NONUNIFORM DISTRIBUTION OF CONCANAVALIN-A RECEPTORS AND SURFACE ANTIGENS ON UROPOD-FORMING THYMOCYTES

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ABSTRACT

Uropods can form spontaneously in a variable fraction of mouse thymocytes incubated for 30-60 min in vitro at temperatures between about 8°C and 37°C. The majority of the cells with a typical uropod are medium and large thymocytes. The "normal" distribution of concanavalin-A receptors and antigens recognized by a rabbit anti-mouse thymocyte serum was studied on these cells by electron microscopy using ferritin-conjugated lectin or antibodies. The cells were fixed with glutaraldehyde or formaldehyde before labeling. The distribution was essentially uniform on spherical cells. On the contrary, on cells which had formed a uropod the labeled receptors and antigens appeared to be preferentially concentrated around the nucleus, and depleted over the uropod, and especially over the constriction at the base of the uropod. Uropod formation and inhomogeneous distribution were inhibited or reversed by cytochalasin B, but not by vinblastine or colchicine. When the same ligands were applied to unfixed cells, the labeled and cross-linked components capped normally towards the cytoplasmic pole of the cell. These observations are described in relation to the ability of receptors and antigens to interact with an intracellular mechanical structure, and to the mechanism of capping.

KEY WORDS concanavalin-A receptors · thymocyte · surface antigens · spontaneous redistribution · immunoferitin · capping · uropod

The concept that the plasma membrane is fluid and that its components are mobile in its plane is now supported by considerable experimental evidence (reviewed in references 7, 19, 26). Theoretical reasons, as well as experimental observations, suggest, however, that the mobility of at least some of these components is restricted, or, in general, controlled by interactions with cytoplasmic structures (3, 11, 12, 19, 21, 31). Interactions of this type, for example, probably take place during the process of ligand-induced capping (11, 12, 23), but are expected to occur also in the absence of external ligands. Thus, for example, nonuniform interactions probably take place on the surface of isolated cells, when the spherical shape of the cell is altered, or during cell movement. If the interactions extend across the membrane, affecting outer surface proteins, they could alter the distribution or the properties of these proteins nonuniformly over the surface. The recent demonstration that membrane immunoglobulins redistribute spontaneously on spleen lymphocytes during cell movement is consistent with this concept (24).

Electron microscopical observations probably corresponding to one of such events were briefly

J. Cell Biology © The Rockefeller University Press · 0021-9525/78/1001-0235$1.00
Volume 79 October 1978 235-251
described in a previous report on the distribution of Theta (θ; Thy.1 according to the present terminology) antigen on the surface of mouse thy-
mocytes labeled with mouse anti-θ alloantibodies
followed by a rabbit anti-mouse Ig-ferritin conjugate (13). The distribution of the θ-antigen-anti-θ
complexes observed in cells prefixed with glutaraldehyde after the addition of the alloantibody,
but before the addition of the conjugate, was
found to be generally uniform on spherical cells,
but not uniform on a minority of cells which had
formed a uropod during incubation. On these
cells, the antigen was concentrated at the nuclear
pole of the cell, i.e., at a site opposite to that at
which it normally accumulates during capping
(29). As the cells had been labeled with anti-θ
antibody at room temperature, we were unable,
however, to exclude the possibility that both the
formation of the uropod and the inhomogeneity
in the distribution had been secondarily induced
by the binding of the antibody. This seemed to be
unlikely, however, considering that antibody was
unable to cap and patch the θ antigen, probably
because the latter was virtually monovalent with
respect to the antiserum (13). To rule out that
possibility, and to determine whether the phenom-
enon was more general, I have extended this study
to the distribution of concanavalin-A (Con-A)
receptors, and of antigens recognized by a rabbit
“anti-lymphocyte” antiserum (16) directed against
mouse thymocytes (hereafter referred to as “ALS-
antigens”). The choice of the Con-A receptors for
this type of study presents the drawback that these
receptors (like the ALS antigens) probably corre-
spond to a heterogeneous population of surface
molecules which may not behave identically, blur-
ing possible differences in the distribution of the
various subpopulations. It has the advantage,
however, that the cells can be pre-fixed with
 glutaraldehyde before the application of the label
(in this case, a Con A-ferritin conjugate), thus
insuring that the observed pattern corresponds to
that preexisting to the binding of the label. Simi-
larly, the anti-lymphocyte serum (ALS) is still
able to react with surface antigens after fixation
with 4% formaldehyde.

The results have confirmed the previous obser-
vations. They have indicated that uropods can
form spontaneously in vitro in a fraction of cells
and that on these cells the distribution of Con-A
receptors and ALS-antigens is inhomogeneous
and resembles that observed with the θ antigen.
The effect of various drugs (colchicine, vinblas-
tine, cytochalasin B) on uropod formation and
receptor distribution suggests that both phenom-
enal are mainly dependent on the action of “micro-
filament” structures, and are essentially independ-
ent of the presence of microtubules.

