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EFFECTS OF MODULATING ERYTHROCYTE MEMBRANE CHOLESTEROL ON Rh_o(D) ANTIGEN EXPRESSION

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SUMMARY

Alteration of Rh_o(D)-antigen expression in Rh-positive erythrocytes after modification of membrane fluidity has recently been demonstrated by indirect fluorescence staining. The isolation of D-antigen from Rh-negative erythrocyte membranes has also recently been claimed. We therefore attempted quantification of D-antigen sites in modified Rh-positive and Rh-negative cells by the direct radiolabelled (^{125}I) antibody uptake technique. The cholesterol/phospholipid molar ratio of erythrocytes was modified with resulting membrane-cholesterol enrichment or depletion. ^{125}I-anti-D uptake doubled in enriched Rh-positive erythrocytes, and was decreased in depleted Rh-positive erythrocytes. No change in anti-D uptake could be shown for Rh-negative erythrocytes similarly modified. If the mechanism for enhanced D-antigen expression in cholesterol enriched erythrocytes is vertical displacement of antigen, our inability to unmask the D-antigen in Rh-negative erythrocytes suggests that it is deeply buried in the lipid matrix.

INTRODUCTION

The detailed chemical structure of the Rh antigens is unknown, but that they are membrane proteins is reasonably well documented (1). The number of Rh_o(D) antigen sites on erythrocytes, estimated by several investigators either by uptake of radiolabelled anti-D (2,3) or by immuno-electron microscopy (4,5), is reported in the vicinity of 10,000-20,000 per erythrocyte. The antigen sites are distributed in an aperiodic manner on the cell surface (5).

Evidence has been presented that interaction of the Rh-carrying molecule with lipids is important for D-antigenic expression (6). In addition Shinitzky et al (7) have shown that D-antigen expression can be modulated by changes in the molar ratio of cholesterol to phospholipid (C/P). According to these authors, when the ratio is increased and membrane lipid microviscosity is thereby increased,

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vertical displacement of membrane proteins may expose previously masked antigenic sites.

Plapp et al. (8) have recently reported that the membranes of Rh-negative erythrocytes actually contain the D-antigen, which is spatially masked from exposure at the external membrane surface. It was suggested that the degree of exposure of the antigen might depend on its hydrophobicity. Based on these observations we considered the possibility that modification of the C/P ratio of Rh negative erythrocytes might expose the D-antigen at the cell surface. In the present study we modified the membrane cholesterol content of Rh-positive and Rh-negative red cells and quantitated their $Rh_o(D)$ binding sites by measuring their ^{125}I-anti-D uptake.

MATERIALS AND METHODS

Human anti-$Rh_o(D)$ (Gamulin Rh, Parke-Davis) was purified by absorption with washed D-negative erythrocytes. Monospecificity was further ascertained by testing against a panel of well-characterized erythrocytes.

Chloramine-T was obtained from Eastman Kodak, Rochester, N.Y. Carrier-free ^{125}I-NaI (20mCi/ml) was obtained from New England Nuclear, Boston, Mass . Cholesterol,egg lecithin and bovine serum albumin (BSA) were Sigma products. The fluorescence probe used was 1,6-diphenyl-1,3,5 hexatriene (DPH) (Aldrich chemicals, Milwaukee, Wisconsin). Sodium metabisulfite and tetrahydrofuran were obtained from Fischer Scientific Company, Fairlawn, New Jersey.

Iodination of Anti-Rh_o (D): Iodination of the pre-absorbed antibody was accomplished by the method of Greenwood et al (9). To 60 μg of anti-$Rh_o(D)$ in 1.0 ml of phosphate-buffered saline (PBS) (pH 7.4) was added 2 mCi of carrier-free ^{125}I-NaI under continuous stirring at 4°C. Next 100 μg of Chloramine-T, dissolved in 0.1 ml PBS, was added dropwise. Ten minutes later an equal amount of sodium metabisulfite was added to neutralize any remaining oxidizing agent and stop the reaction. ^{125}I-anti $Rh_o(D)$ thus prepared was dialyzed against saline for 3-4 days at 4°C with repeated changes of dialysate.

