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Temperature-Induced Variations in the Surface Topology of Cultured Lymphocytes Are Revealed by Scanning Electron Microscopy

(plasma membrane/microvilli/lipid transition/protein transition)

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ABSTRACT **Temperature-induced variations in the surface morphology of cultured lymphocytes were evaluated by scanning electron microscopy. At 25–37° the cells' surfaces are largely obscured by numerous undulating microvilli of various lengths but uniform diameter. Temperature changes alter the number of microvilli, their lengths, diameters, distribution, branching, and fusing. Typically, chilling to 0–4° markedly reduces the number of microvilli and increases the diameter of the survivors in a reversible process. In contrast, heating the cells to about 45° rapidly and irreversibly transforms the ordinarily smooth membrane surface into one with a "cobblestone" morphology. At the same time most microvilli disappear and the few that remain clump into a cap. The data suggest that the low-temperature effects reflect a change in the physical state of membrane lipids, while the high-temperature alterations represent thermotropic protein transitions.**

The surfaces of diverse lymphoid cells bear one or more transducing systems essential to a complete immune response. Of these, membrane-bound immunoglobulins (1, 2), as well as θ, H-2^k antigens of mice (1), and some histocompatibility antigens of humans (3), exhibit a *thermotropic* topological distribution detectable by fluorescence microscopy. Thus, the distribution of membrane immunoglobulins on the cell perimeter of B-type lymphocytes changes gradually with temperature from *diffuse* near 0° to "*spotty*" by 16° (2), the spots fusing by "capping" at physiological temperatures (1, 2). The "diffuse → spot" thermotropism is thought to arise either from increased diffusion rates of membrane macromolecules within the membrane plane at higher temperatures or from temperature-induced phase transitions of membrane lipids, analogous to those reported by various physical methods in model systems (4–8), various microbial membranes (9–13), and mitochondrial membranes isolated from rat liver (14).

Thus, the membranes of *Acholeplasma laidlawii* undergo a broad state transition between 15° and 45°, detectable by thermal studies, nuclear magnetic resonance, and x-ray diffraction (9, 15–17); the exact temperatures depend on the fatty acids incorporated from the medium. Other studies, with functional membrane vesicles from *Escherichia coli* mutants that are unable to synthesize unsaturated fatty acids (11–13), point to two thermotropic changes of state: the first, below 30°, depends on membrane-lipid composition; the second, above 40°, suggests alterations of the permeability or structure of the membranes, secondary to temperature-induced changes in membrane proteins. We note, in this regard, that difference UV spectroscopy (18) can detect reversible temperature-induced changes in the architecture of soluble proteins within the upper range of physiologic temperature.

Membrane thermotropic transitions tend to occur over a broad temperature range; moreover, those that can be securely assigned to membrane lipids have been recorded only from low-cholesterol membranes, e.g., *A. laidlawii*, *E. coli*, and mitochondria. In contrast, cholesterol-containing model and biomembranes show either no low-temperature phase transitions or very diffuse ones (5, 19), because cholesterol inhibits hydrocarbon chain motion in otherwise fluid areas and inhibits crystalline packing at low temperature (6). This fact complicates interpretation of thermotropic transitions in *lymphocyte membranes* in purely lipid terms since these structures exhibit a high molar cholesterol:phospholipid ratio of 1:1 (20–22). Also, prior thermal studies rely upon averaging techniques that cannot take account of possible topologic heterogeneities in membrane lipid and/or protein distribution. We suspect that this caution applies very markedly to plasma membranes of those lymphocytes that bear numerous, pleiomorphic microvilli (23) and thus exhibit a very complicated geography.

We have accordingly applied our techniques of scanning electron microscopy to the matter of thermotropic transitions of lymphoid cell membranes and introduce the results herein. We use the bovine NBC-6 lymphocyte line, maintained in continuous suspension culture, as a model system. These cells offer the following important advantages: (*a*) they are a pure cell line; (*b*) they can be cultured under precisely defined conditions; and (*c*) they can be isolated for study without significant manipulation. We use these cells as one might use a myeloma line in the study of immunoglobulins.

