RELATIONSHIP BETWEEN HL-A ANTIGENS AND β2-MICROGLOBULIN AS STUDIED BY IMMUNOFLUORESCENCE ON THE LYMPHOCYTE MEMBRANE*

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Human β2-microglobulin (β2-μ),[1] a protein of 11,600 mol wt, initially isolated by Berggård and Bearn (1) in the urine of patients with kidney tubular dysfunction, recently attracted particular attention, following two lines of findings. On the one hand, the amino acid sequence of this protein (2–4) displays a partial homology with constant domains of IgG1 heavy chains, which evokes an evolutionary link (2, 3) between the genes coding for β2-μ and Ig, and stimulated speculations concerning a possible biological function of β2-μ. On the other hand, a number of reports demonstrated the ubiquity of the distribution of β2-μ, which was found on the membrane of most human cell types studied, including B (5, 6) and T lymphocytes (7), polymorphonuclear cells (8), and platelets (8) and which was synthesized by both lymphoid (9–11) and nonlymphoid cells (5). However, like HL-A antigens, β2-μ is not detected on erythrocytes (reference 8 and footnote 2).

Purified preparations of papain-solubilized HL-A antigens were shown to contain two noncovalently-bound polypeptide chains (12, 13): one with a mol wt around 30,000, bears the serological HL-A specificities, and the other with a mol wt of 11–12,000 was recently identified (reference 13 and footnote 2) as β2-microglobulin. It is not known, however, if these two polypeptide chains, which are found associated after solubilization procedures, are bound together at the cell membrane. In order to elucidate this point, we have used a "differential redistribution" method of immunofluorescence (IF), already described and applied to the study of the topographical relationships between histocompatibility and other antigens at the cell membrane (14–16). Results

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[1] *Abbreviations used in this paper:* β2-μ, human β2-microglobulin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IF, immunofluorescence; R, rhodamine; TRITC, tetramethyl-rhodamine-isothiocyanate.

[2] Petersen, P. A., L. Rask, and J. B. Lindblom. 1973. Highly purified solubilized HL-A antigens contain β2-microglobulin. *Proc. Natl. Acad. Sci. U. S. A.* In press.

show that the migration of $\beta 2$-μ into spots and caps,[3] induced by specific anti-$\beta 2$-μ antibodies and anti-Ig on the lymphocyte surface, modifies the distribution of HL-A antigens, which become undetectable on the membrane outside the $\beta 2$-μ-anti-$\beta 2$-μ caps formed, thus suggesting an association between HL-A antigens and $\beta 2$-μ. However, the redistribution of HL-A antigens by specific antibodies does not apparently displace all surface $\beta 2$-μ.

Materials and Methods

Cell donors were healthy volunteers of known HL-A phenotype or genotype, most of them of O blood group. Lymphocyte suspensions were prepared from defibrinated blood, as previously described (17) using a Ficoll-triosil method (18) (Pharmacia Fine Chemicals, Inc., Upsalla, Sweden).

The preparation and characteristics of rabbit anti-$\beta 2$-μ sera no. 814 and no. 815 have been described in detail elsewhere.[3] Rabbits were immunized with urinary $\beta 2$-μ purified from a patient of known HL-A phenotype (HL-A3, W28, W15, and W10). Anti-$\beta 2$-μ antibody activity was determined by cytotoxicity (19) on a panel of lymphocytes, by passive hemagglutination of sheep erythrocytes coated with urinary $\beta 2$-μ concentrates,[3] and by indirect IF.[3] After absorptions on human erythrocytes and on human-insolubilized IgG,[3] both anti-$\beta 2$-μ sera appeared monospecific by immunodiffusion, and did not agglutinate human erythrocytes nor IgG-coated sheep red blood cells.[3] Their reactivity in IF was abolished after absorption on human lymphocytes. The lymphocyte-staining capacity of the derived anti-$\beta 2$-μ fluorescent conjugates was blocked by preincubation of cells in unconjugated anti-B2-μ serum or by addition of a highly purified preparation of $\beta 2$-μ,[3] which failed to suppress staining by anti-HL-A conjugates.

