Proc. Natl. Acad. Sci. USA
Vol. 76, No. 6, pp. 2964–2968, June 1979
Medical Sciences

Partial purification of $Rh_o(D)$ antigen from Rh positive and negative erythrocytes

(Rh antigen/affinity chromatography/LW antigen)

FRED V. PLAPP, MARK M. KOWALSKI, LOWELL TILZER, PEGGY J. BROWN, JAMES EVANS, AND MASAHIRO CHIGA

Department of Pathology and Oncology, The University of Kansas Medical Center, Kansas City, Kansas 66103

Communicated by Robert L. Hill, March 16, 1979

ABSTRACT **A rapid method is described for partial purification of $Rh_o(D)$ antigen from sodium deoxycholate-solubilized erythrocyte membranes by affinity chromatography on a column coupled with anti-$Rh_o(D)$ IgG. The $Rh_o(D)$ antigen is a low molecular weight membrane protein that comigrates with the lipid zone of erythrocytes during sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Similar quantities of $Rh_o(D)$ antigen were present in Rh positive and negative erythrocytes. However, in the latter erythrocytes the antigen may not be exposed to the external cell surface, explaining why these cells are not agglutinated by anti-$Rh_o(D)$ antiserum. LW antigen was also present in the fraction containing $Rh_o(D)$ antigen. The genetic implications of these findings are discussed.**

Although much research has been devoted to characterization of blood group antigens, little is known about the chemical structure of the Rh antigens or their relationship to other erythrocyte membrane proteins. Characterization of the Rh antigens has been hampered by the inability to readily purify them. This difficulty has been attributed to the small quantity of Rh antigens present per erythrocyte and the harsh procedures needed to separate these antigens from other membrane components. Only Abraham and Bakerman (1) have isolated Rh antigens that retained their antigenicity. We now report a rapid and gentle method for partial purification of antigenically active $Rh_o(D)$ antigen from human erythrocyte membranes by detergent solubilization and affinity chromatography. This method allowed us to demonstrate that Rh positive and negative erythrocyte membranes contained similar amounts of $Rh_o(D)$ antigen, but that in the latter erythrocyte membranes the antigen appeared to be masked from the external membrane surface.

MATERIALS AND METHODS

Outdated group O, Rh positive and negative human erythrocytes were obtained from the University of Kansas Medical Center blood bank. Erythrocyte membranes were prepared according to Fairbanks *et al.* (2) except that the lysis buffer consisted of 5 mM Tris·HCl (pH 7.5) and 0.1 mM Na_2EDTA and the washing buffer contained 5 mM Tris·HCl (pH 7.5).

Membranes were solubilized according to Juliano and Li (3). Pellets (1 ml) of packed membranes, containing 10–12 mg of protein, were suspended in equal volumes of 0.02 M Tris·HCl (pH 8), and sodium deoxycholate (Sigma) was added to 1%. After incubation for 1 hr at room temperature, suspensions were centrifuged at 54,000 × *g* for 30 min at 4°C in a Spinco type 50 rotor. Supernatants, which contained 70–80% of the membrane proteins, were recovered for affinity chromatography.

Immunoadsorbents were prepared by coupling an IgG fraction of human anti-$Rh_o(D)$ antiserum (Rh_oGam, Ortho Diagnostics, Raritan, NJ) to Affi-Gel 10 agarose (Bio-Rad). IgG (1.5 ml), containing 150 mg of protein per ml and approximately 300 μg of anti-$Rh_o(D)$ IgG, was dialyzed for 24 hr at 4°C against 2 liters of 0.1 M Na_2HPO_4 (pH 7). In some cases 1.5 ml of IgG was adsorbed with 30 ml of washed, packed, Rh negative (*cde/cde*), LW positive erythrocytes for 1.5 hr at 37°C prior to dialysis. Dialysates were diluted up to 25 ml with cold 0.1 M Na_2HPO_4 (pH 7) and added to a 1-g vial of lyophilized Affi-Gel 10. These mixtures were gently shaken for 3 hr and incubated overnight at 4°C. Approximately 60% of the added IgG was coupled, yielding about 6 mg of IgG per ml of packed agarose. Coupled agarose was washed with 500 ml of cold 0.05 M Na_2HPO_4 (pH 7) containing 1 M NaCl and then with 100 ml of 15 mM Na_2HPO_4, pH 7.4/0.9% NaCl (P_i/NaCl). Coupled agarose (1.5 ml) was packed into a 0.7 × 4 cm polypropylene column (Bio-Rad).