MATERIALS AND METHODS

Light microscopy

Cells isolated from thymuses of 5-7-week-old C3H/
HeJ and C57 black mice were washed at 0-4°C, twice
in Dulbecco’s physiological saline (PBS) containing
0.2% bovine serum albumin (BSA), and once in Leibov-
itz’s medium L15 (Flow Laboratories, Inc., Irvine, Scot-
land) containing 0.25% BSA (L15/BSA). They were
resuspended in the same medium at a concentration
of 2 x 10⁶ cells/ml and kept on ice until used. To induce
formation of uropods, 0.1- or 0.2-ml cell samples distrib-
uted in plastic or glass test tubes were warmed in a water
bath to the temperature of the experiment, and incu-
bated for 30-60 min, or as otherwise indicated. Incuba-
tion was stopped by addition of an equal volume of 4-6%
phosphate-buffered glutaraldehyde at the same tem-
perature. In some cases (cf. Table II), the cells were
preincubated for 30-60 min at 37°C before the final
incubation at the selected temperature. Alternatively,
1.4-2.0-ml aliquots of a suspension of 5 x 10⁶ cells/ml
were incubated in 35-mm Falcon plastic tissue culture
dishes (Becton Dickinson AG, Basel, Switzerland). In-
cubation was stopped by careful and gradual addition of
an equal volume of 6% phosphate-buffered glutaralde-
hyde. Under these conditions all cells were in contact
with the plastic substrate, and after fixation virtually
all of them remained attached with unaltered shape to
the bottom of the dish (probably to a layer of adsorbed
BSA). Attachment was firmer at 37°C than at lower
temperatures, and at 37°C the cells could be displaced
only by vigorous pipetting.

Fixed cell suspensions or fixed cells attached to Petri
dishes were examined and photographed under a cover
slip in bright field, using a Leitz Orthoplan microscope
with a 40x dry objective, or a 100x oil immersion
objective. Cells on Petri dishes were also examined
before, during, or after fixation with an inverted micro-
scope and phase optics. For quantitative estimates, the
number of nonspherical cells with either a “typical”
uropod or a marked constriction (see Results) were
counted. To obtain statistically significant percentages,
300-700 cells were counted for each sample.

Electron Microscopy

Incubation conditions for electron microscopy were

Abbreviations used in this paper: ALS, rabbit anti-
mouse lymphocyte serum; BSA, bovine serum albumin;
Con A, concanavalin A; GaRlg, goat anti-rabbit Ig
antibody; Ig, immunoglobulin; PBS, phosphate-buffered
saline.
identical to those used for light microscopy except for the larger size of the samples (25-40 × 10⁶ cells). Various samples were incubated in test tubes at 14°, 24°, and 37°C, or on flat surfaces (Falcon tissue culture dishes or bottles) at 24° and at 37°C. To label Con A receptors, the cells were fixed as described above for 1–2 h with 3% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.4, or, occasionally, for 2 h with freshly prepared 4% formaldehyde in the same buffer. Small aliquots from each sample were taken for light microscopic examination. The cells were exhaustively washed for 2 h or overnight, with three centrifugations in 0.9% NaCl, and incubated for 30–60 min with a Con A-ferritin conjugate (prepared using glutaraldehyde as a cross-linking agent, according to a described procedure [13]). Several conjugates, at different dilutions, gave essentially the same results. Con-A labeling was completely abolished, however, if incubation was carried out in the presence of 0.1 M α-methyl-mannoside. The cells were washed by repeated centrifugations, or by centrifugation through a 5% BSA layer in PBS. In some experiments, the cells were washed with buffer containing 0.075 M NH₄Cl (to block free aldehyde groups), and 0.010 M NH₄Cl was added to the incubation mixture containing the Con A-ferritin conjugate. No difference in the results was noted. After washing, the final pellets were postfixed with cold 1% OsO₄ (1–2 h) in veronal-acetate buffer. They were washed with water, block-stained with 0.5% uranyl-acetate in water (40–60 min), dehydrated in alcohol, and embedded in a mixture of 50% Araldite-50% Epon, or in Epon alone. Thin sections were examined either unstained or stained with 5% uranyl acetate in water and 0.1% lead citrate dissolved in freshly made 0.02 M NaOH.

For labeling experiments with anti-lymphocyte serum (ALS), the cells were preincubated as for the labeling with Con A. They were pre-fixed with 4% formaldehyde in 0.12 M phosphate buffer for 45 min, washed as above, and incubated with ALS diluted 1:50 with PBS for 30 min at room temperature. The ALS antiserum unabsorbed, but heated for 30 min at 56°C to inactivate complement, was a gift from Miss Luciana Forni, and was prepared by three intravenous injections of 10⁹ mouse thymocytes in rabbit according to the procedure of Levey and Medawar (16). The cells were washed three times by centrifugation, resuspended in 150 μl of a goat anti-rabbit immunoglobulin (Ig)-ferritin conjugate (GaRlg-ferritin), and incubated for 30 min at room temperature. The conjugate was prepared and tested according to described procedures (11). The cells were separated from the excess conjugate by centrifugation through a 5% BSA layer, resuspended in 2 ml of PBS, fixed with glutaraldehyde, and processed as above.