Purification of ^{125}I-labelled antibody. In order to remove ^{125}I-labelled non-antibody proteins, a purification procedure similar to that described by Hughes-Jones et al was used (3). 1 ml of washed packed $Rh_o(D)$-positive red blood cells was incubated with 0.5 ml of ^{125}I-anti-$Rh_o(D)$ for an hour at 37°C with gentle stirring. The cells were washed six times in large excess of 1:10 dilution of human serum in PBS at 0°C, quickly frozen (dry ice/acetone or liquid nitrogen) and allowed to lyse by incubation at 37°C for five minutes. Lysis was completed by addition of 20 milliosmolar phosphate buffer (pH 7.4) according to Dodge et al (10). The ^{125}I-labelled ghosts were collected by centrifugation at 25,000 x g (SW 25.1 bucket rotor) in a Beckman ultracentrifuge (model L3-50) at 2-3°C for 45 minutes and washed three times under identical conditions. The ghosts, suspended in 1% solution of BSA in 0.05M phosphate adjusted to pH 4, were kept at 37°C for 5 to 10 minutes in order to facilitate the dissociation of the antigen:antibody complex. The ghosts were then removed by centrifugation and the antibody-containing supernatant solution was dialysed overnight against iso-

tonic saline at 4°C. The specific activity of the labelled antibody was found to be 13.6 x 10^{10} cpm/μ mole, calculated from the molecular weight of IgG as 160,000.

Red cell preparations: Modification of the C/P ratio of erythrocyte membranes was carried out by the method of Shinitzky (11). Blood was drawn into acid-citrate-dextrose solution (ACD) from Rh-positive and Rh-negative individuals and the cell-free plasma was heated for 30 minutes at 56°C. The heat-inactivated plasma, clarified by centrifugation, was then enriched with cholesterol or lecithin by incubation with a dispersion of cholesterol (5mg/ml) or of egg lecithin (2 mg/ml) in a mixture of tetrahydrofuran and aqueous 0.6% KCl (5:1 v/v). One volume of cholesterol or egg-lecithin dispersion was added with rapid mixing to ten volumes of 10% heat-inactivated plasma in PBS (pH = 7.4), containing glucose (2 mg/ml) and potassium penicillin (250 units/ml). The solution was lyophilized and the powder stored at -20°C. Membranes of intact erythrocytes were then enriched with or depleted of cholesterol as follows: Red blood cells (Rh-positive or Rh-negative) were suspended in the plasma preparations after reconstitution with buffer (7) to give a final hematocrit of 8 to 10%. Incubations were carried out in a rotary-shaker water bath at 37°C for 22 hours in the presence of 250 units/ml of potassium penicillin. As a control, erythrocytes were incubated with reconstituted plasma-buffer lacking added cholesterol or lecithin. After incubation, the cells were washed three times in PBS (pH = 7.4). A portion of the washed-cell suspension was set aside for microviscosity determinations (12) and lipid analysis (13,14).

Uptake of ^{125}I-labelled antibody: Preliminary experiments were carried out to determine the optimal concentrations of antibody and of red blood cells for the binding studies, so that cell-surface antigenic receptors would be saturated at the maximum antibody concentration used. Antibody uptake was assayed as follows: Radiolabelled antibody was added at increasing final concentrations to suspensions of known numbers of red blood cells (measured by Coulter Counter) and incubated at 37°C for one hour with agitation (Dubnoff shaker). Cells were then separated by centrifugation, washed three times with PBS at 4°C to remove unbound radiolabelled antibody, and then assayed for radioactivity in a well-type gamma counter. Sources of Rh-positive and Rh-negative erythrocytes were the same two individuals for all experiments.

Fluidity measurement: Lipid fluidity of erythrocyte membranes was determined by fluorescence depolarization measurements (12) using DPH as the probe. The measurements of fluorescence anisotropy were carried out in a fluorescence polarization spectrophotometer (SLM Instruments, Urbana, Illinois) at room temperature, with an excitation wavelength of 360 nm. All measurements were performed within 24 hours after incubation.

RESULTS AND DISCUSSION

Results of experiments with unmodified Rh positive erythrocytes designed to establish the required range of antibody concentration are shown in Fig. 1. The data are presented in Fig. 1a in accordance with the Scatchard (15) equation,

$$b/[A] = Kn - Kb \qquad (i)$$

where b is the amount of antibody taken up by the red cells (moles/liter), [A] the equilibrium concentration of free antibody (moles/liter), n the total moles of antibody that can be bound maximally by one liter of red cells, and K the

