for example, without blocking the phytohemagglutinin receptor. Although the quantitative aspects of anti-β_2m blocking of SRBC rosette formation needs further investigation, it may be that the receptor for SRBC is distinct and distant from that of the β_2m. Poulik *et al.* (*13*) and Peterson *et al.* (*14*) have recently obtained data suggesting a physical association between the serologically defined antigens of HL-A, the major histocompatability complex in man, and β_2m. In that context, our findings support the concept that phenotypic products of the HL-A chromosomal region may function in recognitive processes.

MARILYN L. BACH
SHI-WEN HUANG
RICHARD HONG
Departments of Pediatrics, Pharmacology, and Microbiology, University of Wisconsin, Madison 53706
M. DAVID POULIK
Department of Immunochemistry, Wayne State School of Medicine, William Beaumont Hospital, Royal Oak, Michigan 48072

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Aggregation of HL-A Antigens at the Lymphocyte Surface Induced by Antiserum to β_2-Microglobulin

Abstract. *Rabbit antiserum to human β_2-microglobulin followed by goat antiserum to rabbit immunoglobulin induces aggregation of β_2-microglobulin at the lymphocyte surface, as shown by immunofluorescence and by acquisition of resistance to lysis by complement. This treatment also affects all HL-A antigens of the cell, which cap together with β_2-microglobulin, so that the cell becomes resistant to lysis upon addition of antibodies to HL-A in the presence of complement. These findings suggest that a physical linkage exists between these two classes of polypeptides at the surface of the living cell and is in agreement with recent biochemical data obtained by others with cell membrane preparations.*

It is generally accepted that many protein components of the cell membrane are floating in the lipid bilayer, where they are oriented according to their physical characteristics (*1*). Antibodies directed against some of these products are potent and specific agents for rearranging cell surface components without impairing cell viability. In fact, antibody-mediated aggregation of molecules at the outer layer into "caps" and "spots" has become an important technique for exploring the topography of the cell surface (*2*). With this method we have proved definitively that each one of the three pairs of genes of the HL-A chromosomal region of man, which are expressed as "serologically defined" antigens (SD), are responsible for the synthesis of distinctive physical units that do not interact with each other. Thus the six HL-A antigens (of heterozygotes at the three SD loci) move independently at the cell surface and do not cap together (co-cap) (*3, 4*). Surface immunoglobulins of B (marrow-derived) lymphocytes also do not co-cap with transplantation antigens (*2, 5*). Usually, labeled reagents are employed, and the rearrangement of molecules is visually studied by immunofluorescence microscopy. Bernoco *et al.* (*3*), however, have also observed that formation of complexes of HL-A antigen plus antibody to HL-A plus antiserum to human Ig (immunoglobulin) at the lymphocyte surface make the cells specifically resistant to complement-dependent lysis, even upon addition of new antibody and excess complement, for all HL-A determinants carried by the molecular population which has been aggregated.

By contrast, the cell is normally susceptible to complement-dependent lysis for the residual HL-A antigens. For other systems similar phenomena have been described as "antigenic modulation" (*6, 7*). Actually, fixation in vitro on the surface of living lymphocytes of antibodies to HL-A antigen (and by implication of antibody against some other membrane components) is labile and initiates a complex chain of events (*8*). Of particular significance for this presentation are the following. (i) "Desensitization" of the cells, during incubation at 37°C, becomes progressively resistant to lysis upon addition of complement alone (*9*). This is due in part to microaggregation of the antigen, in part to loss from the cell surface of the antigen-antibody complex by endopynocytosis, shedding, and, later, peptic digestion (*10*). Usually the lost antigen is replaced at the same rate by virgin molecules, and the cell is still lysed by addition of further antibody and complement. (ii) The rapid formation of caps and spots by the two-step procedure (addition of human antiserum to HL-A and then antiserum to human Ig) leaves large areas of the cell surface totally deprived of that antigen, as seen by immunofluorescence, and paralleled by resistance to complement-dependent lysis. (iii) HL-A molecules lost by two-step addition described above are replaced by new ones (recovery) if the cells are incubated in nutrient medium at 37°C for 8 to 12 hours. (iv) Recovery requires active protein synthesis and is inhibited by cycloheximide and other antimetabolites.