MATERIALS AND METHODS

NBC-6 cells were maintained in spinner cultures, in modified McCoy's 5A medium (containing 20% horse serum, heated at 56° for 30 min). For temperature studies, the cells were washed 3 times for 10 min each in serum-free medium at 150 × g_{av}. Aliquots of 2 to 5 × 10^6 washed cells in 0.5 ml of serum-free medium were incubated at 0, 4, 25, 37, 40–50, and 65°. In the range of 40–50° we proceeded in 1° steps. After incubation we fixed the cells with 2% glutaraldehyde in Millonig's buffer (pH 7.3) (24) at the incubation temperature. We then washed the cells three times in serum-free medium and let them settle onto confluent monolayers of African green monkey kidney cells (VERO) (propagated at 37° in McCoy's 5A medium plus 20% fetal-calf serum, under water-saturated

5% CO_2–95% air). These monolayers serve as highly efficient traps for the lymphocytes, (a key to their optimal morphologic observation) and morphologic reference standards. Once the lymphocytes attached (120 min, 25°), glutaraldehyde fixation was repeated, followed by three washes in serum-free medium. The cells were dehydrated in six steps, going from 20 to 100% ethanol and washed twice, 10 min each time, in 100% amyl acetate. After they were dried by Anderson's critical point method (25), with CO_2 as transition fluid, the specimens were coated with a thin layer (about 200 Å) of gold–palladium (60:40) at room temperature (25°) and 10^{-4} to 5×10^{-5} mm of Hg, with a JEOL JEE 4C vacuum evaporator. The specimens were placed on a tilted rotary turntable during coating. Electron microscopy was with a JEOL JSM-U3 scanning instrument operated at 25 kV, with 100-sec scanning periods.

RESULTS AND DISCUSSION

The surface morphology of NBC-6 cells remains relatively constant between 25° and 37°, but otherwise varies drastically with temperature. We will use the surface topology of cells equilibrated at 25° as point of reference (Fig. 1). We find that this appearance is typical also for peripheral lymphoid cells, such as rat splenic and human blood lymphocytes. The cells are grossly spherical and roughly 7 μm in diameter, but at this temperature part of the cell surface is relatively smooth and part is elaborated into microvilli. The villus diameters are rather uniform, about 0.1 μm, but their lengths range from 0.1 to 1.6 μm, with an average of 0.39 μm. Although most of the cells' surfaces are obscured by microvilli, precise evaluation of several hundred cells shows that only about 7% of the cells' surfaces are elaborated into microvilli. This gives about 1.4×10^3 microvilli per cell. Using average values for microvillus diameters, numbers, and lengths, we compute that these processes increase the cells' surface areas by at least 65% over those of smooth spheres. However, the subtle shapes, dimensions, and surface distributions of microvilli can be fully evaluated only by computer-assisted stereological techniques (26). We will deal with these intricacies in subsequent papers, and concentrate here on the major and dramatic changes of state occurring below 25° and above 40°.

