Surface Modification of T-Lymphocytes Observed during Rosetting

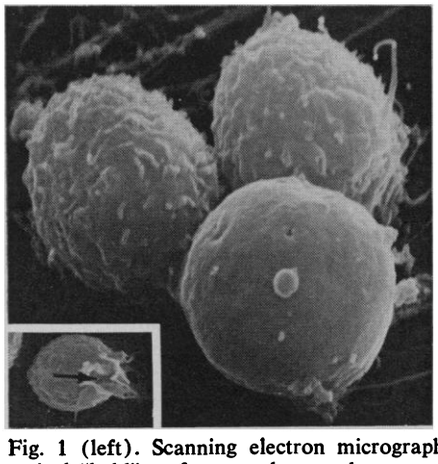
Abstract. *Scanning electron microscopy has been used to characterize alterations of lymphocyte surface topology that occur on contact with erythrocytes during the rosetting reaction. Molt-4 cells, a line of leukemic human lymphocytes, defined as T-cells through their ability to form rosettes with sheep erythrocytes, were used for this. Unreacted Molt-4 cells exhibit surfaces that are virtually smooth and carry few microvilli. In contrast, Molt-4 cells in rosettes display a time-dependent modification of surface topology, characterized by the emergence of numerous, long microvilli, particularly in areas of red cell contact.*

The peripheral lymphocytes of man and diverse experimental animals can be grouped into two categories according to their kinetics, functions, and surface characteristics. The first class, the so-called B-cells, probably enter the circulation directly from the bone marrow and participate principally in antibody production. These lymphocytes usually bear detectable quantities of immunoglobulins and complement receptors on their surfaces.

The second category, the T-cells, probably also originate in the bone marrow, but reach the periphery only after differentiating in the thymus into cells specialized for cell-mediated immunity. These cells bear specific surface antigens different from those of B-cells, become metabolically activated upon surface binding of certain plant lectins, and agglutinate spontaneously with erythrocytes to form rosettes; this last property also typifies isolated human thymocytes and is most marked with sheep red blood cells (SRBC).

The rosetting phenomenon is useful for lymphocyte classification, but its biological significance and the mechanisms involved remain unknown. However, micromorphologic studies (*1*) show that contacts between erythrocytes and T-cells are mediated by the lymphocytes' surface microvilli. This appears puzzling, because isolated, nonrosetted thymocytes bear extremely few microvilli (*2, 3*) and because many lymphocytes in T-cell rosettes exhibit rather "bald" surfaces in regions not involved in contacts with erythrocytes. In contrast, the majority of free peripheral lymphocytes, splenic lymphocytes, and lymphocytes participating in complement-mediated B-rosettes bear numerous, long microvilli (*1–3*). It appears feasible, therefore, that contact with erythrocytes might alter the surface structure of T-cells, and we have explored this possibility.

For this we used Molt-4 cells, a clone of human leukemic lymphocytes, isolated and established in permanent culture by Minowada *et al.* (*4*). Molt-4 cells lack surface immunoglobulins and surface complement receptors, but show the surface topology of isolated thymocytes and form spontaneous rosettes with SRBC. They are thus appropriately defined as T-lymphocytes. They constitute a uniform cell population which can be maintained under well-controlled conditions and can be used to study the rosetting phenomenon, with minimal preliminary manipulation.



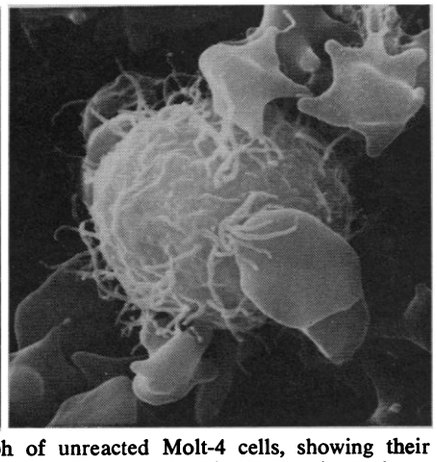


Fig. 1 (left). Scanning electron micrograph of unreacted Molt-4 cells, showing their typical "bald" surface topology and scarce, short microvilli (× 4000). The inset shows a Molt-4 cell with flat pseudopodal extensions (arrow) (× 1300). Fig. 2 (right). Micrograph of a rosette fixed at 3 hours, showing the emergence of numerous long microvilli from a reacted Molt-4 cell. There is extensive and complex contact between the microvilli and the erythrocytes (× 4000).