Prior to affinity chromatography, 10% NaCl was added to 0.9% final concentration to the solubilized membrane supernatants. Supernatants were layered onto an affinity column and the column was washed successively with 10 ml of P_i/NaCl, 3 ml of 1 M acetic acid, and 10 ml of P_i/NaCl. Adsorbed protein was eluted with 4 ml of dimethylformamide (Eastman). All chromatography procedures were performed at room temperature. The dimethylformamide eluate was immediately dialyzed against three 1-liter changes of P_i/NaCl for 24 hr, chilled in ice, and precipitated overnight with 2 vol of cold (−20°C) acetone. The precipiate was dissolved in 50 μl of P_i/NaCl (hereafter referred to as "preparation").

Elution profiles were monitored by labeling membranes with either fluorescamine (Fluram, Roche) or $Na^{125}I$ prior to detergent solubilization. Packed membranes were suspended in 3 vol of 0.05 M NaB_4O_7 (pH 9) and labeled with 3 mg of fluorescamine dissolved in 50 μl of acetone according to Cross and Briggs (4). Fluorescence was measured according to Bohlen *et al.* (5). Membranes were radioiodinated by the chloramine T method (6). Membrane protein (16 μg) was labeled with 15 μl of 20 mCi/ml of $Na^{125}I$ (New England Nuclear) (1 Ci = 3.7×10^{10} becquerels) in a final volume of 50 μl. Labeled membranes were chromatographed on a 1-ml column of Sephadex G-25 and the voided fractions were solubilized and layered directly onto affinity columns.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis was performed in 5% gels at 7 mA per gel for 3 hr according to Weber and Osborn (7).

Hemagglutination inhibition assays were performed in rigid microtiter plates with V-shaped wells (Linbro), using papain-treated Type O, Rh positive, LW positive erythrocytes. P_i/NaCl (50 μl) was added to wells 1 through 11 and 50 μl of 1/1000 diluted anti $Rh_o(D)$ IgG (Ortho Diagnostics) was added to well

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: P_i/NaCl, 15 mM Na_2HPO_4, pH 7.4/0.9% NaCl.

1. Aliquots (50 μl) were serially transferred from wells 1 through 11. P_i/NaCl (100 μl) was added to all wells followed by 50 μl of a 2% suspension of papain-treated erythrocytes. The plate was covered, gently shaken, and incubated at 37°C for 2 hr. Complete effacement of wells by erythrocytes indicated hemagglutination, whereas the small erythrocyte button in the bottom of wells indicated hemagglutination inhibition. To assess the antigenicity of membrane fractions, 50-μl suspensions of erythrocytes, membranes, or preparations were incubated with 50 μl of 1/1000 diluted anti-Rh_o(D) IgG for 1 hr at 37°C. The supernatants obtained after centrifugation were used as the sources of antibody in the assays, and were added in place of the anti-Rh_o(D) IgG mentioned above.

RESULTS

Because Lorusso and Green (8) solubilized membrane fractions containing Rh_o(D) antigen with sodium deoxycholate and Allan *et al.* (9) purified membrane proteins by affinity chromatography in the presence of deoxycholate, this detergent was used to purify the Rh_o(D) antigen by affinity chromatography. When fluorescamine- or ^{125}I-labeled Rh positive membranes were solubilized with deoxycholate and applied to an anti-Rh_o(D) IgG affinity column, preparations were obtained that comprised 0.1–0.7% of the total membrane protein.