With this background information concerning HL-A behavior, we have studied, in the living cell, the behavior of β_2-microglobulin (β_2m) which is present on the surface of many types of cells, including lymphocytes (*11, 12*). Particularly, Poulik (*13*) has shown that rabbit antiserum to human β_2m which has been appropriately absorbed, allows the detection of β_2m at the lymphocyte surface by immunofluorescence (indirect method) and induces cell lysis upon addition of rabbit complement.

For the reagents and techniques used in the experiments presented below see (*14*). Desensitization of lymphocytes coated with antiserum to β_2m (anti-

β_2m) (*9*) closely paralleled desensitization for HL-A antibodies (*8*); that is, a 50 percent reduction of killing upon addition of complement was observed after incubation for 4 to 6 hours at 37°C. Total resistance to lysis by complement was reached after 8 to 12 hours. When, however, the cells were treated with additional anti-β_2m and complement, maximal killing was obtained at any sampling time. Under these conditions anti-β_2m treatment did not modify the sensitivity of cells to lysis by positive antiserum to HL-A. This suggests that aggregation of antigen, loss of immune complexes from the cell surface by interiorization and shedding, replacement with new antigen molecules, follow a similar pattern after rearrangement of the cell surface components brought about by either anti-β_2m or antiserum HL-A.

For the study of cap formation by immunofluorescence microscopy, cells first sensitized with anti-β_2m, were incubated at 37°C for 2 hours with goat antiserum to rabbit Ig, labeled with rhodamine. In fact, cap formation for β_2m requires a longer time than for membrane-bound Ig or HL-A antigens. Under this condition more than 50 percent of the cells showed typical caps, where fluorescence was concentrated in less than one-third of the cell contour. All residual cells showed rims stained by rhodamine in marked spots and patches.

When, however, co-capping for a second antigen must be studied, the long incubation at 37°C allows resynthesis of the antigens sequestered in the cap. To prevent this "recovery," cycloheximide was used. As shown in Fig. 1, when the cells that had been treated with anti-β_2m were subjected to a second sensitization in the cold with antibodies to HL-A and then treated with antiserum to human Ig labeled with fluorescein, complete overlapping of the two fluorochromes was observed. The absence of the fluorescein-labeled antibody to human Ig from the entire cell perimeter except at the cap, despite the addition of an excess of the antiserum, is good evidence that all known HL-A antigens carried by the cell were sequestered in the same areas with the β_2m determinants. Other explanations, however, cannot be excluded (for example, release of the HL-A component from the complex of HL-A and β_2m and hence from the cell surface, because of conformational changes induced by anti-β_2m). By contrast, capping for Ig molecules linked to the cell membrane of B lymphocytes did not remove HL-A antigens.

The technique for studying development of resistance to complement-dependent lysis is described in Table 1. The results confirmed and extended what was observed by immunofluorescence; that is, prior treatment with rabbit anti-β_2m followed by treatment with goat antiserum to rabbit Ig prevented lysis, as assayed by typing for all HL-A antigens carried by the cell, whereas prior treatment with specific antiserum to HL-A only blocked reactivity for the corresponding antigen. Prior treatment with antiserum to HL-A antigen W6 (4b) inhibits lysis for the two antigens of the SD-2 locus, in agreement with the fact that this cross-reacting specificity is carried by both HL-A8 and HL-A W5 molecules. Prior treatment with anti-β_2m does not affect sensitivity to lysis by antilymphocyte serum. Identical results on resistance to complement-dependent lysis were obtained with different cell samples, carrying a variety of HL-A combinations at the three SD loci.