For ligand-induced redistribution experiments, unfixed cells (preincubated, or not, at 24°C) were incubated at 24°C with Con A-ferritin, or with ALS and GaRlg-ferritin, as indicated above, washed through a BSA layer, and then fixed; alternatively, in the case of Con A-ferritin labeling experiments, the cell suspension was fixed without washing by addition of 0.3% glutaraldehyde (final concentration), and immediately spun through a layer of 3% glutaraldehyde in phosphate buffer (excess unbound label remains on top of the layer) to avoid changes in cell shape due to agglutination in the pellet.

Drugs
Vinblastine sulfate (Ely Lilly & Co., Indianapolis, Ind.) and colchicine (Fluka AG, Buchs, Switzerland) 1 mM solutions in PBS/BSA were prepared fresh before use. Cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was dissolved in dimethyl sulfoxide (DMSO). A solution of 100 μg/ml (1.25% in DMSO) in PBS/BSA was made from this stock solution (BSA increases cytochalasin B solubility at low DMSO concentrations), and used for further dilution with L15/BSA.

RESULTS

Light Microscopy

SPONTANEOUS UROPOD FORMATION IN THYMOCYTES: Uropods can develop spontaneously on thymocytes in vitro under various experimental conditions. When thymocytes were incubated for 30–60 min in vitro at any temperature between 8°–10°C and 37°C under the conditions described in Materials and Methods, a variable proportion of cells (between 5 and 15%) became nonspherical, and many of these formed spontaneously a uropod (Fig. 1a and b). In cells isolated in the cold, uropods started to develop after 10–15 min (at 24°C) and reached a maximum after 30–60 min.

The cells with uropod did not represent an average sample of the total population, but corresponded prevalently (20–40%) of the nonspherical cells below 20°C, 50–80% between 20°C and 37°C) to the subpopulation of large and medium thymocytes (with diameter of the fixed cells ≥ 8 μm), which, in the animals used in these experiments, constituted −10–20% of all thymocytes. The typical appearance of these uropod-cells under the light microscope is shown in Fig. 1a and b. The uropod usually appeared as a narrow, cylindrical or conical protrusion, 2–5 μm long, emerging from a roughly spherical body (hereafter also referred to as the “head” of the cell) which is largely occupied by the nucleus (see Figs. 4 and 14). In cells freely rotating in the fluid, the uropod appeared as a relatively stable and rigid structure. We shall refer to these cylindrical or conical uropods as “typical” uropods. Most of the cells with a uropod below 30°C and ~50% of those at 37°C presented these characteristics. At 37°C,
FIGURE 1 Cells with uropods and other nonspherical shapes observed under the conditions described in the text. (a and b) "Typical" uropods formed at 24°C on large and medium thymocytes. On the slide, the uropods point sideways or upwards. (c) "typical" uropod and elongated cell, probably a moving cell, at 37°C. (d) Two small and one large lymphocyte with midbody constriction at 14°C. (a) × 550; (b–d) × 800.

other nonspherical cells had broader uropods or irregular shapes (Fig. 1c). The latter probably correspond to moving cells. Most of the following electron microscopical observations refer to cells with "typical" uropods, which could be identified unambiguously in thin sections. Only relatively few of the small lymphocytes had "typical" uropods. The majority of the small lymphocytes with nonspherical shape had a central constriction which divided them into two approximately spherical parts (Fig. 1d; cf. Fig. 11).

Uropods developed either in test tubes or on flat plastic surfaces. On the latter, their formation was somewhat enhanced, especially at 37°C (although contact with the substrate was not critical, as typical uropods developed also in cells isolated as described and kept in suspension under continuous stirring for 30–60 min at 24°C). In cells incubated in Petri dishes, practically all cells with a typical uropod at any of the temperatures tested (10°C–37°C) appeared to be attached to the substrate. Unexpectedly, however, when these cells were examined before or after fixation without disturbing their contact with the substrate, they appeared to be oriented vertically or obliquely with respect to the latter and attached to it by the tip of the uropod (Fig. 2). This peculiar type of attachment resembles that of lymphocytes in contact with one another, or with other cells (e.g., macrophages) (5, 18, 27). The "head" of the cell did not appear to be in contact with the plastic. Some of the small lymphocytes with a mid-body constriction appeared to have the same orientation. The cells with typical uropod did not appear to move on the substrate. Continuous observation near room temperature (22°C–25°C) showed that the cells did not move or change appreciably in shape for as long as 40–60 min. The behavior of these "mushroomlike" cells (Fig. 2) contrasted with that of the other cells with a simple constriction, or with that of many of the cells with a thicker uropod or irregular shape. The latter cells lay horizontally on the substrate and in many cases were actually seen to be moving over it.

