BBA 76097

BIOCHEMICAL CHARACTERIZATION OF A LIPID-DEPENDENT MEMBRANE PROTEIN ANTIGEN IN HK SHEEP RED CELLS

PETER SHRAGER*, DANIEL C TOSTESON AND PETER K. LAUF

Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N C. 27710 (U S A)

(Received May 26th, 1972)

SUMMARY

This paper reports on several biochemical characteristics of the M antigen in membranes of high-potassium sheep red cells.

1. The M antigen was solubilized by sodium deoxycholate and retained its activity following removal of detergent.

2. Membrane proteins and lipids were separated by gel filtration in deoxycholate and the M antigen was shown to reside only in the protein fraction, but at reduced levels.

3. Antigenic activity was enhanced by recombination with lipid, and both phospholipid and cholesterol were required for full activation.

4. Treatment of whole M-positive membranes with 2-mercaptoethanol and iodoacetamide resulted in a slight decrease of the M-antigenic activity but completely inactivated the M antigen when performed in the presence of 6 M guanidine·hydrochloride.

The results indicate that the antigen is bound to the membrane primarily through hydrophobic forces. We suggest that portions of the antigen are relatively buried in the membrane matrix, and are inaccessible to small molecular weight reagents.

INTRODUCTION

The study of membrane proteins and their interactions with lipid has been hampered by the extreme insolubility generally characteristic of these components. Some techniques have been developed to circumvent this difficulty[1,2] but functional integrity of solubilized preparations is often either lost or no longer measurable. This paper reports on the application of several methods to the characterization of an antigen present in sheep red cell membranes.

The M–L-antigen system in sheep erythrocytes is of particular interest due to its association with cation-transport properties[3–6]. The M antigen is present in high potassium (HK) cells, but not in homozygous low potassium (LK) cells, which

* Present address Department of Physiology, University of Rochester Medical Center, Rochester, N Y, 14642, U S A

contain the L antigen. Heterozygotes (LK) have both the M and L antigens. The M antigen was destroyed by heating to 100 °C (Lauf, P.K., unpublished), but not by neuraminidase[7], suggesting its identity as a protein. However, extraction of lipid from stroma in butanol–water[8] left the membrane protein devoid of M-antigenic activity[4].

The goal of this research was to characterize further the biochemical properties of the M antigen. We used a series of techniques to modify chemically and to solubilize membrane proteins since each technique disrupts different forms of chemical bonds with a different potency. Comparing relative effects of these procedures provided structural information with respect to the locus and stability of the antigen in the membrane Disruption of cell membranes by detergents has been one of the most effective means of solubilizing membrane protein, and the need for tests for structural integrity following solubilization and reaggregation have been stressed by several authors[9–12]. The M antigen has proven to be a useful system for such studies.

A brief preliminary note has appeared[13].

METHODS

Hemoglobin-free membranes

Sheep blood was drawn into heparin by jugular puncture. Red cells were washed 4 times in 153 mM NaCl, 2 mM Tris–HCl, pH 7.6. Hemoglobin-free membranes were prepared by the method of Dodge *et al.*[14], using Tris–HCl, 10 mM, pH 7.6, in place of phosphate buffer. If membranes were not to be used immediately, they were washed once in 1 mM tris–HCl, pH 7.6 and either stored frozen at −68 °C or lyophilized and stored at 4 °C.

Hemolytic tests

(*A*) *Serum titer.* Complement hemolysis of HK red cells exposed to M antibody was measured as in Lauf and Tosteson[4]. HK red cells were washed 4 times in 153 mM NaCl, 2 mM Tris–HCl, pH 7.6, and a suspension with a hematocrit of 0.5 % was made in veronal-buffered saline[15], with 5 mM KCl substituted 1:1 for NaCl. Veronal-buffered saline was used as the diluant in all succeeding steps. 0.5 ml of cells was mixed with 0.5 ml of stepwise dilutions of M antiserum (S31B, provided by Dr Ben Rasmusen, University of Illinois, Urbana, Ill.). 0.25 ml of a 1/10 dilution of whole guinea pig serum complement was added and samples were incubated at 37 °C for 90 min with frequent mixing. Samples were placed on ice, diluted by addition of 2 5 ml of cold veronal-buffered saline, and centrifuged. Absorbances of supernatants were read at 414 nm. Controls included samples with cells alone, cells and serum (highest concentration used) only, and cells and complement only. Calculations of percent hemolysis were corrected for the small amount of lysis (usually less than 10 %) due to complement alone.