In preliminary experiments this anti-β_2m was extensively absorbed with sheep red blood cells to remove any heterophile activity. This absorbed serum produced identical results to those de-

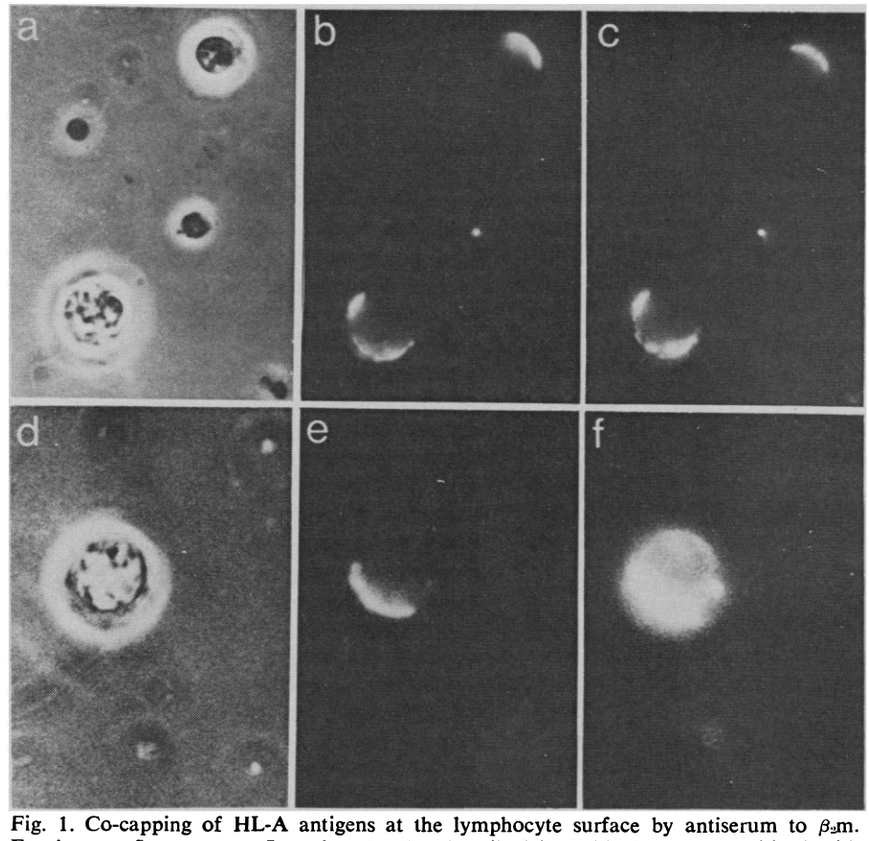

Fig. 1. Co-capping of HL-A antigens at the lymphocyte surface by antiserum to β_2m. For immunofluorescence: Lymphocytes (as described in Table 1) were sensitized with rabbit antiserum to human β_2m and then mixed with rabbit antiserum to human Ig, in the presence of cycloheximide (100 μg/ml per 2×10^6 cells); they were kept for 2 hours at 20°C, washed three times, incubated 2 hours at 37°C with goat antiserum to rabbit Ig coupled with rhodamine, in the presence of cycloheximide (100 μg/ml per 2×10^6 cells), washed three times, and cooled at 0°C. They were sensitized for 2 hours at 0°C with a mixture of the five antiserums to HL-A, which were used for typing in the experiment in Table 1. They were then washed three times at 0°C and incubated for 45 minutes at 0°C with rabbit antiserum to human Ig, coupled with fluorescein. The preparations were washed three times at 0°C, mounted on slides, and read on the refrigerated stage of a Leitz Ortholux microscope with selective filters (*15*). (b and c) The same cells, as shown in (a) by phase contrast, are identical (fluorescent cap, black rim), thus showing that treatment with rabbit antiserum to β_2m followed by antiserum to rabbit Ig also removed from the cell surface all HL-A molecules. For comparison, the pictures in the lower row show a B lymphocyte, as seen by phase microscopy in (d), from the same cell population which, however, has been treated in the first step at 37°C with rabbit antiserum to human Ig (rhodamine) only, then in the second step at 0°C with a mixture of the five antiserums to HL-A followed by rabbit antiserums to human Ig (fluorescein): the sharp difference between (e) and (f) shows that capping of Ig does not remove HL-A.