EFFECT OF DRUGS ON UROPOD FORMATION: The effects of drugs were tested at 24°C and 37°C. 12–20 mM of sodium azide severely inhibited the formation of uropods (Table I) (although azide induced other independent morphological alterations, such as formation of microvilli, to be discussed elsewhere).

Cytochalasin B at doses of 3–15 µg/ml inhibited uropod formation virtually completely (Table II), or caused, in 10–20 min, previously formed uropods to revert. The cells became perfectly spherical (only a very small protuberance was observed occasionally on some of them) (Fig. 3a).

Microtubule-disrupting drugs (2 × 10⁻⁵ or 1 × 10⁻⁶ M colchicine; 10⁻⁶ M vinblastine) did not inhibit formation of uropods and constrictions but somewhat enhanced it (Table II; Fig. 3b). The uropods were somewhat shorter and rounder. In Petri dishes, many of them were present on "mushroomlike" cells as in the absence of the drugs.

DISTRIBUTION OF CON-A RECEPTOR ON PREFIXED THYMOCYTES: The "undisturbed" distribution of Con A receptors was examined on nine thymocyte samples incubated for 30–60 min at 14°C, 24°C, or 37°C. Similar observations were
Figure 2. Appearance of glutaraldehyde-fixed cells with typical uropods still attached to the bottom of a Petri dish and photographed with the microscope focused at different distances from the plastic surface. (a) Plane of focus near the surface and the tip of the uropod (the head of the cell is completely out of focus). (b) The same, ~2 μm above the plane of a. (c) The same, ~4 μm above the plane of a (the edge of the "head" is in focus, the uropod out of focus). Note that the edge is not perfectly smooth, and that "stress lines" (?) are present in b in the transition area (constriction). (d and e) Group of cells with typical uropods as in a: in d they are focused close to the surface and to the tip of the uropod, in e at the level of the "head". One vertically oriented small lymphocyte with constriction is also visible in this section (arrow). × 800.

Table I

| Inhibition of Uropod Formation by Sodium Azide and Cytochalasin B* |
|-----------------|-----------------|-----------------|
|                 | t = 5 min       | t = 45 min      |
|-----------------|-----------------|-----------------|
| A. Control      | 2.4             | 10.1            |
| Sodium azide (20 mM) | 3.0             | 2.5†             |
| B. Control (no DMSO) | 3.2             | 10.5            |
| Control (0.125% DMSO) | 3.0             | 11.1§            |
| Cytochalasin B† | 0.2             | 0.6             |

* Percentage of cells with uropod or constriction. Cells in PBS/BSA (for azide inhibition) and in L15/BSA (for cytochalasin inhibition) incubated at 24°C for the times indicated. The drugs were added at the beginning of incubation (t = 0).

† Irregular nonspherical shapes. Percentage significantly different from controls (P < 0.001, normal deviate z test).

§ Not significantly different from the control without DMSO (P = 0.67).

Cytochalasin B (0.25% DMSO) added at time t = 0. Control and experimental percentages after 15 min not significantly different (P = 0.20), but significantly different from those at 0-5 min (P < 0.02%).

Table II

| Uropod Formation at 24°C in the Presence of l × 10⁻⁶ M Vinblastine |
|--------------------------------|-----------------|-----------------|
|                               | 35 min          | 60 min          |
| A.* Control                   | 5.9             | 10.0            |
| Vinblastine                   | 9.9             | 10.9            |
| B† Control                    | 2.8             | 2.3             |
| Vinblastine                   | 3.4             | 4.3             |
|                               | 15 min          | 45 min          |
|                               | 4.5             | 11.6            |
|                               | 7.3             | 10.5            |

* Cells incubated for 1 h at 37°C with or without vinblastine, and then at 24°C for the time indicated (with, or without vinblastine). Samples used for electron microscopy.

† Cells not preincubated at 37°C. Vinblastine added at time t = 0. Control and experimental percentages after 45 min not significantly different (P = 0.20), but significantly different from those at 0-5 min (P < 0.02%).

Carried out on five samples incubated at 24°C in the presence of colchicine, vinblastine, or cytochalasin B (see below). The cells were pre-fixed with glutaraldehyde before being labeled with Con A-ferritin. In all normal samples, thymocytes with typical uropods were present in varying proportions (Fig. 4) in addition to some cells showing constrictions or more irregular shapes, the latter being more common at 37°C. Most of the cells with uropod had the characteristics (abundant cytoplasm, nucleus with dispersed chromatin) of medium or large thymocytes (Fig. 4). Most of the large "head" of these cells was occupied by the nucleus, which was surrounded by a relatively thin
layer of cytoplasm. This consisted of an outer thin, and sometimes poorly visible, network of filaments separating the membrane from the cytoplasmic matrix which contained many ribosomes but practically no other cellular organelles (Fig. 4a). Most of the cytoplasm, with ribosomes, microtubules (Fig. 4b) and all the cellular organelles, was localized in the uropod. The “head” of the cell was separated from the uropod by a marked constriction, or tapered off into a conical uropod along a relatively straight surface (cf. Figs. 7 and 9b).