Polyspecific anti-HL-A (T2) serum and anti-HL-A2, anti-HL-A5, and anti-W5 sera from multiparous women or polytransfused subjects were kindly provided by J. Dausset (Hôpital Saint Louis, Paris, France) and their titers determined by lymphocytotoxicity (19). A goat antirabbit IgG antiserum was purchased from Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif.

Fluorescent conjugates were prepared as previously described (14) using either fluorescein isothiocyanate (FITC) (Isomer I, Sigma Chemical Co., St. Louis, Mo.) or tetramethylrhodamine-isothiocyanate (TRITC) (Baltimore Biological Laboratories, Baltimore, Md.). The characteristics of conjugates are given in Table I. All conjugates were further absorbed on packed human erythrocytes (1 vol/2 vol conjugate) for 1 h at 37°C and 1 h at 0°C. Monospecific anti-HL-A conjugates were absorbed on lymphocytes devoid of the corresponding antigens (10^8 cells/ml conjugate).

The other conjugates used were a TRITC-conjugated goat antirabbit IgG and a commercial FITC-conjugated goat antirabbit Ig (Hyland Div., Travenol Laboratories, Inc.), both absorbed on human erythrocytes and lymphocytes, a FITC-conjugated goat anti-human γ-chain, antihuman Ig (Hyland Div., Travenol Laboratories, Inc.), and antihuman μ-chain (Meloy Laboratories Inc., Springfield, Va.).

Procedures for inducing the redistribution of all detectable $\beta 2$-μ at the cell surface have been described in detail elsewhere.[3] 1.5×10^6 lymphocytes were incubated three times at 0°C for 15 min in 50 μl of anti-$\beta 2$-μ serum 814 or 815 at a 1:2 dilution, washed three times at 0°C with Eagle's minimal essential medium (MEM) supplemented with 15% fetal calf serum

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[3] Bismuth, A., C. Neauport-Sautes, F. M. Kourilsky, Y. Manuel, T. Greenland, and D. Silvestre. Distribution and mobility of $\beta 2$-microglobulin on the human lymphocyte membrane: immunofluorescence and immunoferritin studies. *J. Immunol.* In press.

TABLE I

Characteristics of Conjugates

Conjugate	Isolation of IgG	F/P** ratio	Absorbed with:	Antibody titer	Dilution used in IF	% labeled cells
FITC anti-HL-A2 (MEN . . .)	$(NH_4)_2SO_4$	2.4	Human erythrocytes and HL-A1, 3, 7, Da 31 Lymphocytes	1/32*	1/2	91
TRITC anti-HL-A2 (MEN . . .)	$(NH_4)_2SO_4$	2.2	Human erythrocytes and HL-A3, 11, 7, W22 Lymphocytes	1/32*	1/2	70
FITC anti-HL-A5 (CLE . . .)	$(NH_4)_2SO_4$	2.4	Human erythrocytes and HL-A2, 10, W10 Lymphocytes	1/8*	1/2	91
TRITC anti-HL-A5 (CLE . . .)	$(NH_4)_2SO_4$	2.2	Human erythrocytes and HL-A2, 2, W10, Da 30 Lymphocytes	1/5*	1/2	69
FITC anti-HL-A polyspecific (T2)	DEAE	1.5	Human erythrocytes	1/3*	1/1	50
TRITC anti-HL-A polyspecific (T2)	DEAE	0.9	Human erythrocytes	1/12*	1/2	90
FITC rabbit‡ Anti-$\beta 2$-μ (814 F)	$(NH_4)_2SO_4$	1.6	Human erythrocytes	1/640§	1/1	50‖
TRITC rabbit‡ Anti-$\beta 2$-μ (814 R)	$(NH_4)_2SO_4$	2.7	Human erythrocytes	1/320§	1/1	90‖
FITC rabbit‡ Anti-$\beta 2$-μ (815 F)	$(NH_4)_2SO_4$	1.2	Human erythrocytes	1/160§	1/1	<50‖
TRITC rabbit‡ Anti-$\beta 2$-μ (815 R)	$(NH_4)_2SO_4$	2.7	Human erythrocytes	1/80§	1/1	80‖

* Antibody titer determined by microlymphocytotoxicity (20).