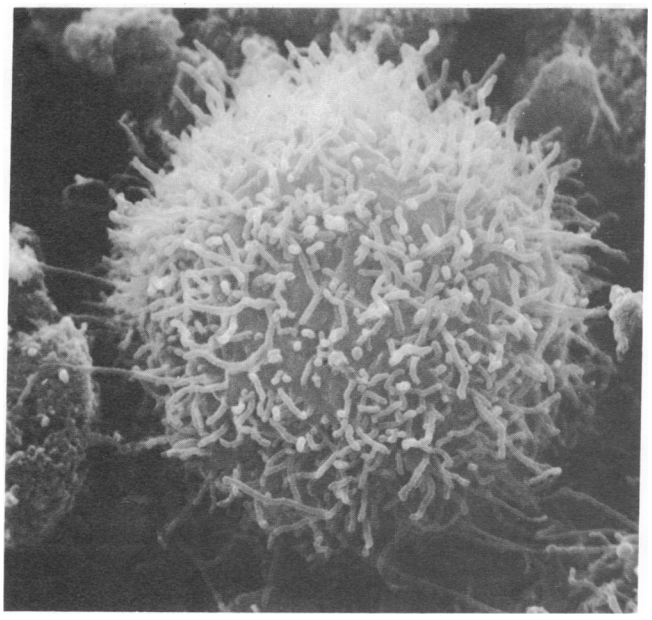

FIG. 1. Surface morphology of an NBC-6 cell equilibrated at 25° for 2 hr. The bare surface is relatively smooth, as is that of the uniform microvilli. ×7600.

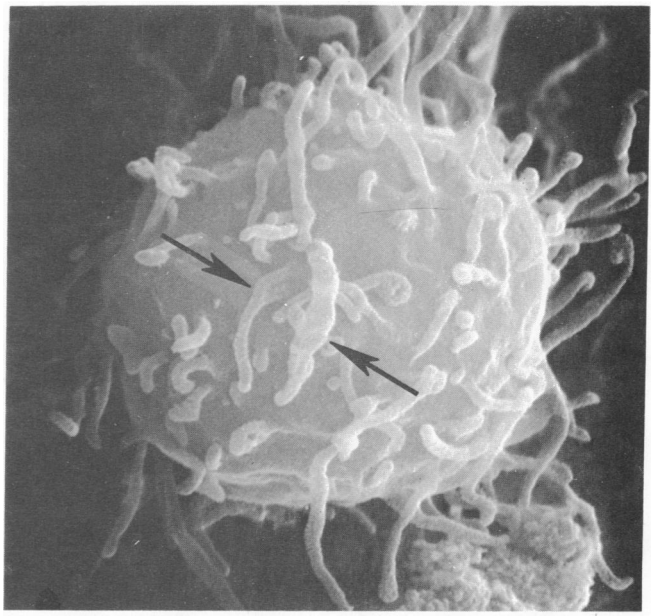

FIG. 2. Surface morphology of an NBC-6 cell transferred from 37 to 0° and maintained at 0° for 20 min. Note enlargement of the microvilli and diminution in their number. Fusion of microvilli is indicated by *arrows*. ×9500.

Changes induced by chilling

At low temperature (Fig. 2) the microvilli decrease in number and increase in diameter 2-fold or more; also, the average length of the remaining microvilli is above average. A transition to 4° from 37° or 25° produces similar changes, but the lower the final temperature and the longer the cells are maintained at it, the more the cells appear as smooth spheres. The mean diameter of the chilled cells, excluding microvilli, approaches 8 μm. This increase in diameter over that at 25–37° is consonant with the notion that the surfaces of microvilli at 37° partly merge into the smooth surfaces of the chilled cells. The topologic alterations induced by chilling are fully reversible, and reversal is not blocked by 10 mM sodium azide.

The surface changes at low temperature are most simply attributed to changes in lipid state in a membrane with high cholesterol content. However, since our preparation for scanning electron microscopy must still be done at *room temperature*, our ability to observe the surface morphology at low temperature derives from the fact that the surface topology, at a given temperature, can be *fixed by glutaraldehyde*, a reagent that does not react appreciably with membrane lipids (27). Hence, its ability to block return to the "25°" surface morphology cannot be attributed to a direct interference with lipid phase changes. Other possibilities are: (*i*) lipid phase changes do occur on a molecular scale, but cannot be detected at our level of resolution, because fixation of the cell as a whole blocks surface mobility; (*ii*) lipid phase transitions of membrane are prevented by crosslinking of membrane proteins, such as produced by glutaraldehyde (28); (*iii*) the restructuring of the cell surface at low temperature requires metabolic