Uropods were present also in samples incubated with vinblastine (10^{-5} M, 1 h at 24°C) or colchicine (2 \times 10^{-5} M, 1 h at 24°C). The appearance of the uropods in thin sections was similar to that of the control cells (Fig. 8) except for the absence of microtubules (only exceptionally a few ill-defined microtubules were visible near the centrioles in colchicine-treated cells) and the presence of large tubulin crystals in vinblastine-treated cells (cf. Fig. 11). On the contrary, in samples treated with 10 \mu g/ml of cytochalasin B (45 min, 24°C), uropods did not form, and all the cells remained spherical. Similarly, uropods previously formed in 45 min at 24°C reverted to the spherical shape (occasionally with a more irregular edge along the side in contact with the substrate) upon addition of 10 \mu g/ml of cytochalasin B for 30 min at 24°C.

In agreement with previous observations (reviewed in reference 10), on spherical cells of all samples, including all those treated with cytochalasin B, the distribution of Con A-ferritin was essentially uniform over the entire surface. In the majority of small thymocytes the ferritin molecules formed an almost continuous layer, whereas on larger cells the distribution was more sparse, suggesting that the concentration of receptors of comparable affinity was lower on the latter cells.

On the contrary, on practically all the cells with a uropod the distribution was nonuniform. Most of the ferritin molecules were concentrated around the anterior part of the cell, whereas they were more sparse on the uropod, especially in the region of the constriction (or transition between “head” and uropod) (Figs. 4–7). The ferritin concentration was lowest on the constriction where, in different cells, it was \( \frac{1}{2}-\frac{1}{3} \) of that on the “head” (on which the concentration was estimated to be of the order of 1-2 \times 10^4 molecules/\mu m^2). Relatively more ferritin molecules were present on the uropod, where they were often irregularly distributed in loose “patches” separated by gaps containing very little ferritin. The nonuniform distribution of the Con-A receptors was characteristic of the cells with uropods, irrespective of the incubation temperature (14°C, 24°C, or 37°C), and was found on cells pre-fixed either with glutaraldehyde (Figs. 5 and 6) or with formaldehyde (Fig. 7). An identical distribution was observed on uropod-cells incubated with colchicine or vinblastine (Fig. 9).

The transition from the anterior area of high Con-A receptor density to the constriction area of low receptor density was relatively sharp, and often (but not always) was paralleled by a change in the membrane profile. This profile was straight or concave with rare undulations in the area of the constriction, whereas it was more wavy around the “head” (Figs. 7 and 14). In several sections, the cortical microfilament layer appeared thicker or more compact (and less penetrated by ribosomes) in the low ferritin constriction area than in the anterior part of the cell (Figs. 9 and 10). The filaments, \( \approx 6 \) nm in diameter, seemed to have different orientations, but generally ran parallel to the membrane and, at least in some of the sections, in an anterior-posterior direction (Fig. 9). Although this submembrane filamentous layer was always present, a clear morphological transition between head and constriction was obvious only in a fraction of all sections (not more than a third), even among sections of the same cell. This could be due to variations in the orientation of the section, to the high density of the cytoplasmic matrix, and to distortions introduced by fixation, but it could also reflect the existence of structural
Figure 4  Cell with a "typical" uropod in thin section. Sample incubated at 24°C and labeled with Con A-ferritin. (a) General view. Bar, 1 μm. × 14,800. (b) Details of the centrosomal area showing the presence of microtubules (arrows). Bar, 0.2 μm. × 64,000.
inhomogeneities in the cortical layer of the constriction.

A nonuniform ferritin distribution was also occasionally observed on cells without a visible uropod. As judged from their general appearance, these cells most likely corresponded to medium-large lymphocytes with a uropod in which the plane of the section had passed through the base of the uropod, leaving the uropod itself out of the section. The label was invariably less concentrated on the cytoplasmic side of the cell and denser around the nucleus.

On the contrary, the nonuniform distribution was not usually observed on the small thymocytes in which a marked constriction subdivided the cell into approximately equal parts (Fig. 11). In these cells, the nucleus occupied almost the entire volume of one-half of the cell where it was surrounded by a very thin rim of cytoplasm (Figs. 11 and 12), and extended partially through the constriction into the other half of the cell. The Con A-ferritin was continuously distributed and only occasionally showed a reduced density in the constriction area.

