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Passive modulation of blood-group antigens

(antigen projection/lipid fluidity/D antigen/A antigen/erythrocyte membrane)

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ABSTRACT **Rh-positive human erythrocytes were enriched and depleted of membrane cholesterol, and the mediated change in the degree of exposure of the D antigens was determined by fluorescence-activated cell sorting, using indirect fluorescent antibody labeling. The results are compatible with a model in which the expression of the D antigens can be modulated significantly by the lipid microviscosity ($\bar{\eta}$). At a high cholesterol-to-phospholipid ratio (C/PL) of 1.55, which corresponds to $\bar{\eta}$ (25°C) = 7.5 poise (1 poise = 0.1 Pa·sec), the relative detectable number of D antigens was about double than that at C/PL = 0.65, $\bar{\eta}$ (25°C) = 4.1 poise. In analogous experiments similar fluidity changes resulted in only about 20% modulation of expression of the A_1 antigen, suggesting that in the native state this antigen is already well exposed on the erythrocyte surface. This type of antigenic modulation may also operate *in vivo*, and may thus bear some fundamental implications on tumor immunology and autoimmune diseases.**

Integral proteins of the cell membrane can be divided into those that freely diffuse in the lipid matrix and those for which mobility is determined by the state of polymerization of the cytoskeleton network to which they are anchored (1–3). The equilibrium position of the freely diffusing proteins can be described formally as the state of minimum free energy of all intermolecular interactions of the protein with the ambient aqueous and lipid phases. Hence, this position has the quality of partitioning which, in principle, can be shifted by perturbations in the immediate lipid domain (4, 5). Based on experimental evidence and thermodynamic arguments, it has been asserted that upon increase in the membrane lipid microviscosity the protein–lipid interaction will decrease and the equilibrium position of the protein will be shifted more towards the aqueous domains. Decrease in the lipid microviscosity will result in the converse shift in equilibrium position (6, 7). In this study, we have demonstrated that the projection of the blood-group antigens D and A_1 can be modulated passively and reversibly, though to different extents, by changes in the erythrocyte membrane microviscosity.

MATERIALS AND METHODS

Freshly-drawn human erythrocytes (type O^+ or A_1^-) were first subjected to treatments for changing the membrane cholesterol level and then the availability of the blood-group antigens was monitored. For mild changes in cholesterol content treatments with lipid-modified sera were employed as described (8). Greater cholesterol changes were achieved by treatment with mixed liposomes of egg lecithin and cholesterol (9). Chemical analysis of cholesterol (10) and phospholipids (11), as well as determination of the lipid microviscosity (12), were carried out on the isolated erythrocyte membranes (13). The antigenic modulation was correlated with the mole ratio of cholesterol to phospholipids (C/PL), with the degree of fluorescence polarization (P) of the lipid fluidity probe 1,6-diphenyl-1,3,5-hexatriene (12), and with the corresponding lipid microviscosity ($\bar{\eta}$).

Assessment of the number of detectable D antigens on the treated erythrocytes was carried out with a fluorescence-activated cell sorter (B-D model II). Because direct labeling with fluoresceinated anti-D antigen resulted in a rather weak fluorescence tagging, we employed an indirect fluorescence staining. After cholesterol enrichment or depletion, Rh-positive erythrocytes were washed three times with phosphate-buffered saline and then incubated with an excess of anti-D serum (OrthoDiagnostics, Raritan, NJ; 1:10 titer), which has been verified before to be sufficient for maximum binding of Rh-specific antibodies. The treated cells were washed three times with phosphate-buffered saline and then mixed with rabbit anti-human IgG, fluorescently labeled with 3.3 mol of fluorescein isothiocyanate per mol of protein (Cappel, Cochranville, PA; 1:4 titer). Thereafter, the fluorescently labeled cells were washed twice with phosphate-buffered saline and dispersed at 10^6 per ml of phosphate-buffered saline for analysis of fluorescence intensity distribution with the cell sorter. In this method the fluorescence intensity of individual cells is registered, and the accumulated profile essentially represents the relative distribution of number of antigens per cell (14). Analogous procedures were used for monitoring the distribution profiles of the A_1 antigens.

RESULTS AND DISCUSSION

The D antigen of the Rh antigenic system, Rh(D), is an integral membrane protein with a high affinity for phosphatidylcholine and phosphatidylethanolamine (15, 16). Its number generally varies in the range of 10,000–30,000 per erythrocyte, according to the Rh genotype (17, 18). Fig. 1 presents the antigen-distribution profiles for Rh(D) erythrocytes enriched with cholesterol (C/PL = 1.55) and depleted of cholesterol (C/PL = 0.65). With unmodified cells (C/PL = 0.95), the obtained profiles appeared in between the ones presented in the figure (see Fig. 2). The antigenic distribution patterns for treated and untreated cells from different sources appeared to obey a Gaussian distribution of a constant width at the half of the fluorescence peak channel, F_{max}, of approximately $F_{max}/2$. Both the shape and the position of the obtained profiles could be reversibly manipulated by changing the C/PL of the membrane in any direction.