Table 1. Resistance to lysis (modulation) for HL-A antigens of lymphocytes previously treated with antiserum to β_2m. (i) Human lymphocytes of the peripheral blood, suspended in RPMI medium supplemented with 10 percent normal rabbit serum that was absorbed and inactivated were sensitized for 2 hours at 20°C with the first antiserum (that is, human antiserum to HL-A or rabbit antiserum to human β_2m) and washed three times. (ii) The cells were then incubated at 37°C with rabbit antiserum to human Ig or goat antiserum to rabbit Ig, and washed three times. During steps (i) and (ii), cycloheximide (100 μg/ml per 2×10^6 cells) was added in order to prevent "recovery" of modulated antigens (*8*). (iii) The cells were then typed by lymphocytotoxicity and scored (*14*) as follows: **1**, ≤ 5 percent dead cells (negative); **2**, between 5 and 20 percent dead cells (negative, high background); **8**, between 80 and 100 percent dead cells (strong positive). Four cytotoxic units of antibody were used for the preliminary treatment and for typing (*8*). Abbreviations: C, rabbit complement (8 units added for typing); ALS, horse antiserum to human lymphocyte; T4, new HL-A allele at SD-3 (*4*); W6 (4b), cross-reacting specificity shared by HL-A8 and W5 (at SD-2). HL-A genotype of the cells at the three SD loci: A1; A8; —/A2; W5; T4. Preliminary treatment with antiserum to HL-A8 and antiserum to HL-AW5 prevents lysis only for the corresponding specificity. Antiserum to W6 prevents lysis for both A8 and W5. By contrast, antiserum to β_2m followed by antiserum to rabbit Ig induces resistance to lysis for all five HL-A antigens, but not for ALS.

Treatment with antiserum to		Scores of typing after treatment with							
First	Second	C	A1	A2	A8	W5	T4	β_2m	ALS
None	None	1	8	8	8	8	8	8	8
Human Ig	None	1	8	8	8	8	8	8	8
Rabbit Ig	None	1	8	8	8	8	8	8	8
HL-A8	Human Ig	2	8	8	1	8	8	8	8
HL-W5	Human Ig	1	8	8	8	2	8	8	8
HL-AW6	Human Ig	1	8	8	2	1	8	8	8
Human β_2m	Rabbit Ig	2	2	2	2	2	2	2	8

scribed in Table 1. Further, the lack of any effect of rabbit antiserums aainst human Ig added to lymphocytes argues strongly that the phenomenon observed is not due to a naturally occurring antibody in rabbit serum.

After treatment with anti-β_2m followed by antiserum to rabbit Ig, the cells were washed, suspended in nutrient medium, and incubated at 37°C. Recovery, that is, the new expression of HL-A antigens after acquisition of resistance to lysis, was studied by sampling the cells at various times and testing for lysis in the presence of excess antibody and complement. Recovery of β_2m determinants followed the same pattern previously observed for HL-A antigens (*8*). In fact, the new determinants reached the original levels after 6 to 8 hours. Moreover, return of β_2m at the cell surface was closely paralleled, within the accuracy of the method, by return to lysis susceptibility for all HL-A specificities. Recovery for both β_2m determinants and for HL-A antigens was completely blocked by cycloheximide (100 μg/ml), which inhibits more than 95 percent of protein synthesis (as measured by [^{3}H]leucine incorporation). Incubation at 37°C of fresh untreated lymphocytes with this dose of cycloheximide, followed up to 24 hours, did not modify the expression of β_2m, as measured by complement-dependent lysis. By contrast, at this time expression of SD-3 antigens is completely suppressed, expression of SD-2 antigens is significantly reduced, and even expression of SD-1 antigens begins to weaken (*4*).