CAPPING OF CON A-FERRITIN: Unfixed thymocytes were labeled with Con A-ferritin at different concentrations for 15 or 45 min at 24°C, or for 20 min at 24°C after being preincubated in tissue culture medium for 45 min at 24°C to induce formation of uropods. A variable number of cells in the various preparations showed the formation of a cap often associated with the presence of a uropod. The caps were always localized on the uropod (or, respectively, on the cytoplasmic pole in cells without a distinct uropod), leaving the “head” of the cell free, or almost completely free, of molecules (Fig. 13). In particular, the large-medium lymphocytes either showed a normal localization of the cap or failed to cap and maintained a roughly spherical form, with Con A-ferritin distributed almost uniformly or in ill-defined patches.

SPONTANEOUS AND LIGAND-INDUCED REDISTRIBUTION OF ALS ANTIGENS: The distribution of ALS antigens on formaldehyde-pre-fixed thymocytes labeled with ALS followed by a goat anti-rabbit Ig-ferritin conjugate was essentially identical to that of the Con A receptors. On spherical cells, the ferritin distribution was essentially uniform. On cells with a uropod, the antigen was distributed prevalently around the “head” of the cell with only irregular and sparse labeling on the constriction area and on the uropod (Fig. 14).
Figure 6 Details of the front of the cell (a) and the uropod area (b) of a cell incubated at 37°C on a flat surface. Fixed and labeled as in Fig. 5. The relative orientation of detail b with respect to a in the actual cell section is indicated by arrows. Unstained section. Bar, 0.2 μm. × 52,000.

Figure 7 Details of the transitions area between "head" and uropod of a cell incubated at 24°C, fixed with formaldehyde and labeled with Con A-ferritin. Note the wavy profile of the membrane around the nucleus and its straighter outline on the uropod. Bar, 0.2 μm. × 42,000.
If the reagents (ALS followed by the anti-Ig conjugate) were added under the same conditions to unfixed cells, typical caps formed in a considerable proportion of these cells. The cap was localized around the Golgi area, or on a uropod, whereas the "head" of the cell remained free of ferritin.

DISCUSSION

The "normal" distribution of Con-A receptors and several antigens as observed on pre-fixed cells is essentially dispersed and uniform (although probably not strictly random [1]) in a variety of cells including lymphocytes (reviewed in reference 10). A uniform distribution was observed also on thymocytes in the present investigation, but only on the cells which remained spherical. In those cells which developed a uropod, the majority of the labeled Con-A receptors and ALS antigens were concentrated over the rim of the cytoplasm surrounding the nucleus, whereas less label was present over the uropod and in particular over the constriction at the base of the uropod. This nonuniform distribution was reversible and was correlated with the change in cell shape. Because it was not locally induced by extracellular factors (such as a ligand) and extended over regions of the cell not in contact with the substrate, the distribution most likely reflected some changes in the receptor molecules themselves, or in their membrane environment, which were induced by transmembrane interaction with intracellular structures, and which were probably dependent on the same process that determines the change in cell shape. The precise nature of the process is unknown, but it is probably based on the activation of a contractile system of the actomyosin type. This is suggested by the morphological characteristics of uropod formation, by its active character (shown by the inhibitory effect of azide), and by its sensitivity to a microfilament-affecting drug such as cytochalasin B. By contrast, uropod formation and membrane molecule redistribution were not inhibited by microtubule-disrupting agents such as vinblastine and colchicine. Although the overall shape of the uropod seems to be slightly modified (e.g., shortened) in the presence of the latter drugs, microtubules do not seem to be needed for inducing the observed changes in cell shape and in the distribution of the labeled components. This is consistent with the view that microtubules might only effect function and mobility of surface components indirectly, by imposing...
some additional organization on the membrane-displacing mechanical system (and thus modulating its activity), or by interacting with relatively few molecules at selected points in the membrane (cf. references 3, 31, 10).

The morphological process of uropod formation apparently results in the separation of the cell into two regions, one of which is “contracted” into a narrowed shape, and the other of which remains essentially “relaxed” and spherical. This morphology is similar to that of the “hand-mirror-shaped” lymphocytes moving on a
substrate (e.g., references 4, 8, 17), in which the anterior part appears to be dynamically more active and plastic, while the uropod behaves like a rigid rod. In several thymocytes with a uropod the membrane around the "head" of the cell appeared to be finely undulated, in contrast with its tauter profile over the uropod and especially over the area of the postnuclear constriction. A thickened layer of filaments was often seen in the latter areas whereas the filament network was more irregular and looser around the nucleus. The scarcity of filaments around the nucleus was particularly striking in several small thymocytes with a mid-body constriction, as in these cells almost no cytoplasm remained between the plasma membrane (carrying a high concentration of Con-A receptors) and the nuclear membrane (cf. Fig. 12). The filament layer in the constriction area of uropod-cells might be the structure responsible for the formation of the constriction. This would be consistent with the observation that a constriction ring can be induced in this region by addition of ATP to glycerinated lymphocyte models (20). It seems very likely that, in this region as well as on the uropod, at least some membrane proteins are connected, or somehow interacting, with cytoskeletal elements which apply to them (and indirectly to the membrane as a whole) a tension with a component normal to the membrane. This would account for the concave shape and the very pronounced curvature of the membrane often observed in this area.