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of these preparations (Fig. 1, lane b) revealed a single protein band of approximately 7000 daltons, which comigrated with the leading edge of the lipid zone of erythrocyte membranes (Fig 1, lane a) described by Fairbanks *et al.* (2). Electrophoresis of the anti-Rh_o(D) IgG suspension used for coupling to the affinity column (Fig. 1, lane c) revealed predominantly IgG and no low molecular weight proteins, indicating that leakage of coupled proteins was not producing the protein in the preparations. The faintly stained band near the bottom of the gel is sodium dodecyl sulfate.

To determine whether the adsorbed proteins from Rh positive membranes retained their Rh_o(D) antigenicity after elution, preparations were tested in hemaglutination inhibition assays. As seen in Fig. 2, row e, these preparations completely inhibited hemagglutination of Rh positive erythrocytes by anti-Rh_o(D) IgG as compared to the control (row a).

Because Rh negative erythrocytes are not agglutinated by anti-Rh_o(D) IgG and adsorb 1/10th the amount of antibody as Rh positive erythrocytes (10), preparations from these erythrocyte membranes were expected to contain little Rh_o(D) antigen activity. Surprisingly, however, preparations from Rh negative cell membranes also inhibited hemagglutination (Fig. 2, row d) and contained a single protein band upon electrophoresis (Fig. 1, lane d), which comigrated with the preparation from Rh positive membranes (Fig. 1, lane b).

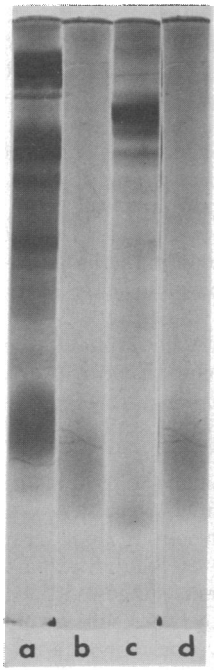

FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Lanes: a, 50 μg of Rh positive erythrocyte membrane protein; b, 10 μg of protein from affinity chromatography of Rh positive erythrocyte membranes; c, 20 μg of anti-Rh_o(D) IgG; d, 10 μg of protein from affinity chromatography of Rh negative erythrocyte membranes. Gels were stained with Coomassie blue.

To determine whether the agglutination that was being inhibited in the hemagglutination inhibition assays was really due to anti-Rh_o(D) IgG and not to antibodies reacting with membrane antigens common to Rh positive and negative erythrocytes, the antisera used in these assays were adsorbed with Rh negative erythrocytes. As seen in Fig. 2, row b, the adsorbed anti-Rh_o(D) titer remained the same as unadsorbed anti-Rh_o(D) (Fig. 2, row a), indicating that agglutination was due to anti-Rh_o(D) IgG. Also, Rh negative erythrocytes were not agglutinated by anti-Rh_o(D) IgG under these experimental conditions (Fig. 3, row f).

Previous investigators have suggested that Rh_o(D) antigen may be closely related to LW antigen, which is present on both Rh positive and negative erythrocyte membranes (11). Therefore, it was determined whether the anti-Rh_o(D) IgG coupled to agarose also contained anti-LW IgG, because this antibody would bind LW antigen that might crossreact with anti-Rh_o(D) IgG and inhibit hemagglutination. An affinity column was prepared with anti-Rh_o(D) IgG that was previously adsorbed with Rh negative, LW positive erythrocytes. Chromatography of solubilized membranes prepared from the same batch of Rh negative cells used for adsorption still yielded preparations that inhibited hemagglutination (Fig. 2, row g). Similarly, preparations from Rh positive erythrocyte membranes inhibited hemagglutination (Fig. 2, row h). Thus the affinity column, which should have contained only anti-Rh_o(D) IgG, still bound antigen from Rh negative erythrocyte membranes capable of inhibiting the agglutination of Rh positive erythrocytes by anti-Rh_o(D) IgG.