‡ Antisera were previously absorbed with human erythrocytes and polymerized Ig.

§ Antibody titer determined by passive hemagglutination.[2]

‖ Mean values. Percentages of labeled cells varied with different individual lymphocytes.

** Fluorochrome/protein molar ratio.

(FCS), and labeled 30 min at 0°C with 0.05 ml of FITC or TRITC-conjugated goat anti-rabbit Ig at a 1:20 dilution. After three washings, labeled cells were incubated 1 h at 37°C in 0.5 ml of culture medium to allow redistribution of antigens. The time evolution of redistribution was determined, as previously described,[3] by sampling cells at time intervals during the incubation period. In order to restain the cells after redistribution, cells were cooled and labeled for 30 min at 0°C with anti-HL-A, anti-β2-μ, or anti-Ig, conjugated with the opposite fluorochrome, and washed three times at 0°C.

For redistribution of HL-A antigens, lymphoid cells were incubated at 37°C for 30 min with 50 μl of anti-HL-A fluorescent conjugate, washed three times, and further incubated at 37°C for 45 min in 0.5 ml of culture medium. After, redistribution, cells were cooled and restained with anti-β2-μ or anti-HL-A conjugates at 0°C. In some experiments, in order to saturate and redistribute all the available HL-A antigenic determinants, three successive incubations at 0°C for 15 min were performed in each anti-HL-A antiserum diluted 1:2, followed by labeling with goat antihuman γ-chain conjugate, and redistribution was allowed at 37°C during 30 min. Cells were fixed as previously described[3] and slides were examined with a Leitz Ortholux fluorescence microscope (E. Leitz, Wetzlar, West Germany), equipped with a Ploem type epi-illumination system and with the proper combination of interferential filters for discriminating between fluorescein and rhodamine.[3]

RESULTS

Comparative Redistribution of β2-Microglobulin and HL-A Antigens.—As described elsewhere,[3] the redistribution of β2-μ cannot be induced by anti-β2-μ antibodies alone but requires the addition of anti-Ig to the anti-β2-μ antibodies. On the surface of lymphocytes stained with a fluorescent anti-β2-μ conjugate and treated with a goat antirabbit Ig at 0°C, the membrane fluorescence is initially diffuse. After incubation at 37°C, fluorescence clusters in spots on all cells, and forms polar caps on 15–60% of cells.[3] The evolution of the redistribution of β2-μ during a 2-h incubation period is illustrated in Fig. 1.

By comparison, Fig. 2 illustrates the evolution of redistribution of HL-A antigens on the same lymphocyte suspension used in Fig. 1. Lymphocytes are stained with a fluorescent anti-HL-A conjugate, and treated or not treated with antihuman Ig antibodies. As shown previously (17) the redistribution of HL-A antigens can be induced by anti-HL-A antibodies alone, and is accelerated by the addition of anti-Ig antibodies.