During uropod formation, therefore, the concentration of labeled components is apparently reduced over an area which is relatively immobilized by interactions with cytoplasmic structures, and increased over a part of the surface which is less constrained and more "fluid". The precise cause of the differential labeling remains, however, uncertain. The difference could reflect either changes in the ability of the membrane components to bind their respective ligands on different parts of the cell (because of changes in
accessibility, or because of masking by other components), or an actual difference in concentration resulting from displacement of molecules from the uropod to the front of the cell. The first explanation faces the difficulty that the hypothetical change in accessibility or affinity would have to affect several components (reacting with different ligands) which presumably have different structural and chemical characteristics. This applies, in particular, to the Con-A receptor sites which are located on the exposed and flexible carbohydrate side chains. The change cannot be accounted for by mere immobilization, as all proteins are immobilized on prefixed cells. Conversely, it is not secondarily affected by fixation, as it is also observed on cells pre-fixed with formaldehyde (which does not decrease appreciably the binding of Con A to its receptors [15]), or even on unfixed cells (as in the case of θ antigens [13]). It seems more likely, therefore, that the change actually corresponds to a displacement of surface molecules from the uropod. This type of redistribution would suggest that ALS antigens and Con-A-binding molecules (at least the majority of them) do not take part in the interactions between membrane molecules and cytoskeletal structures which are responsible for uropod formation. This conclusion would not be unreasonable because, in view of the great variety in structural and functional characteristics of membrane proteins, it is unlikely that all the proteins could react directly with cytoplasmic structures responsible for a very specific function, i.e., a mechanical function. If this is so, the simplest hypothesis for the mechanism of redistribution would be that the components are passively excluded from the “immobilized” areas of the membrane as other (unknown) membrane molecules which interact with cytoplasmic structures are preferentially concentrated in the same areas (Fig. 15a). The mechanism is plausible if the latter are present at a relatively high concentration, or if the size of the molecules involved is relatively large. It is possible that other factors, such as changes in the physical state of the lipids (probably secondary to local alterations in the state of the protein components), or changes in the density of fixed surface charges, may also play a role (although, as regards the latter, no

2 An estimate of the order of magnitude of this effect can be obtained by assuming that molecules (or molecular complexes) A covering a circular area of radius $a$ are immobilized at random (without overlapping) at a concentration $\lambda$ in the plane of the membrane, and by calculating, for different $\lambda$'s, the area which remains available for inserting molecules B of radius $b$. For example, to obtain a fourfold difference in residual free area between “relaxed” and “contracted” regions, the concentration of the immobilized B molecules in the latter should not be less than $\sim 10,200/\mu m^2$ for $a=b=3$ nm, and not less than $\sim 5,700/\mu m^2$ for $a=b=4$ nm (by comparison the concentration of Con-A molecules bound at saturation to the lymphocyte surface is of the order of 10,000-15,000/μm²). These values have been calculated using an approximate distribution function for a random distribution of discs (14).

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consistent difference in the distribution of anionic sites on the uropod of fixed cells has been so far detected using cationized ferritin [S. de Petris. Unpublished results]). The possibility that some of the proteins interacting with underlying structures in the constriction area and on the uropod are Con-A-binding molecules cannot be excluded, because some ferritin molecules are bound in these areas, albeit without a definite pattern. Although a difference in labeling between head and uropod was observed at any concentration of the label, only a minority of Con-A receptors was probably labeled with the conjugate, similar to what has been observed in other experimental situations (2, 30). The labeling was probably representative of the average distribution of the majority of Con-A receptors and in particular of those of high affinity (presumably glycoproteins [22]), but not necessarily of classes of receptors of lower affinity.

A more continuous labeling was observed on small thymocytes with a central constriction, but without a typical uropod. There is some indication, though not conclusive, that also in these cells there may be a lower density of receptors in the constriction area. The smaller size of the area involved, and the fact that Con A-ferritin may give a less than proportional labeling in the areas of higher Con-A receptor density (2, 30), might have blurred the possible differences.