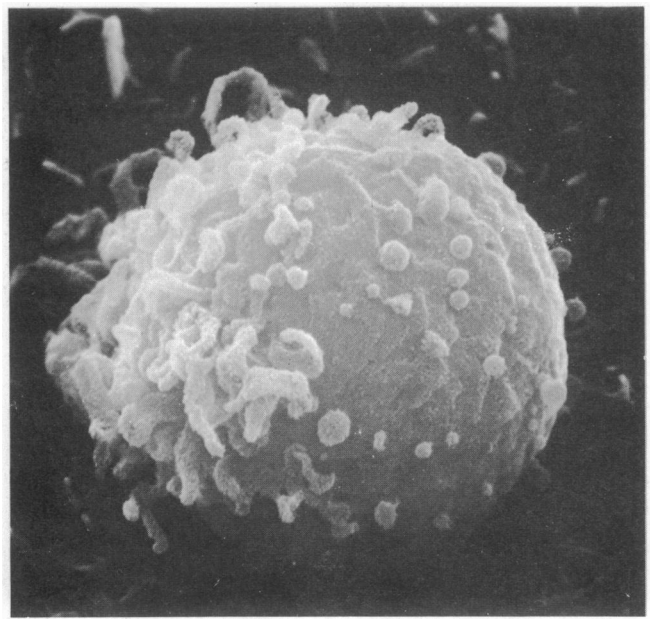

FIG. 3. Surface morphology of an NBC-6 cell exposed to 50° for 20 min. This micrograph shows the limit of the high-temperature transition. In contrast to its appearance at <45°, the surface now exhibits contiguous "cobblestone" protrusions, a few large nodules, and some short microvilli, clumped in one region. ×9500.

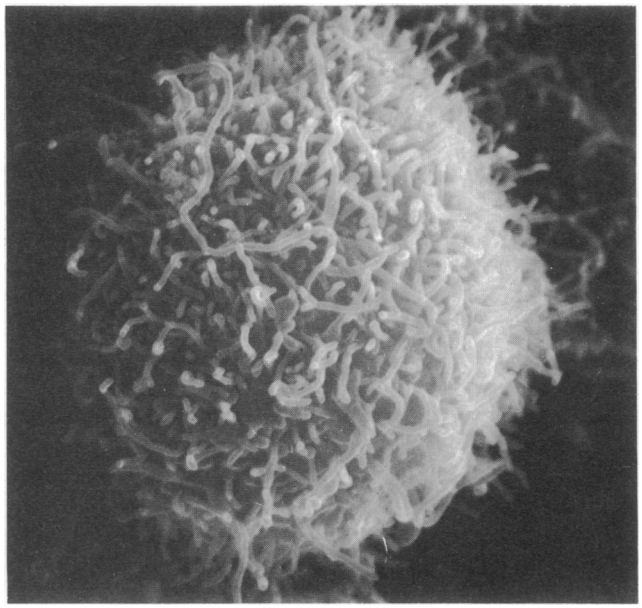

FIG. 4. Surface morphology of an NBC-6 lymphocyte equilibrated at 25° for 2 hr, fixed with glutaraldehyde, and then incubated at 50° for 20 min. ×9300. The cell's surface characteristics are the same as those found without heating (see Fig. 1).

processes (other than oxidative phosphorylation) that are impaired by glutaraldehyde fixation.

Changes induced by heating

Lymphocyte membrane topology changes very little as the incubation temperature is raised from 25 to 44°. However, above 45°, dramatic changes occur (Fig. 3). The microvilli decrease in number and shorten, and the entire cell surface, including remaining microvilli, becomes "pebbly" (Figs. 3 and 5). Some of these effects are seen already at 45°; by 46° only few microvilli remain, which usually cluster together. The changes induced by temperatures above 45° can be blocked by fixation of cells with glutaraldehyde at 25°, before the temperature is raised; such cells show the characteristic morphology, topology, and surface smoothness of cells at 25°, even after extended exposure to 50° (Fig. 4).