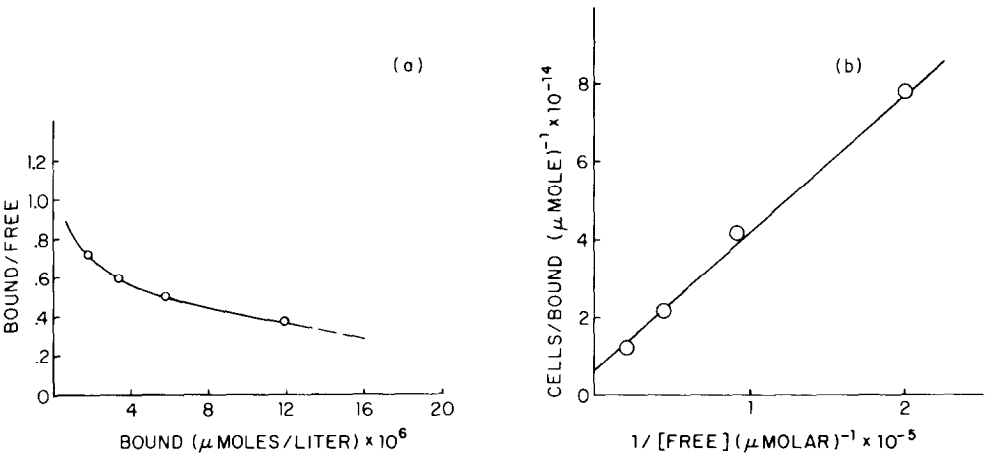

Fig. 1. (a) Scatchard plot showing the equilibrium relationship between the amount of antibody bound/liter of red cells and the ratio of bound/free antibody.

(b) Double reciprocal plot, according to Klotz equation, showing the equilibrium relationship between the amount of antibody bound per red cell and the equilibrium concentration of free antibody in moles/liter.

association constant (liter/mole). n is obtained by plotting b/ [A] against b and extrapolating the curve to zero value of b/[A] . The same binding data were also plotted by the double reciprocal method of Klotz (16). Assuming no interaction between the binding sites, the Klotz equation is

$$\frac{1}{b'} = \frac{1}{n'} + \frac{K'}{n'[A]} \qquad \text{(ii)}$$

where b' is the moles of antibody bound per red cell, K' the dissociation constant (moles/liter), and n' the number of moles of antibody that can be maximally bound per red cell. Typical double reciprocal plots are shown in Fig. 1b. The reciprocal of the vertical-axis intercept gives the maximum number of binding sites. The number of binding sites obtained from the two equations is very similar (6,000-7,000 sites/erythrocyte). The double-reciprocal plot was used for all subsequent experiments. An example of this approach,for modified Rh-positive cells,is shown in Figure 2.

All results of binding experiments are summarized in Table 1. The number of D-antigen sites on control Rh-positive erythrocytes was found to be 5,000-6,000 per erythrocyte, a number similar to that reported by other investigators (2-6).

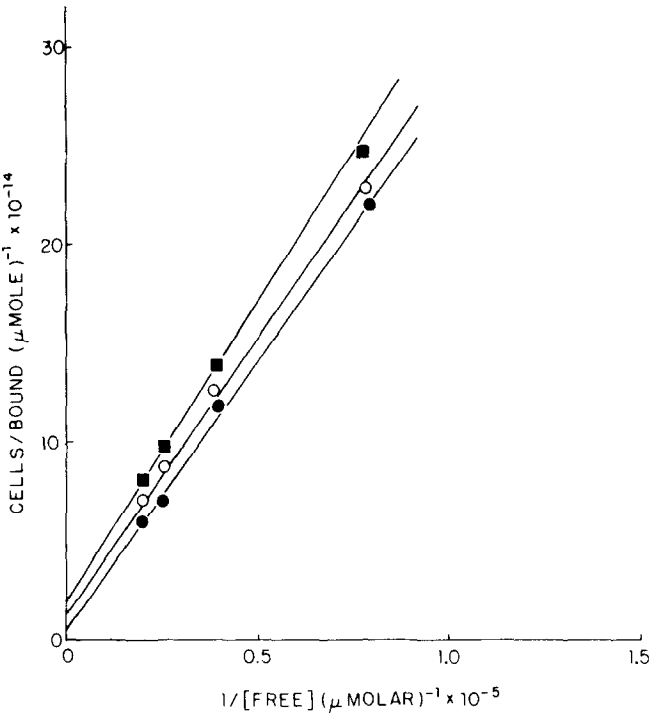

Fig. 2. Double reciprocal plots of the binding data obtained from the experiment with Rh positive modified human erythrocytes. Cholesterol enriched (●), cholesterol depleted (■) and control (O).

Cholesterol enriched Rh-positive red cells showed increased binding of ^{125}I-anti-D, with an estimated number of 12,000 to 14,000 sites per cell, whereas cholesterol-depleted Rh-positive cells had a lower number of binding sites, 3,000 - 3,500. The values of the equilibrium dissociation constant K' (moles/liter), for cholesterol enriched, control, and cholesterol depleted Rh-positive erythrocytes were found to be 5.0×10^{-10}, 2.5×10^{-10}, and 1.67×10^{-10} respectively.