The Molt-4 cells were propagated and the rosetting reaction carried out at 22°C, as described in (*4*). The cells were cultured in RPMI 1640 medium (10 percent heat-inactivated fetal calf serum), washed once with Veronal-buffered isotonic saline solution (VBS), and resuspended in VBS (1×10^7 cells per milliliter); 0.1 ml of the suspension was mixed with 0.2 ml of SRBC (1 percent by volume in VBS) in test tubes (12 by 75 mm) without shaking. Control Molt-4 cells were treated identically, except that we added 0.2 ml of VBS instead of the SRBC suspension. Samples of control cells and Molt-4–SRBC mixtures were removed at 30-minute intervals and immediately fixed by addition of 5 ml of 2 percent glutaraldehyde in Millonig buffer, *p*H 7.3 (*2*). After fixation for at least 12 hours, the cells were washed three times in serum-free medium and allowed to settle onto confluent monolayers of African green monkey kidney cells (propagated at 37°C in McCoy's 5A medium plus 10 percent fetal calf serum, under a water-saturated mixture of 5 percent CO_2 and 95 percent air). After the lymphocytes had attached (120 minutes at 25°C) we repeated the glutaraldehyde fixation and washed the cells twice in serum-free medium. The cells were dehydrated in six steps, going from 20 to 100 percent ethanol; washed twice, for 10 minutes each time, in 100 percent amyl acetate; dried by Anderson's critical point method with CO_2 as transition fluid; and coated with a thin layer (about 200 Å) of gold-palladium (60 : 40) at 10^{-4} to 5×10^{-5} mm-Hg by using a JEOL JEE 4B vacuum evaporator with a tilted rotary turntable. For electron microscopy a JEOL JSM-U3 scanning electron microscope was operated at 25 kv with 50-second scanning periods.

The surface topology of Molt-4 cells resembles that reported for thymic lymphocytes (*2, 3*). Most of the cells are spherical, with some crestlike or moundlike surface protrusions and a few stubby microvilli (Fig. 1). Rare cells show long microvilli, and then only a few per cell. However, many of the Molt-4 cells exhibit large, flat pseudopodal extensions (see Fig. 1, inset).

The bland surface topology of Molt-4 lymphocytes changes dramatically during contact with SRBC in the rosetting process. With specimens fixed after 30 and 60 minutes, numerous, usually

short, microvilli appear in regions of red cell contact, and with increasing time (2 to 3 hours) the microvilli become numerous, long (up to 6 μm), undulating, and branched (Fig. 2). Indeed, at this stage, rosetted Molt-4 cells exhibit a surface topology barely distinguishable from that of "B-type" lymphocytes (*1–3*). However, the regions with abundant long microvilli occur only near sites of contact with SRBC; other regions of the lymphoid cell periphery may continue to be quite bald. Moreover, we do not observe the drastic distortion of erythrocyte surfaces that characterizes complement-dependent B-cell rosettes (*1*). In contrast, unrosetted Molt-4 cells, as well as cells maintained for up to 3 hours under rosetting conditions but without SRBC, exhibit no change in surface topology.

In more than 90 percent of more than 500 rosetted Molt-4 cells counted in four series, the lymphocytes' surface topologies were clearly distinguishable from the resting state. Furthermore, the contacts between Molt-4 cells and SRBC are mediated by microvilli, as reported before for conventional rosetting systems (*1*). In general the microvilli interact mostly with the edges of the rosetted erythrocytes, but very complex contacts can be observed. Quite frequently, for example, the lymphocyte microvilli seem to grasp the erythrocytes in tentacle fashion.