Cells exposed to temperatures above 45° are spherical and almost devoid of projections, save for a few large nodules and some short microvilli clumped in one region, but their entire surface is covered with contiguous "cobblestone" projections (Fig. 3). The average radii of these protuberances are near 0.3 μm, and our calculations, assuming the mean numbers and lengths of the microvilli at 25° as well as the average cell diameter at that temperature, indicate that the entire surface area of the microvilli could be accommodated in the increase of surface area represented by "cobblestone" projections seen at high temperature. (The overall cell diameter at 47° is not significantly larger than that at 37°.) This notion is supported by examination of thin sections of the material used for scanning electron microscopy fixed by glutaraldehyde and OsO_4; these data (unpublished) clearly show that the disappearance of microvilli is not compensated by increased amounts of intracellular membrane and/or intracellular lipid drops. Thus, exposure to high temperature converts most microvilli into small, circumscribed surface projections. The effects at high temperature are only partially reversible morphologically. Thus, if cells exposed to 47° for 20 min are then cooled to 25° and held at this temperature for 20 min, extended microvilli reappear, *but* all of the microvilli as well as the free cell surface retain the "cobblestone" appearance already described.

Interestingly, standard dye-exclusion tests indicate a diminution of cell viability of only 20% at 50°, although normal surface topology is irreversibly eradicated by exposure to this temperature. Moreover, fluorescence microscopy of cells, incubated with 5 μg/ml of fluorescein dipropionate (29) at 50° for 20 min or at 37° after a previous incubation at 50° for 20 min, shows normal fluorochromasia, i.e., normal permeability to fluorescein and fluorescein dipropionate.

The state transition at high temperature occurs rapidly (Fig. 5 *B–E*). Incubation of 25°-equilibrated cells at 47° induces the "cobblestone" irregularity of the microvillous and other surface domains already after 2 min. By 5 min, these alterations are more prominent and the microvilli decrease in number statistically. By 20 min, the cells are essentially spherical, with few short villi remaining. Most of these appear clumped in a single cap, but some appear as large isolated nodular protrusions.

The changes observed above 45° are most rationally attributed to alterations of membrane proteins. This is concordant with observations on membrane state changes induced at high temperatures in microbial and rat-liver mitochondrial membranes (9–17), as well as the results obtained with thermal perturbation difference spectroscopy of proteins (18). The latter results indicate that many proteins change state even at the *higher physiologic range*. The fact that the high-temperature transition, including the changes of surface detail, can be blocked by glutaraldehyde, is consonant with suggestions that the plasma membrane is a large scale, possibly plastic, lipid–protein mosaic.