To determine whether these preparations were nonspecifically inhibiting hemagglutination, preparations from rabbit erythrocytes, which do not contain Rh antigens (11), were prepared on the same affinity column. As seen in Fig. 2, row c, these preparations did not inhibit hemagglutination. Also, preparations obtained from chromatography of solubilized, human Rh positive erythrocyte membranes on the column coupled with pooled human IgG did not inhibit hemagglutination (Fig. 2, row f). Thus, these preparations were not inhibiting hemagglutination due to nonspecific IgG.

Furthermore, preparations obtained from chromatography of solubilized Rh positive and negative membranes on an anti-Rh_o(D) IgG affinity column did not inhibit the agglutination of M and Rh(E) positive erythrocytes by anti-M or anti-Rh(E) antisera. Thus, these preparations were not nonspecifically inhibiting the interaction of erythrocyte antigens with antibodies.

Altogether, the above experiments indicated that preparations obtained from both Rh positive and negative erythrocytes contained Rh_o(D) antigen that specifically inhibited the agglutination of Rh positive erythrocytes by anti-Rh_o(D) IgG. To estimate the relative amounts of Rh_o(D) antigens present in Rh positive and negative erythrocyte membranes, the degrees of hemagglutination inhibition by the preparations from equal volumes of Rh positive and negative packed erythrocytes were compared. As seen in Fig. 3, rows b and c, preparations from Rh positive and negative membranes inhibited hemagglutination to the same degree, indicating that they contained similar quantities of Rh_o(D) antigen.