Contact between SRBC and Molt-4 cells induces a dramatic increase in the number, lengths, and complexity of the lymphocytes' surface microvilli. It seems reasonable to assume that contact with the erythrocytes produces an initially localized, but vigorous increase in surface rearrangement. The resulting extensive microvilli produce a lymphocyte morphology very similar to that seen in B-rosettes (*1*), and we therefore suggest caution in attempts to classify T- and B-cells on the basis of morphologic criteria alone.

If Molt-4 cells constitute an adequate T-cell model, our findings can explain the curious variability of lymphocyte surface topology reported for T-cell rosettes (*1*). The number and lengths of microvilli increase (within limits) with the duration and extent of red cell contact; neither of these environmental variables is usually well defined.

However, we suspect more fundamental implications and propose that the bald and hairy surface topologies of lymphocytes represent separate stages of differentiation. Accordingly, the bland surface features of native thymocytes and freshly cultured Molt-4 cells would reflect a quiescent state. Contact with appropriate surfaces, such as with erythrocytes on exit from the thymus, then induces a surface activation manifested through the development of elaborate microvilli. The biochemical and immunological correlates of the surface activation phenomenon must be explored in order to establish its biological significance.

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Genetic Mapping of Ir Locus in Man: Linkage to Second Locus of HL-A

Abstract. *Fifty-seven members of a family that spanned three generations were studied for antigen E and ragweed skin sensitivity and HL-A antigens. There was significant association between the haplotype HL-A 2-12 and antigen E skin hypersensitivity ($F = .22$ to $.26$) in this family. The map order is first locus of HL-A, second locus of HL-A, and IrE. These determinants are considered to be part of the linkage group HL-1.*

The histocompatibility complex of man, HL-1, includes genes determining certain leukocyte and tissue (HL-A) antigens and the ability to stimulate in mixed lymphocyte culture (MLR-S) (*1*). It is here proposed that HL-1 includes a determinant for hypersensitivity to ragweed antigen E (IrE) and the map order is the first locus of HL-A, second locus of HL-A, and IrE.

This report is based on studies of 57 subjects of a large Minnesota family. Thirteen members of the B. family had histories of respiratory symptoms of asthma or rhinitis (or both) during the ragweed season in Minnesota (August through Sepember). Intradermal tests were performed with 1 : 500 dilutions of ragweed (Hollister-Stier) (*2*). Antigen E, a purified protein derived from ragweed pollen extract, was used for skin testing at concentrations from 1×10^{-12} to 1×10^{-1} mg/ml, and the end point dilution with a positive reaction was scored. The skin tests were read for immediate wheal and flare reactions at 20 minutes. They were graded on a 0 to 4+ scale (*3*). A significant positive reaction was considered to be 2+ or greater. The antigen E preparation (*4*) gave negative reactions in 14 normal, nonatopic individuals ranging in age from 14 to 40 years with dilutions of 10^{-12} to 10^{-1} mg/ml (for all tests 0.02 ml of each dilution was used). Forty-eight ragweed-sensitive individuals ranging from 18 to 48 years of age gave positive reactions to the antigen E preparations, with end point dilu-

Table 1. Estimated frequency of recombination between loci for ragweed sensitivity and HL-A.

Parent phenotype	F*	S.E.	P†	Sample size
HL-A 2 + IrE (a)	.26	.08	$<.05$	27
HL-A 12 + IrE (b)	.22	.08	$<.01$	27
HL-A 3-W18 + IrE (c)	.89	.10	$<.05$	9
HL-A 9 + IrE (d)	.71	.12	$>.10$	14
W15 + IrE (e)	.79	.11	$<.10$	14
HL-A 9-W18 + IrE (f)	.60	.09	$>.10$	5

* Observed frequency of recombinants in offspring. † Probability under the null hypothesis of no linkage between HL-A and IrE. $P < .05$ and $F < .5$ indicate (lines a and b) that a particular HL-A haplotype is linked to the allele for ragweed sensitivity (IrE) (coupling); $P < .05$ and $F > .5$ (line c) indicate that HL-A is linked to a different allele at the locus for IrE sensitivity (repulsion); and $P > .05$ indicates independent segregation (lines d, e, and f).