Other laboratories have reported the association of a low-molecular-weight polypeptide, identical or very similar to urinary β_2m, with fractions of cell membrane preparations carrying HL-A specificities (*16, 17*). It is important to note that this "invariant" small polypeptide has been found to be associated with many different HL-A allelic variants controlled by both the SD-1 (LA) and the SD-2 (Four) loci. Association with membrane preparations that could be assigned to SD-3 (AJ) has not yet been studied. This and other considerations have suggested to Peterson and co-workers (*17*) the hypothesis that HL-A antigens are in fact composed of two subunits: a heavier polypeptide, controlled by the polymorphic different SD loci, and a smaller polypeptide, probably corresponding to β_2m, controlled by an apparently nonpolymorphic cistron.

Our data on the effect of anti-β_2m serums upon HL-A antigens at the cell surface greatly support such a model, although many aspects of the phenomenon remain to be investigated, and alternative explanations should be considered. The significant homology between β_2m and C_H3 (heavy chains of constant region) domain of γ1 chains of immunoglobulin G (*18, 19*) adds interest to the association with products of genes located in the HL-A region, because it may imply a recognition function for these products, possibly related to the genetic control of the immune response, to organogenesis, or to functional collaboration between cells (*20*) as suggested by the data of Bach *et al.* (*21*). The geneticist, however, should not be surprised at finding significant homologies even between proteins with apparently unrelated functions. There is little doubt that the precursors of genes now endowed to present organisms are relatively few, because the easiest way for creating new genes is gene doubling followed by differential evolution according to the Darwinian "descent with modification." Homology maintained over long times, however, implies similarity in function. It has been hypothesized that homology between β_2m and constant region of the heavy chains domains of γ1-immunoglobulin may be related to the ability shared by these products to bind to the cell surface (*19*). We suggest that β_2m may increase the stability in the membrane of the associated HL-A antigens.

M. D. POULIK
Department of Immunochemistry, Wayne State School of Medicine, William Beaumont Hospital, Royal Oak, Michigan 48072

MARIETTA BERNOCO
D. BERNOCO, R. CEPPELLINI
Basel Institute for Immunology, Grenzacherstrasse 487, CH-4058 Basel, Switzerland

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14. The reagents were as follows. Rabbit antiserums to human β_2m have been described (*13*); they had been exhaustively absorbed

with red cell stroma and human serum and did not react with either human Ig or human red cells. The majority of antiserums to HL-A were supplied by the Istituto di Genetica Medica, Torino. For HL-A terminology see "Joint Report" in *Histocompatibility Testing 1972* (Munksgaard, Copenhagen, 1973), p. 619. For the third SD (AJ) locus see (*4*). Rabbit antiserums to human Ig and goat antiserum to rabbit Ig, with and without fluorescent label, were prepared by Dr. G. Trinchieri, according to the technique described by L. Amante and M. Giuriani [*Boll. Ist Sieroterapico Milanese* **48**, 407 (1969)]. Horse antiserum to human lymphocytes (ALS) was a gift of Schweiz. Serum & Impfinstitut, Bern. Nutrient medium was RPMI, supplemented with 10 percent normal rabbit serum, repeatedly absorbed with human red cells and buffy coat. Lymphocytes from defibrinated peripheral blood were purified by centrifugation on Urografin and adherent and phagocytic cells were eliminated according to technique of Mayr *et al.* (*4*). Lymphocytotoxicity was carried out according to the NIH microtechnique [D. L. Brand *et al.*, *Histocompatibility Testing 1970* (Munksgaard, Copenhagen, 1970), p. 357].

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Acute Lymphoblastic Leukemic Cells with T (Thymus-Derived) Lymphocyte Markers

Abstract. *Five of nine children with acute lymphoblastic leukemia had lymphoblasts that bound sheep erythrocytes or reacted with antiserum to thymocytes, suggesting involvement of T (thymus-derived) cells. When lymphoblasts from all patients were examined by immunofluorescence they were found to lack a marker for B (bone marrow or bursa-equivalent) cells, that is, the presence of surface immunoglobulins.*