Table 1: Effects of modulating membrane cholesterol on the number of D antigen sites on Rh-positive and Rh-negative human erythrocytes.

modified human erythrocytes	cholesterol / phospholipid	$\left(\frac{r_o}{r}-1\right)^{-1}_{24^o}$	^{125}I-anti-D binding sites per cell	
			Rh-negative	Rh-positive
cholesterol enriched	1.5 ± 0.1	2.6 ± 0.1	500-600	12,000 - 14,000
control	0.9 ± 0.1	2.1 ± 0.1	500-550	5,000 - 6,000
cholesterol depleted	0.6 ± 0.1	1.8 ± 0.1	500-550	3,000 - 3,500

Rh-negative cells bound a relatively small number of anti-D molecules ($\sim$ 500), and this number remained unchanged after cholesterol enrichment or depletion of erythrocyte membranes. Nicolson et al (5) detected approximately the same number of ^{125}I-anti-D molecules on Rh-negative erythrocytes.

Membrane lipid microviscosity measured by fluorescence depolarization is proportional to the term $\left(\frac{r_o}{r} - 1\right)^{-1}$ where r_o is the maximal limiting fluorescence anisotropy of the probe (for DPH 0.362) and r is the fluorescence anisotropy (12). The microviscosity parameter thus calculated of Rh-positive and Rh-negative erythrocytes did not differ and decrease as the C/P ratio of modulated erythrocytes decreased (Table 1).

Our work with ^{125}I-anti-D antibody confirms an earlier report (7) that cholesterol enrichment enhances expression of D antigen on Rh-positive erythrocytes. Modulation of membrane cholesterol, however, had no effect on the D antigen expression of Rh-negative erythrocytes. Cholesterol enriched and depleted Rh-negative cells also served as controls to assess the effect of our lipid treatment on non-specific antibody binding. In contrast to the previous study in which a fluorescence-labelled secondary antibody was employed to measure relative differences in antibody uptake, we have used a radio-labelled primary antibody. Therefore we were able to titer the number of D antigens on each cell type.

If vertical displacement of the Rh-protein molecule were indeed the mechanism leading to unmasking of antigen sites as suggested by Shinitzky and Souroujon (7), one would have to assume that in Rh-negative erythrocytes the displacement was, in our studies, insufficient to unmask the antigen. It is conceivable that more extreme C/P ratios would have accomplished this.

As an alternative to vertical displacement, the enhancement of antigen expression seen in cholesterol-enriched Rh-positive cells could also be due to changes in Rh-antigen distribution or to lateral mobility in the membrane. Although the surface distribution of D antigen was found to be random in untreated red cells, papain-treated erythrocytes showed an aggregated distribution of sites (17 Such a change in antigen distribution,possibly the result of lateral mobility,

might alter the number of binding sites measured by antibody uptake. One would not expect antigen redistribution to affect antibody binding in Rh-negative cells since the antigen is presumably buried within the lipid matrix.

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REFERENCES

1. Abraham, C.V., and Bakerman, S. (1975) Clinica Chimica Acta, 60, 33-43.
2. Masouredis, S.P. (1959) J. Clin. Invest. 38, 279-290.
3. Hughes-Jones, N.C., Gardner, B., and Telford, R. (1963) Biochem. J. 88, 435-440.
4. Lee, R.E. and Feldman, J.D. (1964) J. Cell Biol. 23, 396-401.
5. Nicolson, G.L., Masouredis, S.P., and Singer, S.J. (1971) Proc. Natl. Acad. Sci. 68, 1416-1420.
6. Green, F.A. (1968) J. Biol. Chem. 243, 5519-5521.
7. Shinitzky, M. and Souroujon, M. (1979) Proc. Natl. Acad. Sci. 76, 4438-4440.
8. Plapp, F.V., Kowalski, M.M., Tilzer, L., Brown, P.J., Evans, J., and Chiga, M. (1979) Proc. Natl. Acad. Sci. 76, 2964-2968.
9. Greenwood, F.G., Hunter, W.M., and Glover, J.S. (1963) Biochem. J. 89, 114-123.
10. Dodge, J.T., Mitchell, C., and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119-130.
11. Shinitzky, M. (1978) Febs. Lett., 85, 317-320.
12. Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652-2657.
13. Cooper, R.A., Arner, E.C., Wiley, J.S., and Shattil, S.J. (1975) J. Clin. Invest. 55, 115-126.
14. Brown, H.H., Zlatkis, A., Zak, B., and Boyle, A.J. (1954) Anal. Chem. 26, 397-399.
15. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672.
16. Klotz, I.M. (1947) Chem. Rev. 41, 373-399.
17. Romano, E.L., Stolinski, C. and Hughes-Jones, N.C. (1975) Brit. Jour. Haem. 30, 507-516.