The ALS antigen and Con-A receptor distribution described here is substantially identical to the distribution of the \( \theta \) antigen previously observed on thymocytes which had formed uropods during labeling with anti-\( \theta \) antiserum (13). The \( \theta \) antigen may in fact correspond to a subclass of Con-A receptors (9, 31) and, in light of the present results and the fact that the antigen was probably functionally monovalent with respect to the alloantibody, it seems virtually certain that the observed distribution represented its "unperturbed" distribution. Since in those experiments the alloantibody was added to unfixed cells at temperatures (22°-24°C) where redistribution can occur, it seems clear that divalent binding of the antibody to two antigen molecules was not sufficient to modify the spontaneous distribution pattern. However, if the antibody was further cross-linked on unfixed cells by an anti-mouse IgG antibody, patches and caps readily formed, and in the capping cell all the cross-linked molecules were transported to the uropod, or to the cytoplasmic pole, as usual (reference 13 and S. de Petris and M. C. Raff. Unpublished observations). The same result was obtained in the present experiments when the ligand (Con A-ferritin, or ALS plus anti-mouse Ig antibody conjugate) was bound to unfixed cells. Thus, molecules which are probably excluded from the uropod during spontaneous redistribution, move...
in the opposite direction when they are extensively cross-linked by an external ligand. According to a current view of capping (the countercurrent model [10, 11, 12]; see, however, reference 6), the capping molecules would be transported towards the back of the cell by interactions with cytoplasmic structures. This postulated mechanism seems to be in contrast with the interpretation of spontaneous redistribution suggested above, which is essentially based on the assumption that the displaced molecules (including all or the majority of the Con-A receptors, the ALS, and θ antigens) do not interact directly with the cytoplasmic structures responsible for mechanical functions. The two concepts can be reconciled, however, if the mechanism of capping is supposed to be indirect (see also reference 10). According to this view, the cytoplasmic mechanical system responsible for surface movements would only interact directly with, and drive backwards with respect to a cellular framework (11, 12), one or a few specialized, and still unknown, classes of protein molecules. This would occur during uropod formation (and cell locomotion, see below) as well as during capping. In the former two cases, however, isolated protein molecules which are free to diffuse in the lipid bilayer would not be affected to any great extent by the backward movement of the cytoplasm-driven molecules, or they would even partially show a counterflow in the opposite direction if the latter were to accumulate on the posterior part of the cell (Fig. 15a). On the contrary, if the “free” molecules were extensively cross-linked in a lattice by a ligand, the cytoplasmic-driven molecules would be able to drag them
FIGURE 15 Proposed model for spontaneous (A) and ligand-induced (B) redistribution of surface components not directly connected to the cytoplasmic mechanical system. Membrane molecules (or molecular complexes) connected to, or directly interacting with, the latter are schematically indicated as cross-hatched rods, whereas nonconnected molecules are represented by open symbols. The connection is probably not permanent. (A) In "relaxed" spherical cells (a) both connected and nonconnected molecules are uniformly dispersed in the membrane. On formation of a uropod (b) the connected molecules "contract" in the cortical area of the constriction and on the uropod, and might exclude unconnected molecules from the area. The process is reversible. (B) On binding of a ligand to unconnected molecules (c) cross-linked patches are randomly formed which might trap unrelated molecules in their lattice. If capping is triggered (d), the coordinate backwards movement (over the entire surface) of the molecules connected with the mechanical system, and "trapped" in the lattice of cross-linked molecules drags the latter towards the cytoplasmic pole of the cell. Dragging could occur also without actual trapping, for example, by the effect of very weak attractive interactions between "free" and "connected" molecules, which are negligible when the molecules are isolated but become appreciable when they act cooperatively between multivalent aggregates. The coordinate movement of the connected membrane molecules may be accompanied by polymerization, or by other changes in their organization, as part of the triggering signal for capping. Molecules not cross-linked may flow in the opposite direction as in the case (A).

backwards by acting as a kind of mobile rake in the bilayer (Fig. 15b). The suggested mechanism is consistent with the observation that myosin tends to accumulate on the uropod both on capping and on noncapping motile lymphocytes (25).

The present observations and their suggested explanation could be relevant to the observation of Stackpole et al. (28), who observed, on several thymocytes, that "caps" of \( \theta \) and H-2 antigens formed at the front of the cell rather than at the cytoplasmic pole. Since these authors labeled the cells using a hybrid antibody method, which does not cross-link surface antigens very efficiently, in their experiments (as in our case with the \( \theta \) antigen [13]) the degree of cross-linking might have been insufficient to trigger a true process of capping, and the "reverse caps" might have corresponded to the "patching" of molecules transported towards the anterior part of the cell by a spontaneous redistribution process.

The cells unambiguously identified in this study on which redistribution was observed, i.e., cells with "typical" uropods, were apparently not moving. Some of the cells showing redistribution in samples incubated on plastic surfaces at 37\(^\circ\)C were probably motile, but this could not be conclusively proven by the present data. Recent observations under slightly different experimental conditions have confirmed, however, that Con-A receptors and ALS antigens can accumulate on the anterior part of the cell, also on moving thymocytes (S. de Petris. Unpublished results).

The technical assistance of Miss Margaretha Lindroth and Miss Ursula Baumgartner is gratefully acknowledged.

Received for publication 6 July 1977, and in revised form 15 June 1978.

Note added in proof: Since this manuscript was submitted, a model for membrane proteins-cytoskeleton interactions and capping similar to that discussed here and in reference 10 has been proposed by L. Y. W. Bourguignon and S. J. Singer (1977, Proc. Natl. Acad. Sci. U. S. A. 74:5031–5035).

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