(*B*) *Antibody-adsorption assay.* The M antigen was assayed by quantitation of the ability of a preparation to adsorb M antibody selectively From the above measurement of serum titer, an M-antiserum dilution just adequate for 100 % lysis was chosen. The sample to be tested for the M antigen was assayed for protein concentration. Stepwise dilutions of the sample were incubated with the chosen antiserum concentration for 1 h at 37 °C. Samples were spun at 13000 rev./min for 15 min in a Sorvall SM24 rotor. 0.5 ml of each supernatant was then tested for remaining

M-antibody activity by addition of fresh HK cells and complement as in Part A above. Controls to exclude non-specific adsorption of M antibody or complement were run with samples derived from homozygous LK (M-negative) membranes. Controls testing for non-immune auto-hemolysis were run in the absence of complement. Unless otherwise noted below, all controls were found to be negative. In general, data from repeated experiments could not be averaged directly due to differences in titer in different batches of S31B sera used and differences in protein concentrations of samples. All samples to be tested were either suspended in or dialyzed against veronal-buffered saline prior to incubation with antiserum.

Reduction–alkylation

Reduction–alkylation of membrane protein was carried out by the method of Fleishman *et al.*[16,17] as described by Poulik and Lauf[18]. Membranes were suspended in 0 55 M Tris–HCl, pH 8 1, 0.2 M β-mercaptoethanol at a protein concentration of 1 mg/ml and allowed to stand at room temperature for 1 h. Solid iodoacetamide was added with stirring to a final concentration of 0.3 M The pH of the reaction mixture was monitored and adjusted to 8.1 with small amounts of solid Tris base. After 30 min samples were dialyzed overnight into 100 vol. of 0.1 M Tris–HCl, pH 7 8 at 4 °C, then into 10 mM Tris-HCl, pH 7.6 for 36 h with frequent changes of buffer, and finally into veronal-buffered saline. When desired, the reduction and alkylation was carried out in the presence of 6 M guanidine–HCl, followed by dialysis as described above. Control aliquots were removed before addition of β-mercaptoethanol or iodoacetamide, and dialyzed

Gel filtration in deoxycholate

Sodium deoxycholate (Schwarz–Mann) was dissolved to 9 % (0.22 M) in 75 mM Tris–HCl, pH 8.0, by slowly adding the detergent under vigorous stirring. The solution was filtered once through Whatman No. 1 and once through Whatman No. 3 paper. 240 ml of Sephadex G-100 (Pharmacia) swollen in distilled water were mixed with 480 ml of the above 9 % deoxycholate solution, allowed to stand 4 h, and degassed. The gel was poured to a final bed height of 27 cm, diameter 2.5 cm, and eluted with deoxycholate 6 %, 50 mM Tris–HCl, pH 8.0.

6 ml of the above 9 % deoxycholate solution were added to 125 mg lyophilized membranes and stirred gently for 3 h at room temperature. The slightly cloudy sample was then centrifuged at 35000 rev./min for 2 h in a Spinco SW39 rotor. 5 ml of the resulting clear supernatant were applied to the column, and eluted as above.

All samples in sodium deoxycholate, including column fractions, were dialyzed into 100 vol. of buffer for 5 days at 4 °C. The buffer for the first 3 days was 10 mM NaCl, 10 mM Tris–HCl, pH 7.6, with daily changes, and for the fourth and fifth days, 2 mM NaCl, 2 mM Tris–HCl, pH 7 6 and 2 mM Tris–HCl, pH 7.6, respectively. Samples were then dialyzed overnight into veronal-buffered saline for antibody-adsorption tests.

Columns with Sephadex G-200 were run in similar fashion, but at a bed height of 40 cm, diameter 2.5 cm Sepharose 4B was also used as above, but with column dimensions of 76 cm × 1.5 cm

DEAE-Sephadex A50 was swollen in distilled water and washed alternatively in 0.1 M NaOH and 0.1 M HCl. Sufficient urea and buffer were added to the gel in

distilled water to make the final concentrations 6 M urea, 20 mM Tris–HCl, pH 7. The gel was poured to a height of 27 cm, diameter 1.5 cm. Initial eluent was 6 M urea, 20 mM Tris–HCl, pH 7. After about 90 ml had been collected, the eluent was changed to a gradient increasing in ionic strength to a final level of 6 M urea, 20 mM Tris–HCl, pH 7, 1 M NaCl.

emical assays

Ch Protein was assayed by the method of Lowry *et al.*[19] with bovine serum albumin as standard. Interference by deoxycholate was negligible at the concentrations used.