A heterogeneous population of lymphocytes are present in the peripheral blood. Bursa-equivalent or bone marrow–derived lymphocytes (B cells) are characterized by surface immunoglobulins and receptors for a complement component (C3) and aggregated immunoglobulin (*1–3*). Human T (thymus-derived) lymphocytes lack the above receptors but bind washed sheep red blood cells (SRBC's) in a rosette distribution (*3, 4*). Using these markers, other investigators have studied the lymphocytes of human leukemia. Chronic lymphatic leukemia (CLL) represents, in most cases, a monoclonal proliferation of B lymphocytes (*5, 6*). Burkitt's lymphoma of both African (*7*) and non-African (*8*) types often arises in B lymphocytes. Similarly, some leukemias and lymphomas could be expected to be of T cell origin. The cells of a patient with malignant lymphoma that appeared to originate in the thymus were of T origin, as shown by the SRBC rosette technique (*9*). Additionally, a cell line comprised of T lymphoid cells was established in continuous culture from a patient with acute lymphatic leukemia (*10*).

In order to study the possibility that acute lymphoblastic leukemia (ALL), the most common malignancy of childhood, involves T cells, we studied lymphoblasts directly after removal from peripheral blood. We now report that lymphoblasts from some of these children form rosettes with SRBC's and react with antiserum to thymocytes and thus carry membrane markers for T cells.

Patients with ALL or CLL confirmed by biopsies of bone marrow, and normal persons as controls, were included in the study. X-ray evaluation of the mediastinum was performed on all patients with ALL. Chronic lymphatic leukemia patients were adults with significant lymphocytosis (more than 30,000 lymphocytes per cubic millimeter). At the time the children with ALL were studied, they were not on treatment and had a significant percentage of lymphoblasts (46 to 99 percent) in the peripheral blood. Peripheral blood was separated on a Ficoll-Hypaque gradient. The separated mononuclear cells were washed three times and preincubated with latex particles so that contaminating monocytes, which phagocytosed the particles, could be distinguished from lymphocytes and lymphoblasts. These mononuclear cells were then resuspended in phosphate-buffered saline, mixed with washed SRBC's, and incubated at 4°C for 1 hour according to the method of Jondal *et al.* (*3*). Cells were considered to be positive when three or more SRBC's were bound to the surface of latex-negative cells. Confirmatory SRBC rosette data were obtained by using cytocentrifuge-prepared, stained slides.

Antiserum to human thymocytes was produced by multiple immunizations of a goat with viable human thymocytes. The antiserum was subsequently absorbed with human erythrocytes and lymphocytes from a patient with CLL of B lymphocyte origin, in which more than 90 percent of cells were bearing surface immunoglobulins. After absorption, the antiserum was used at a 1 : 100 dilution in a cytotoxicity assay. Equal volumes of antiserum, undiluted rabbit complement, and lymphocytes were incubated at 37°C for 45 minutes, atfer which the cells positive for trypan blue were determined. Complement controls were subtracted to give the reported figures.

Lymphocyte surface immunoglobulins were detected by using fluorescein conjugated monovalent antiserums against μ, γ, and α heavy chains and κ and λ light chains in a previously described method (*11*). Reported results are totaled from the heavy or light chain determinations.

In peripheral blood of normal individuals, 60 determinations yielded 50 to 78 percent lymphocytes forming rosettes with SRBC's (Table 1). In three persons with CLL, 1 to 6 percent of peripheral lymphocytes bound SRBC's, indicating that the majority of the lymphoid cells were not of T cell

Table 1. T and B surface markers from peripheral blood lymphoid cells from normal controls and from patients with leukemia. Numbers in parentheses indicate the number of individuals tested.

Patients	T cells		B cells
	Rosette-forming cells	Thymocyte antigen (% cytotoxicity)	Surface immunoglobulins* (%)
Normal controls	50–78% (60) (1100–2800/mm³)	50–70 (13)	14–33 (10)
Chronic lymphatic leukemia	1–6% (3) (1200–2700/mm³)	0–5 (6)	0–99 (12)
Acute lymphoblastic leukemia	1–68% (9) (660–151,500/mm³)	3–69 (4)	1–24 (7)

* Determined by immunofluorescence.