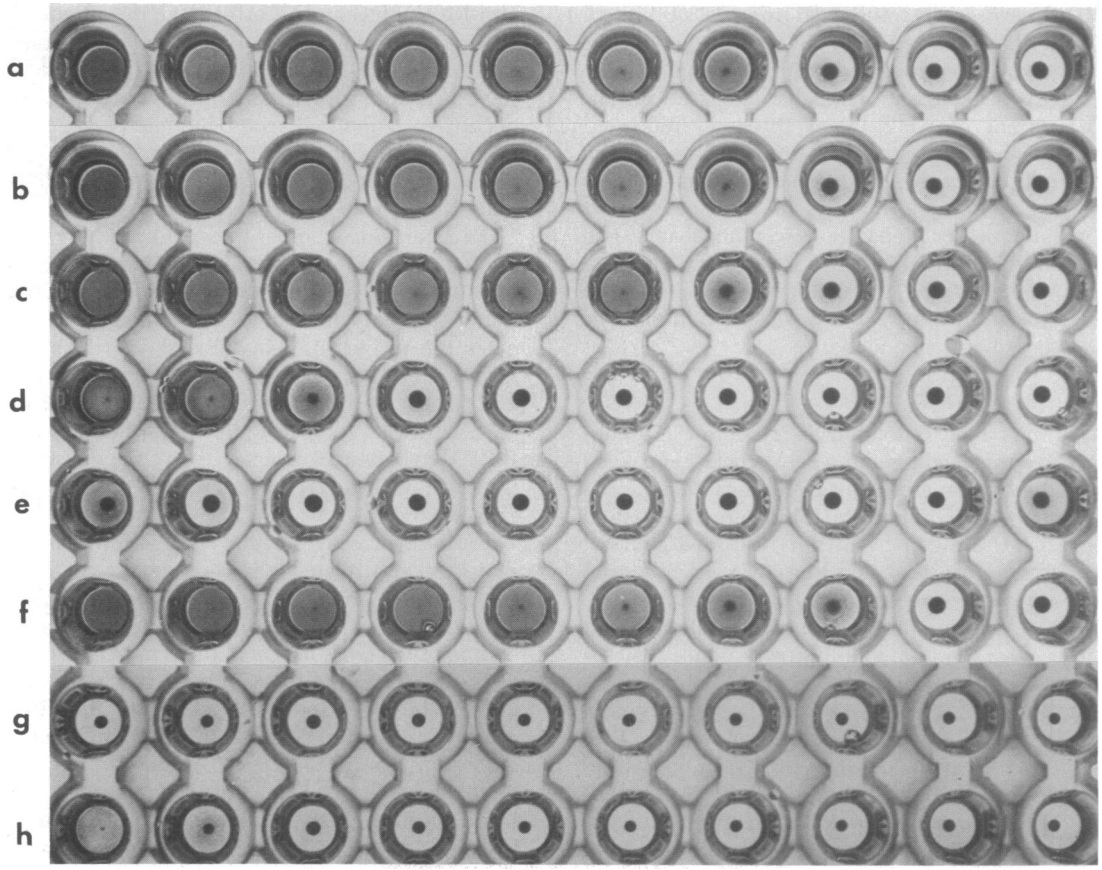

FIG. 2. Hemagglutination inhibition assay. Anti-Rh_o(D) IgG was adsorbed with the following. Rows: a, nothing; b, Rh negative erythrocytes; c, preparation obtained from rabbit erythrocytes; d, preparation obtained from human Rh negative erythrocytes; e, preparation obtained from human Rh positive erythrocytes; f, preparation obtained from human Rh positive erythrocytes chromatographed on pooled human IgG affinity column; g, preparation obtained from human Rh negative erythrocytes chromatographed on adsorbed anti-Rh_o(D) affinity column; h, preparation obtained from human Rh positive erythrocytes chromatographed on adsorbed anti-Rh_o(D) affinity columns.

Although solubilized Rh positive and negative erythrocyte membranes appeared to contain similar quantities of Rh_o(D) antigen, intact Rh negative erythrocytes reacted as though they did not contain Rh_o(D) antigen. Because papain treatment enhances the binding of anti-Rh_o(D) IgG to Rh positive erythrocytes (10), Rh negative erythrocytes were also treated with papain. As seen in Fig. 3, row g, hemagglutination was significantly inhibited by preincubation of anti-Rh_o(D) IgG with papain-treated Rh positive erythrocytes, but was not inhibited by preincubation with papain-treated Rh negative erythrocytes (Fig. 3, row f). Also, preincubation of anti-Rh_o(D) IgG with Rh positive membranes completely inhibited hemagglutination (Fig. 3, row d), whereas Rh negative membranes only slightly inhibited hemagglutination (Fig. 3, row e). Thus, Rh_o(D) antigen in Rh negative membranes was not exposed to antibodies until membranes were solubilized.

Because it has been hypothesized that Rh antigen may be related to LW antigen (11), the preparations from Rh positive and negative erythrocyte membranes prepared on the column coupled with anti-Rh_o(D) IgG that had been previously adsorbed with Rh negative, LW positive erythrocytes were tested for LW antigen activity. Fig. 4 demonstrates that Rh positive (Fig. 4, row b) and negative (Fig. 4, row c) erythrocyte membranes and the preparations obtained from these membranes (Fig. 4, rows d and e) all inhibited hemagglutination by anti LW (Group A Bigelow) antisera, indicating that the preparations contained LW antigens.

DISCUSSION

This paper describes a rapid method to partially purify Rh_o(D) antigen from small volumes of erythrocytes. The purified Rh_o(D) antigen has a molecular weight of approximately 7000, which was confirmed (12) by the sodium dodecyl sulfate/urea/polyacrylamide gel electrophoresis method of Swank and Munkres (13). During electrophoresis the Rh_o(D) antigen comigrated with the lipid zone of erythrocytes. These results agreed with our previous work, which demonstrated a specific precipitant band in the same gel region after incubation of gels containing Rh positive erythrocyte membrane proteins with anti-Rh_o(D) IgG (12). Rh_o(D) antigen appeared to be predominantly protein because it stained with Coomassie blue and its antigenicity was destroyed by trypsin (unpublished results). Also, the antigen did not stain with periodic acid Schiff or osmium tetroxide stains (12). These results agreed with the findings of Abraham and Bakerman, who estimated the molecular weight to be 10,000 (1) and found that only proteolytic enzymes destroyed antigenicity (14).