Lipid phosphorus was measured by the method of King[20].

Cholesterol was determined as in Glick *et al.*[21].

Extractions with organic solvents

Lipid was extracted from hemoglobin-free membranes by the procedure of Bligh and Dyer[22]. For extraction of cholesterol, an aliquot of these lipids in chloroform was added slowly to 25 vol. of acetone at 0 °C. The mixture was chilled to −15 °C for 30 min, then centrifuged at 10000 rev./min at 0 °C for 10 min in a Sorvall SS34 rotor. The supernatant was discarded and the pellet dried under nitrogen and taken up in chloroform. The procedure was then repeated. The resulting preparation contained less than 1 % cholesterol.

Gel electrophoresis

Polyacrylamide gels were prepared according to the methods of Davis[23] and Summers *et al.*[24] with some minor modifications. The gels contained 7.5 % acrylamide (Lot No. X 5521, Eastman, Rochester, N.Y.), 0.04 % *N,N'*-methylene-*bis*-acrylamide (Lot No. 8383, Eastman, Rochester, N.Y.), 5.3 M urea, 0.02 M EDTA, 0.1 M sodium phosphate buffer, pH 7.6, and were activated with 0.02 % ammonium persulfate and 0.0024 ml per ml *N,N,N',N'*-tetramethylethylenediamine (Temed, Lot No. 8178, Eastman, Rochester, N.Y.). The electrophoresis buffer contained 0.1 M sodium phosphate, pH 7.6, 0.02 M EDTA and 0.1 % sodium dodecyl sulfate. Samples (100–300 μg membrane protein) were dissolved in 0.1 ml of a splitting solution consisting of 0.1 M sodium phosphate buffer pH 7.6, 1 % sodium dodecyl sulfate, 8 M urea and 0.2 M 2-mercaptoethanol. The dissolved samples were applied to the gels which had been prerun for 1 h at 2–3 mA per gel tube (dimensions 15 cm × 0.5 cm) to remove any oxidants originated during polymerization of the gels. Using bromophenol blue as a marker, gels were run for 1 h at 3 mA per gel tube, subsequently fixed in cold 10 % trichloroacetic acid for 2 h, and stained with Coomassie blue for 1 h followed by destaining in 7 % acetic acid overnight.

RESULTS

Antibody adsorption assay for the M antigen

The technique used for a quantitative measure of M-antigenic activity in membranes and solubilized components is illustrated in Fig. 1. Fig. 1a shows complement hemolysis of HK red cells as a function of M-antiserum dilution for one serum used. As described in Methods, M-antigenic activity was assayed by first incubating stepwise dilutions of HK and homozygous LK membranes with the dilution of anti-M

serum just adequate for 100 % lysis (in this case 1/80). The remaining unadsorbed serum was then tested for M-antibody activity on fresh HK cells (Fig. 1b). Fresh HK membranes were highly active in binding M antiserum and thereby inhibiting hemolysis, while M-negative membranes from homozygous LK red cells showed no adsorption

In earlier work, using M antiserum S11, it was found that lyophilization of HK membranes rendered the M antigen inactive[4]. With serum S31B, however, membranes suspended in 1 mM Tris–HCl, pH 7.6, and lyophilized, were fully active in adsorbing M antibody. This difference may be at least partially attributed to different affinities of the M antibodies used since S11 activity appeared to be mainly of IgM nature, while activity of S31B was of IgG nature (Lauf, P.K., unpublished).

Variation of ionic strength, reduction and chelation

A series of experiments was conducted aimed at solubilization of the M antigen by alteration of ionic strength and by addition of reducing and chelating agents. Mitchell and Hanahan[25] have shown that up to 20 % of the human red cell membrane protein is released into solution by suspension in 1 M NaCl. HK sheep red cell membranes were suspended in 100 vol. of 1 M NaCl, both unbuffered, and buffered to pH 7.6 with 10 mM Tris–HCl, and incubated at 4 °C for 27 h. Samples were spun at 17250 rev./min for 45 min in a Sorvall SS34 rotor, and supernatants and residues tested for protein concentration and antigenic acitivity. Only 8–10 % of the original membrane protein was solubilized by 1 M NaCl (buffered or unbuffered). Results