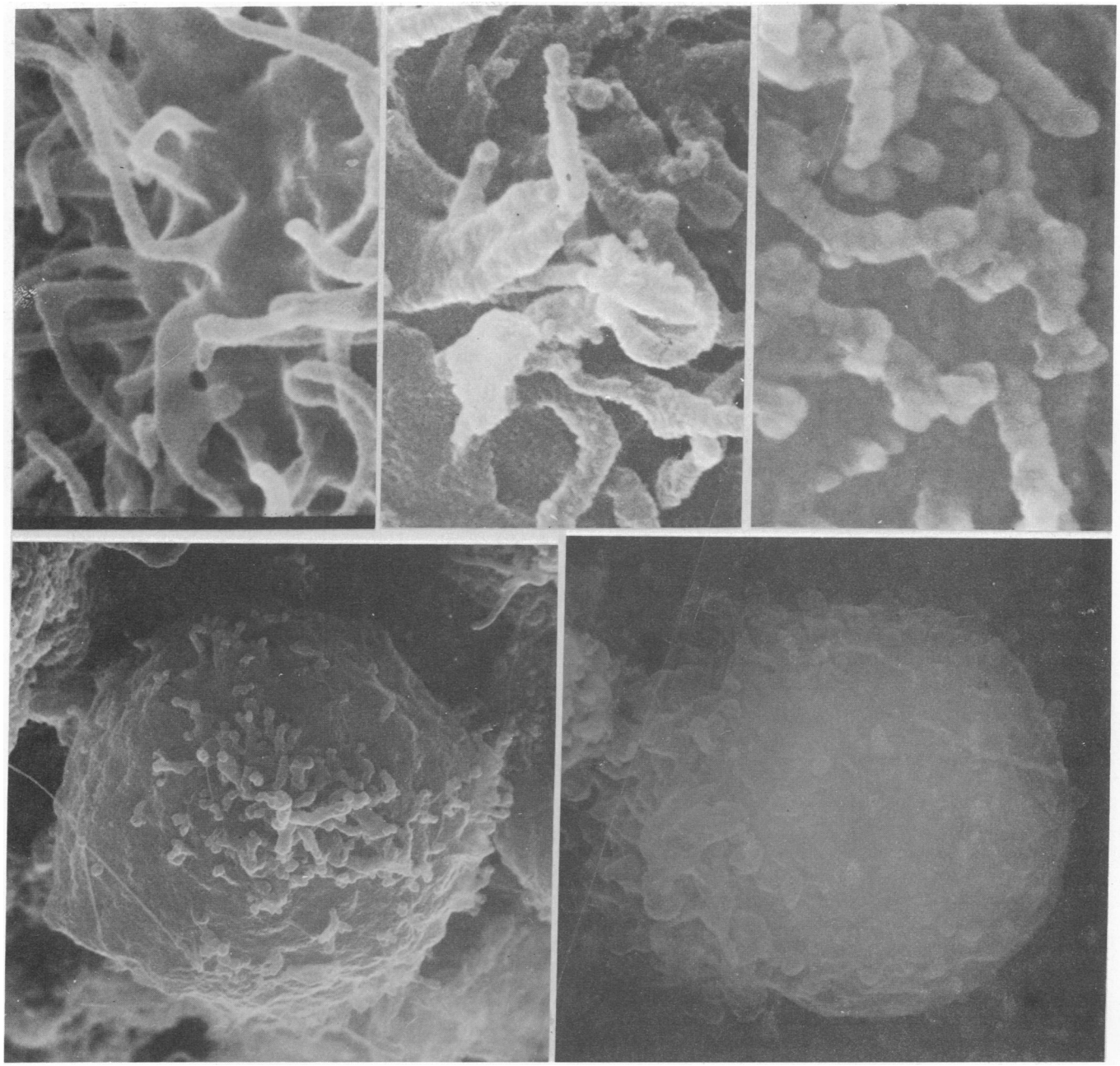

FIG. 5. Surface morphology of NBC-6 cells incubated at 47° for various times. (*A*) *Top left:* control (25°, 20 min), ×29,700. (*B*) *Top center:* 2 min, ×29,700. (*C*) *Top right:* 5 min, ×49,500. (*D*) *Bottom left:* 10 min, ×9,900. (*E*) *Bottom right:* 20 min, ×9,900. Note that the contiguous "cobblestone" irregularities are visible within 2 min and prominent by 5 min.

Our data have some rather immediate implications: *First*, lymphocyte plasma-membrane fractionations (20–22) will depend dramatically on the temperature at which these manipulations are done and on whether equilibration has been achieved before cell rupture. *Second*, the topologic distribution of lymphocyte surface functions and the interactions of lymphocytes with other cells must be reinvestigated in the light of micromorphologic data; present interpretation would appear too simplistic. *Third*, molecular interpretations of the distribution of lymphocyte surface antigens and other receptors must take account of the gross membrane heterogeneities and thermotropism described herein.

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