Deoxycholate was used in the present study to solubilize membranes because it could be readily removed from solubilized proteins by acetone precipitation of the proteins and did not interfere with hemagglutination inhibition assays. We have also solubilized Rh_o(D) antigen with sodium dodecyl sulfate (12), Triton X-100, and Nonidet P-40. Solubilization of the Rh_o(D) antigen by nonionic detergents suggested they were integral membrane proteins (15). However, if they were integral proteins, then they should be glycoproteins exposed to the

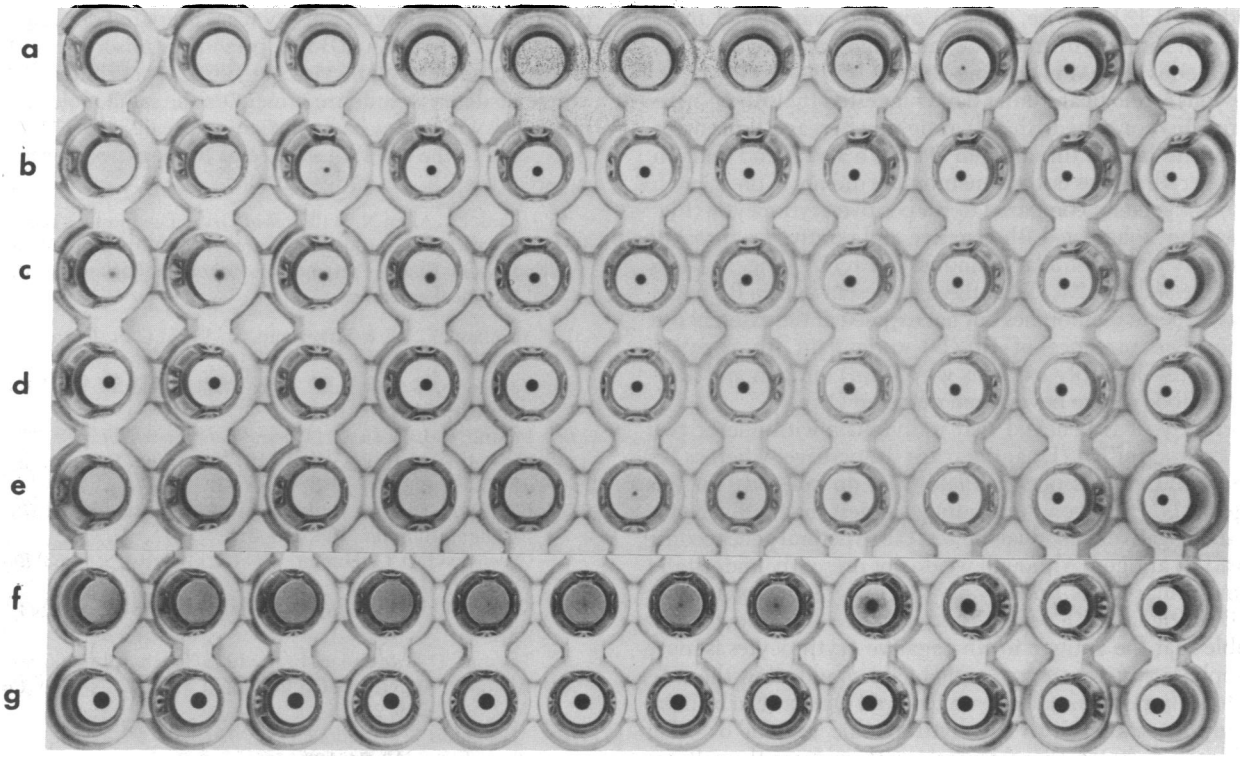

FIG. 3. Hemagglutination inhibition assay. Anti-$Rh_o(D)$ IgG was adsorbed with the following. Rows: a, nothing; b, preparation from Rh positive erythrocytes; c, preparation from Rh negative erythrocytes; d, membranes from Rh positive erythrocytes; e, membranes from Rh negative erythrocytes; f, papain-treated Rh negative erythrocytes; g, papain-treated Rh positive erythrocytes.

external membrane surface (15). $Rh_o(D)$ antigen is exposed to the external surface in Rh positive erythrocytes because they are labeled by anti-$Rh_o(D)$ IgG (11).