The dependence of average number of detectable Rh antigens on the C/PL of the erythrocyte membranes, presented in Fig. 1, is shown in Fig. 2, curve A. Analogous and separate results, obtained with Rh-positive erythrocytes containing fewer D antigens, are given in Fig. 2, curve B. As shown, for the two samples of erythrocytes the number of detectable Rh(D) antigens increased by about 2-fold between the extreme cholesterol levels of C/PL = 0.65 and 1.55. The antigen distribution profiles obtained with the second sample also obeyed the same

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Abbreviation: C/PL, cholesterol-to-phospholipid mole ratio.

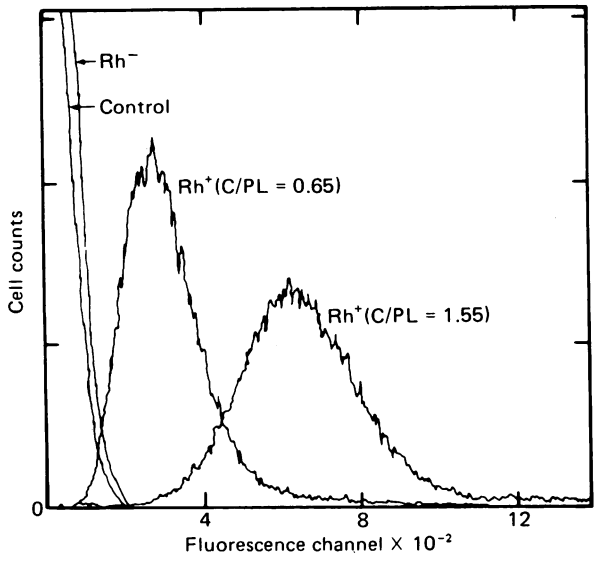

FIG. 1. Distribution of D antigens in Rh^+ human erythrocytes (type O) simulated by fluorescence intensity profiles of attached fluorescent antibodies. Erythrocytes were treated by liposomes for cholesterol enrichment or depletion (9) for 22 hr at 37°C. C/PL in the cholesterol-enriched sample was 1.55 and the corresponding membrane microviscosity (12), $\bar{\eta}$, at 25°C was 7.5 poise (1 poise = 0.1 Pa·sec) (P = 0.363). In the cholesterol-depleted erythrocytes these parameters were C/PL = 0.65 and $\bar{\eta}$ (25°C) = 4.1 poise (P = 0.309). After cholesterol modulation the erythrocytes were first treated with a large excess of anti-D, washed, and then treated with fluoresceinated rabbit anti-human IgG. The fluorescently labeled cells (over 99% of the total) were analyzed immediately in a cell sorter, and the distribution profiles were recorded with 50,000 cells. In the control experiment the same erythrocytes were directly incubated with the fluoresceinated rabbit anti-human IgG without preincubation with anti-D. In another control experiment Rh^- human erythrocytes (type O) were treated identically to the analyzed ones. The recorded profiles for these experiments are also presented.

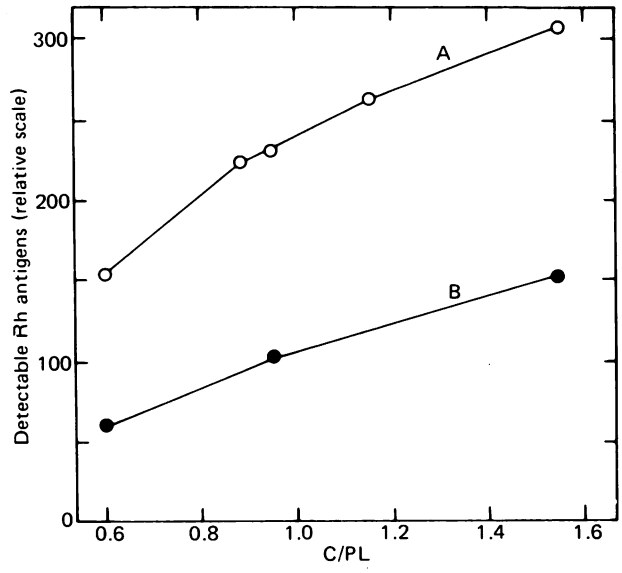

FIG. 2. Average number of detectable D antigens in two samples of Rh-positive human erythrocytes (type O) of different membrane cholesterol levels. Membranes of curve B contain fewer D antigens than those of curve A. The presented points are the fluorescence peak channel, F_{max}, obtained with a cell sorter (see legend to Fig. 1). The extreme points in both samples were obtained after treatments with liposomes (9) for 22 hr at 37°C, whereas all the middle points were obtained after treatments for up to 10 hr at 37°C with lipid-modified sera (8).