Distribution of HL-A Antigens after Redistribution of β2-Microglobulin.—Conditions were preliminarily defined in order to induce the redistribution of all detectable β2-μ molecules at the cell surface[3]: lymphocytes were saturated by three successive incubations in rabbit anti-β2-μ serum 814 or 815 and redistribution induced at 37°C after labeling with FITC-conjugated goat anti-rabbit Ig. In such conditions, TRITC-conjugated anti-β2-μ 814 rhodamine (R) or 815 R failed to detect β2-μ outside the green spots and caps already formed, and the redistribution was considered to be complete, involving all β2-μ molecules (Fig. 3). Using these experimental conditions, after staining and migration of β2-μ at 37°C, lymphocytes were labeled with the various TRITC anti-HL-A conjugates at 0°C and the respective location of rhodamine and fluorescein labeling was determined on about 100 capped individual cells in each experi-

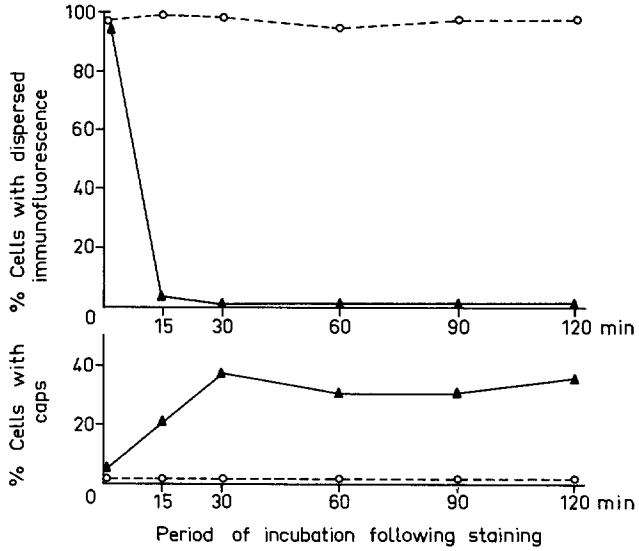

FIG. 1. Redistribution of $\beta 2$-μ. Lymphocytes labeled at 0°C with TRITC-conjugated rabbit anti-$\beta 2$-μ 814 R alone (O- - -O) or with 814 R and goat antirabbit IgG diluted 1:10 (▲——▲) and incubated at 37°C. Only cells with diffuse fluorescence and caps, but not cells with patchy fluorescence, are plotted. Redistribution occurs only after incubation in anti-Ig.

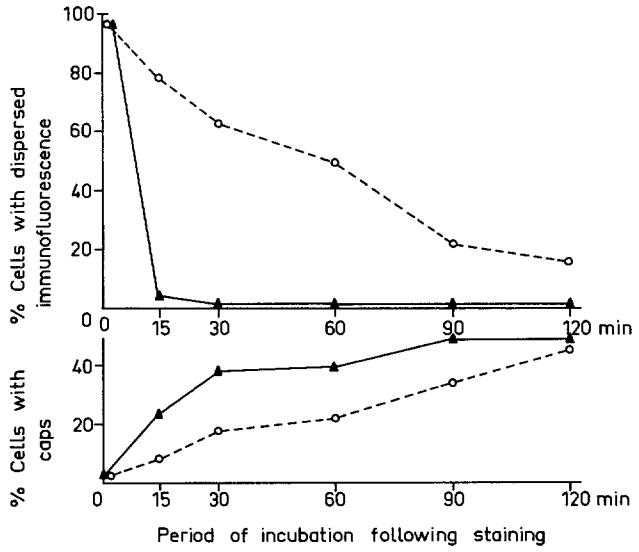

FIG. 2. Redistribution of HL-A antigens. Same lymphocytes as in Fig. 1, labeled with TRITC-conjugated human anti-HL-A (T2 R) alone (O- - -O) or with T2 R and goat anti-human IgG serum diluted 1:10 (▲——▲). Redistribution occurs with anti-HL-A antibodies alone, and is accelerated by a second layer of anti-Ig.

ment. No rhodamine labeling (anti-HL-A) could be seen outside the green anti-β2-μ spots and caps on almost all cells, as illustrated in Fig. 4 and Table II. The same results were obtained using different lymphocytes and the different anti-HL-A conjugates. In contrast, upon restaining of the same cells with anti-Ig conjugates after redistribution of anti-β2-μ anti-Ig complexes, surface Ig was found diffuse on B cells and labeled the cell surface outside the β2-μ caps (Fig. 5), confirming previous results (20). In some experiments, in order not to redistribute all β2-μ molecules, lymphocytes were coated with non-saturating dilutions of anti-β2-μ sera. In these cases, after the redistribution of anti-β2-μ antibodies with antirabbit Ig conjugates, restaining of the cell membrane could be obtained with anti-HL-A as well as with anti-β2-μ conjugates, outside the β2-μ caps previously formed.