Masouredis *et al.* (10) have estimated that there are 25,000–40,000 $Rh_o(D)$ antigen molecules per Rh positive erythrocyte membrane. Assuming 40,000 molecules per membrane and a molecular weight of 7000, there would be about 0.5 fg of $Rh_o(D)$ antigen per Rh positive erythrocyte membrane. Because the membrane of an erythrocyte contains approximately 0.6 pg of protein (15), $Rh_o(D)$ antigens should comprise approximately 0.1% of the total membrane protein. Because the preparations from Rh positive erythrocytes contained up to 0.7% of the total membrane protein, they probably contained other proteins in addition to $Rh_o(D)$ antigen, even though electrophoresis revealed a single band (Fig 1, lane b). Furthermore, similar percentages of total proteins were present in preparations of Rh positive membranes chromatographed on a column coupled with pooled human IgG, even though no $Rh_o(D)$ antigen activity was present (Fig. 2, row f). No protein was adsorbed to uncoupled agarose, indicating that contaminating proteins were nonspecifically adsorbed to coupled IgG. This nonspecific adsorption was most likely due to hydrophobic binding because proteins were eluted only with dimethylformamide and not with 1 M acetic acid, 8 M urea, or 6 M

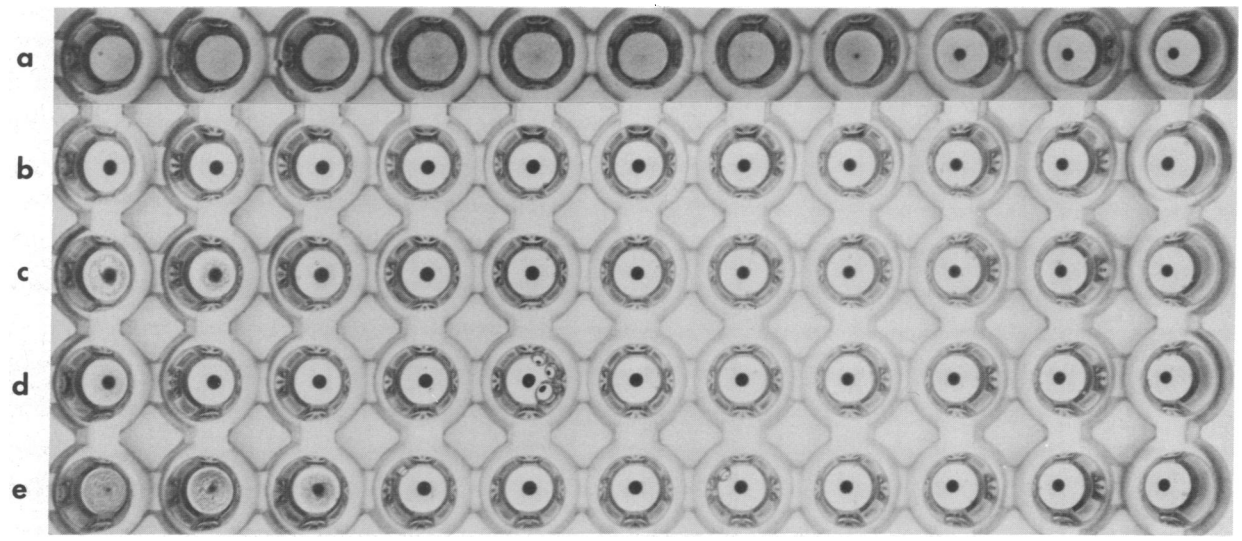

FIG. 4. Hemagglutination inhibition assay. Anti-LW antisera (Group A Bigelow) were adsorbed with the following. Rows: a, nothing; b, Rh positive erythrocytes; c, Rh negative erythrocytes; d, preparation from Rh positive erythrocytes; e, preparation from Rh negative erythrocytes.

guanidine·HCl. Likewise, $Rh_o(D)$ antigen was eluted most easily with dimethylformamide, although variable amounts were eluted with 6 M guanidine·HCl.