empirical Gaussian distribution obtained with sample A. The above findings therefore suggest that the D antigens act independently, although each antigen senses in a similar manner the dynamics of the membrane lipids. This conclusion is in line with the observation that D antigens in human erythrocytes of various Rh genotypes are evenly distributed on the plane of the membrane (18).

Fluorescence-activated cell sorting was carried out analogously with erythrocyte type A_1. The current notion of the A, B, and H antigens is of a branched polysaccharide attached to a sphingolipid, which is firmly associated with a glycoprotein that by itself does not exhibit any blood-group-specific activity (19, 20). The number of antigenic sites of this group varies widely among individuals, in the range of 10^5–10^6 antigens per erythrocyte (21), as is classified in genotypic subgroups. After establishment of conditions for minimum agglutination and maximum binding, the cholesterol-modified cells were first treated with anti-A human serum (Ortho, titer 1:10) and then with the fluoresceinated rabbit anti-human IgG, as in the previous experiments. However, the effect of cholesterol enrichment or depletion on the distribution profiles was small and the shift in F_{max} from the control value, although in the same directions as was observed for the D antigens, never exceeded 20%. Furthermore, when Rh-positive A_1 erythrocytes were monitored simultaneously for exposure of D and A antigens, the modulation of the D antigens by C/PL was much more pronounced than the modulation of the A antigens. The changes in C/PL in the A_1 erythrocytes also affected their agglutinability with anti-A_1. A typical example of agglutination titers is given in Table 1. The changes in agglutinability indicate a small but consistent increase in the availability of the blood-group A antigens upon increase of C/PL in the erythrocyte membrane. The above observations suggest that, unlike the partial exposure of the Rh antigens, the antigenic determinants of the A, B, and H blood-groups are well exposed to the aqueous surrounding and are only slightly affected by changes in membrane fluidity. The high number of detectable antigens of this group in untreated erythrocytes (21) supports this suggestion.

It has previously been proposed (4–6) and verified (6, 7) that upon increase in the membrane lipid viscosity the bulk of the membrane proteins are vertically displaced towards the aqueous layers on both sides of the membrane, and vice versa. The lipid fluidity modulation of any membrane-associated function was termed "passive modulation" (5), because it is spontaneous and does not require metabolic energy. The alterations in the projection of blood-groups D and A_1, which were presented in this study, are typical cases of passive antigenic modulation. The increase in projection of these antigens with the cholesterol level, which is the main physiological determinant of lipid microviscosity in the erythrocyte membrane

Table 1. Correlation between C/PL, membrane microviscosity ($\bar{\eta}$), and agglutinability of type A_1 erythrocytes.

C/PL	$\bar{\eta}$, poise	P	Agglutination titer
0.65	4.1	0.307	1/140
0.95	5.3	0.332	1/280
1.45	7.1	0.358	1/560

Washed erythrocytes were incubated in lipid-modified sera (8) for 12 hr at 37°C. C/PL was evaluated by direct determination of cholesterol and organic phosphate (10, 11). The lipid microviscosity, $\bar{\eta}$, was determined at 25°C by the degree of fluorescence polarization (P) of embedded 1,6-diphenyl-1,3,5-hexatriene (12). Agglutination was performed in a microtiter plate and was assessed microscopically.

(22), is in line with our vertical displacement hypothesis (4–7). Secondary effects, like changes in the membrane surface area or local rearrangements of protein assemblies, may also contribute to the observed passive modulation. However, because a similar modulation of projection of the D antigen was also observed after incorporation of stearic acid, as a membrane rigidifier, and oleic acid, as a membrane fluidizer (data not shown), it may be suggested that the vertical displacement is the major process that accounts for the passive antigenic modulation described in this study. It is also plausible that the marked increase in availability of some blood-group antigens (e.g., I antigen) at a low temperature also originates from increase in exposure, which is imposed by the increase in the lipid viscosity.

Passive modulation of membrane antigens was also observed by us with the θ antigen and the H-2 antigen in lymphocytes (unpublished results). It is intriguing to hypothesize that such processes may take place under physiological conditions, and may therefore have important clinical implications, particularly in autoimmune diseases and tumor immunology (23).

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