphate masking. The cells in (c—f) were incubated for 5 min at 37°C. (c) Ferritin is located over one pole of the cells; the remainder of the cell surface is unlabeled. (d—f) The same as above followed by ATPase histochemistry. Note that the lead phosphate deposits could either precipitate at the Ig cap zone (d) or at the cap role but with additional deposits (e,f); note the internalized ferritin in the cell cytoplasm (f). Each bar represents 1 μm.