Labeling of β2-Microglobulin after Redistribution of HL-A Antigens.— Lymphocytes were labeled at 37°C with FITC-conjugated anti-HL-A2 antiserum, washed, incubated at 37°C in order to induce redistribution of HL-A antigens, and then labeled at 0°C with TRITC anti-β2-μ conjugates 814 R or 815 R. The rhodamine labeling was found diffuse outside the green anti-HL-A clusters as shown in Table III. Under these same conditions, a second staining with the same anti-HL-A2 serum, TRITC conjugated, failed to label the membrane outside the green caps and was even blocked by the previously fixed conjugate, indicating that all detectable HL-A2 antigenic sites had been redistributed. Similar results were obtained with anti-HL-A5 antibodies. (Table III).

In order to better insure the saturation of HL-A antigenic sites by the anti-HL-A antibodies, lymphocytes were incubated three times for 15 min at 37°C in anti-HL-A2 or polyspecific anti-HL-A (T2) sera diluted 1:2, and redistribu-

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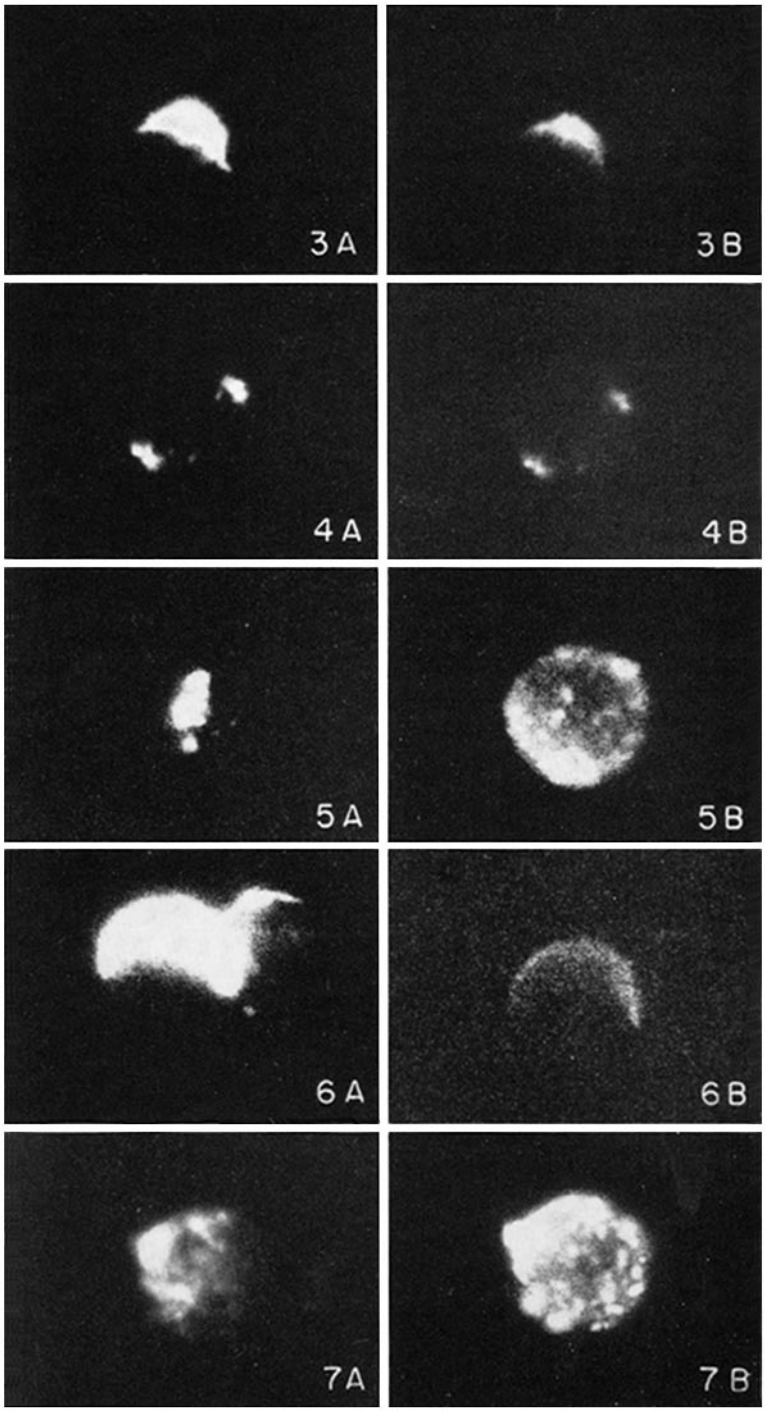
FIGS. 3–7. (3) Lymphocyte labeled with rabbit anti-β2-μ 814 serum and FITC-conjugated goat antirabbit IgG, then incubated at 37°C, and finally stained at 0°C with the TRITC-conjugated anti-β2-μ 814. 3A, clusters of fluorescein (anti-β2-μ); 3B, no rhodamine labeling (anti-β2-μ) is seen outside the green caps. (4) Lymphocyte labeled with rabbit anti-β2-μ 814 antiserum and FITC-conjugated goat antirabbit Ig, then incubated at 37°C, and finally stained at 0°C with the R anti-HL-A (T2) conjugate. 