Previous serological studies have suggested that Rh positive erythrocytes contained $Rh_o(D)$ antigen whereas Rh negative erythrocytes lacked this antigen (11). We also found that Rh negative erythrocytes or their membranes did not express much $Rh_o(D)$ antigenicity compared to Rh positive erythrocytes (Fig. 3). However, solubilized Rh negative and positive erythrocyte membranes yielded similar quantities of $Rh_o(D)$ antigen. These data suggested that $Rh_o(D)$ antigen is present within Rh negative erythrocyte membranes but not exposed at the external cell surface. The presence of $Rh_o(D)$ antigen in Rh negative erythrocytes probably explains why Rh(d) antigen and anti-Rh(d) antibody have never been detected (11).

Currently, it is believed that individuals with one or two genes coding for $Rh_o(D)$ antigen are Rh positive, whereas Rh negative individuals possess alleles that do not code for this antigen (11). However, our findings suggest that both Rh positive and negative individuals possess the structural genes coding for $Rh_o(D)$ antigen. Although we do not presently understand the molecular basis underlying the different exposures of $Rh_o(D)$ antigen in Rh positive and negative erythrocytes, it is possible that the antigen in Rh negative erythrocytes is more hydrophobic. Increased hydrophobicity would allow the antigens to be buried more deeply in the lipid matrix or to be more strongly associated with the hydrophobic regions of other membrane proteins, thereby masking them from the cell surface. Other investigators have reported that histocompatability antigens can be modified so that they aggregate with other membrane proteins and are no longer exposed to the external cell surface (16). If our hypothesis is true, then the genetic difference between Rh positive and negative erythrocytes is interesting because it has been hypothesized that $Rh_o(D)$ antigen is the precursor of LW antigen (11). We could not presently determine if both $Rh_o(D)$ and LW antigenic determinants are present on the same molecules, because the $Rh_o(D)$ antigen has not been adequately purified. However, the preparations containing LW antigen activity were prepared on the column coupled with anti-$Rh_o(D)$ IgG previously adsorbed with LW antigen positive erythrocytes suggesting a possibility that both antigenic determinants are present on the same molecule. This possibility will be especially intriguing because the LW antigen is exposed to the external surface of Rh negative erythrocytes whereas the $Rh_o(D)$ antigen is not.

We thank Dr. Allan N. Williams of Ortho Diagnostic's Blood Bank Research Laboratory for his gifts of anti-$Rh_o(D)$ and anti-LW antisera. We thank Mrs. Elaine Fast and Mr. Robert McFarland for their excellent technical assistance.

1. Abraham, C. V. & Bakerman, S. (1975) *Clin. Chim. Acta* **60,** 33–43.
2. Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10,** 2606–2617.
3. Juliano, R. L. & Li, G. (1978) *Biochemistry* **17,** 678–683.
4. Cross, J. W. & Briggs, W. R. (1977) *Biochim. Biophys. Acta* **471,** 67–77.
5. Bohlen, P., Stein, S., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155,** 213–220.
6. Greenwood, D. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89,** 114–123.
7. Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244,** 4406–4412.
8. Lorusso, D. J. & Green, F. A. (1975) *Science* **188,** 66–67.
9. Allan, D., Auger, J. & Crumpton, M. J. (1972) *Nature (London) New Biol.* **236,** 23–25.
10. Masouredis, S. P., Sudora, E. J., Mahan, L. & Victoria, E. J. (1976) *Transfusion* **16,** 94–106.
11. Issitt, P. D. & Issitt, C. H. (1975) *Applied Blood Group Serology* (Spectra Biologicals, Oxnard, CA), pp. 115–157.
12. Plapp, F. V., Kowalski, M., Tilzer, L., Brown, P. J. & Chiga, M. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37,** 442 (abstr.).
13. Swank, R. T. & Munkres, K. D. (1971) *Anal. Biochem.* **39,** 462–477.
14. Litten, J. & Bakerman, S. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37,** 910 (abstr.).
15. Steck, T. L. (1974) *J. Cell Biol.* **62,** 1–19.
16. Callahan, G. W. & Allison, J. P. (1978) *Nature (London)* **271,** 165–166.