4A, cap of fluorescein (anti-β2-μ); 4B, no rhodamine labeling (anti-HL-A) is found outside the cap. (5) Lymphocyte labeled with rabbit anti-β2-μ 814 serum and TRITC-conjugated goat antirabbit Ig, then incubated at 37°C, and finally stained at 0°C with FITC-conjugated goat antihuman μ-chain conjugate. 5A, Clusters of rhodamine (anti-β2-μ); 5B, diffuse fluorescein labeling (anti-μ) of μ-chains on the same cell. (6) Lymphocytes labeled with anti-HL-A (T2) serum and FITC-conjugated goat antihuman γ-chain, then incubated at 37°C, and finally stained at 0°C with TRITC conjugated anti-HL-A (T2). 6A, Cap of fluorescein (anti-HL-A); 6B, no rhodamine labeling (anti-HL-A) is seen outside the green cap. Restaining of the green cap is probably due at least in part, to the presence of goat anti-γ-chain antibodies. (7) Lymphocyte labeled with anti-HL-A (T2) serum and FITC-conjugated goat antihuman γ-chain, then incubated at 37°C, and finally stained at 0°C with anti-β2-μ antiserum and TRITC-conjugated goat antirabbit Ig. 7A, Cap of fluorescein (anti-HL-A); 7B, rhodamine labeling (anti-β2-μ) is seen outside the anti-HL-A cap.


3A
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tion induced at 37°C after treatment with antihuman γ-chain conjugate. As shown in Table III and Fig. 7, β2-μ was still detectable on the cell membrane outside the green caps (HL-A) upon staining at 0°C with TRITC anti-β2-μ conjugates or with anti-β2-μ serum and anti-Ig conjugate, whereas HL-A antigens were no more detectable outside the HL-A caps previously formed (Fig. 6). In some experiments, however, using polyspecific serum T2, a concomitant redistribution of HL-A and β2-μ labeling was observed on 10–30% of lymphocytes.

Since different HL-A antigens migrate independently on the cell surface (16), the above experiments were unlikely to involve all serologically detectable HL-A antigens of the lymphocyte surface, even using multispecific antisera. Another experiment was performed using cells from a donor genotyped through family studies, who was homozygote at the two HL-A loci and whose HL-A type was HL-A2, HL-A2/W5, W5. The lymphocytes were incubated three times at 37°C for 15 min with the anti-HL-A2 (MEN . . .) and then with the anti-W5 (SAL . . .) sera diluted 1:2, stained with a FITC-conjugated goat antihuman Ig, and further incubated at 37°C to allow redistribution. Restaining at 0°C with TRITC-conjugated anti-HL-A2 (MEN . . .) failed to detect residual HL-A2 antigen outside green caps, whereas β2-μ was still readily detectable all over the cell membrane on most lymphocytes, after staining with either anti-β2-μ conjugate 814 R or 815 R serum and TRITC-conjugated anti-rabbit Ig. These data indicate that in our experimental conditions, the redistribution of HL-A antigens did not provoke the redistribution of all the β2-μ molecules of the lymphocyte membrane.

DISCUSSION

Most membrane proteins are presently conceived as mobile units in the fluid lipid bilayer of the cell membrane (21), and alteration of their random distribution on living cells can be induced in certain conditions by cross-linking agents, such as antibodies (22). Some antigens, such as surface Ig (22, 23) or histocompatibility antigens (14, 17) can be aggregated into spots and polar caps by bivalent antibodies alone, whereas other antigens, such as θ-antigen (22), antilymphocytic serum antigens (17), or β2-μ[2] require the addition of anti-Ig antibodies to be mobilized. The experiments reported here utilize this antibody-induced redistribution process to study the relationships between β2-μ and HL-A antigens on the surface of human lymphocytes, according to the following principle: if noncross-reactive antigens are associated on the same molecule or structure at the cell surface, one can expect that the redistribution of one antigen induced by the corresponding antibody, as detected by IF, will redistribute also the second antigen, and vice versa. This method was already described and applied to the study of the molecular relationships between surface antigens of the lymphocyte membrane (14, 16, 20).

Our results indicate that after the redistribution of all detectable β2-μ with rabbit anti-β2-μ antibodies and fluorescent antirabbit Ig conjugate, the lym-

TABLE II

Effect of the Redistribution of β2-μ Molecules on the Distribution of HL-A Antigens

First labeling at 37°C	Second labeling at 0°C	% of cells where both labelings are aggregated at the same place	% of cells where the first labeling is aggregated and the second diffuse
Rabbit anti-β2-μ and FITC goat antirabbit Ig	TRITC anti-β2-μ (814)	92	8
	TRITC anti-β2-μ (815)	93	7
	TRITC anti-HL-A polyspecific (T2)	96	4
	TRITC anti-HL-A2 (MEN. . .)	90	10
Rabbit anti-β2-μ and TRITC goat anti-rabbit Ig	FITC anti-β2-μ (815)	100	0
	FITC anti-HL-A (T2)	92	8
	FITC anti-HL-A2 (MEN. . .)	100	0
	FITC antihuman Ig	0	100
	FITC anti-human μ-chain	20	80

phocyte surface could be stained no more by anti-HL-A conjugates outside the β2-μ caps already formed. The simplest interpretation is that HL-A antigens and β2-μ are bound together at the cell surface, and that the redistribution of β2-μ has therefore provoked the simultaneous migration of HL-A antigens. However, such an interpretation needs to be discussed and placed within the limits of the technique used. The restaining by anti-HL-A conjugates of the caps where β2-μ is aggregated (cocapping) is of little value in our experience since nonspecific adsorption of irrelevant anti-HL-A or normal human Ig conjugates was observed on such antigen-antibody anti-Ig complexes, even in the absence of detectable immunological cross-reactivity between the reagents used. Therefore, the only relevant feature is the absence of staining of the remaining membrane by anti-HL-A conjugates, after capping of β2-μ. In the same conditions, surface Ig, which were shown to migrate independently of β2-μ,[3] were diffusely stained on B lymphocytes by antihuman Ig conjugates. In our experimental conditions, all detectable β2-μ and anti-β2-μ antibodies were redistributed in the spots and caps, and were not detectable outside. The concomittant alterations of the distribution of β2-μ and HL-A antigens are unlikely to be explained by a simple cross-reactivity between these two antigens since purified β2-μ blocked the staining capacity of anti-β2-μ, but not that of an anti-HL-A conjugate used, and since some target lymphocytes selected had no HL-A antigens in common with the donor of β2-μ used for immunization of

TABLE III

Effect of the Redistribution of HL-A Antigens on the Distribution of β2-μ

First labeling at 37°C	Second labeling at 0°C	% of cells where both labelings are aggregated at the same place	% of cells where the first labeling is aggregated and the second diffuse
FITC anti-HL-A2	TRITC anti-β2-μ (814)	0	100
	TRITC anti-β2-μ (815)	0	100
FITC anti-HL-A5	TRITC anti-β2-μ (814)	0	100
	TRITC anti-β2-μ (815)	0	100
Anti-HL-A2 and F goat antihuman γ-chain	TRITC anti-HL-A2 (MEN. . .)	100	0
	Anti-β2-μ (814) and TRITC goat anti-rabbit IgG	24	76
Anti-HL-A polyspecific (T2) and F goat antihuman γ-chain	TRITC anti-HL-A (T2)	100	0
	TRITC anti-HL-A2 (MEN. . .)	99	1
	TRITC anti-β2-μ (814)	8	92
	Anti-β2-μ (814) and TRITC goat anti-rabbit IgG	14	86

rabbits. Nevertheless it cannot be totally excluded that anti-β2-μ rabbit sera react with a "constant" portion of the HL-A polypeptide chain, although such cross-reaction was not detected.[3]

After redistribution of HL-A antigens with anti-HL-A antibodies, all β2-μ molecules were not redistributed in the aggregates and caps formed. This result is apparently not simply due to the fact that all independently migrating (16, 24) HL-A antigens were not moved by the anti-HL-A antibodies used, since failure to redistribute all β2-μ persisted upon careful saturation and redistribution of the four main serologically detectable HL-A antigens on lymphocytes from a genotyped donor. These data may therefore suggest that all β2-μ is not bound to HL-A antigens, or that the HL-A polypeptide chains are loosely bound to β2-μ, and plucked off by antibody in the process of redistribution.

In spite of all the above mentioned restrictions, the data presented here are compatible with the concept that HL-A polypeptide chains are associated with β2-μ molecules on the lymphocyte membrane, as suggested from chemical

studies (13), but apparently indicate that all $\beta 2$-μ molecules are not bound to HL-A antigens. The significance of such an association is far from being clear as yet. Beside the fact that it is not known if all $\beta 2$-μ found on the lymphocyte membrane is synthesized by the cell or adsorbed on it, a partial association between $\beta 2$-μ and HL-A antigen may bear very different meanings. $\beta 2$-μ may represent either a specific ligand for HL-A polypeptide chains at the cell surface, or a molecule with peculiar binding capacities, capable to attach to various molecules at the cell surface, among which are HL-A antigens, but eventually also other substances (25).

SUMMARY

The antibody-induced redistribution of $\beta 2$-microglobulin ($\beta 2$-μ) and HL-A antigens on the surface of living lymphocytes was studied by immunofluorescence. When all $\beta 2$-μ was redistributed on the lymphocyte membrane by specific rabbit antibodies and goat antirabbit Ig conjugates, the HL-A antigens were no more detectable with anti-HL-A conjugates outside the $\beta 2$-μ caps already formed. However, the redistribution of HL-A antigens fails to provoke the redistribution of all detectable $\beta 2$-μ molecules. These results suggest that HL-A antigens may be associated with $\beta 2$-μ at the cell surface, but that all $\beta 2$-μ molecules are not bound to HL-A antigens.

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