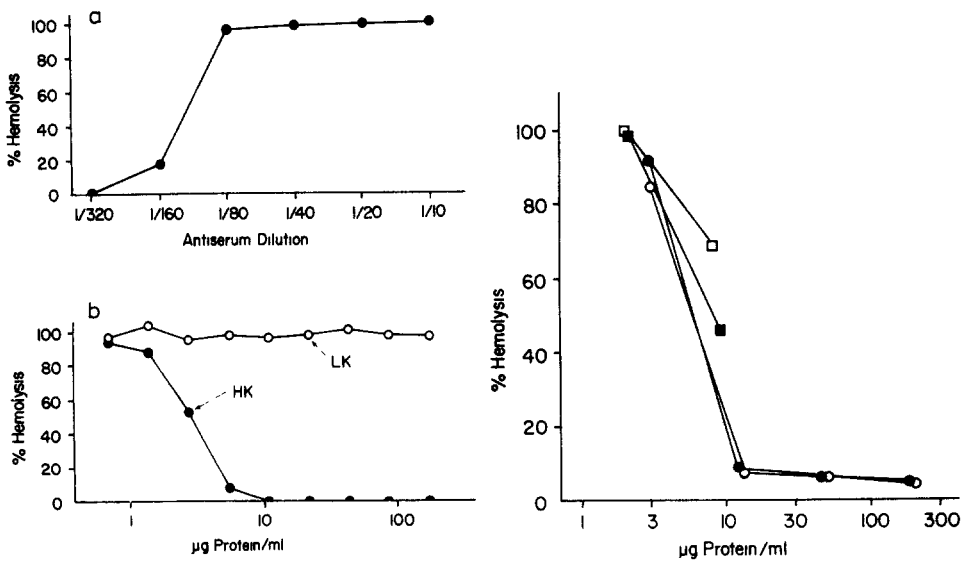

Fig 1 a Complement hemolysis of HK sheep erythrocytes by dilutions of anti-M serum. Incubation 90 min at 37 °C b Binding of M antiserum by HK (●) and homozygous LK (○) membranes Protein concentrations refer to membrane protein present during the antibody adsorption step. Antiserum dilution during adsorption was 1/80

Fig 2 M-antigenic activity after treatment with 1 M NaCl Antibody adsorption tested as in Fig 1b □, supernatant after incubation in 1 M NaCl and centrifugation, ○, residue after 1 M NaCl treatment Filled symbols. same, but 1 M NaCl buffered to pH 7 6 with 10 mM Tris–HCl

are shown in Fig. 2. While amounts of solubilized material were insufficient for complete inhibition, antibody binding per μg solubilized protein was similar to that for residue material. The data indicate, therefore, that solubilization of membrane components in 1 M NaCl is relatively non-specific with respect to the M antigen.

We have further investigated sequential solubilization similar to that used on human red cell stroma by Rosenberg and Guidotti[26], and following work by Mitchell and Hanahan[25], and Marchesi and Steers[27]. Membranes were exposed first to low, and later to high ionic strength solutions. Membranes were dialyzed against 1 mM Na_4EDTA, 50 mM β-mercaptoethanol for 5 days at 4 °C, and centrifuged at 17000 rev./min for 1 h. Residues were suspended in 1 M NaCl, 10 mM Tris–HCl, pH 7.6, and centrifuged at 16000 rev./min for 75 min. 40 % of the membrane protein was solubilized in chelating–reducing medium, but this fraction was devoid of M-antigenic activity. The insoluble material was active, though at reduced levels. Further solubilization of this residue with high NaCl concentrations gave results similar to those obtained before, without pre-treatment. Only 5–6 % of the membrane protein was solubilized, and antigenic activity (per mg protein) was similar in solubilized and residue fractions.

Chaotropic anions

Certain anions, including I^- and SCN^-, promote the transfer of hydrophobic groups from an apolar environment to the aqueous medium[28,29]. Hatefi and Hanstein[29] have used high concentrations of these chaotropic ions to disrupt mitochondria membranes and release complexes of the electron-transport system and other membrane proteins. We have attempted a solubilization of the M antigen using this technique. Membranes were suspended in either 2 M NaI or 4 M NaSCN, stirred for 24 h at 4 °C, and spun at 17500 rev./min for 1 h in a Sorvall SM24 rotor. A thin, milky band appeared at the top of each tube, and the lower supernatant phase was clear. The clear supernatants were desalted on a Sephadex G-10 column and added to 1/4 vol. of a 5 times concentrated solution of veronal-buffered saline. The insoluble band was resuspended in veronal-buffered saline and dialyzed against it. 5–10 % of the membrane protein was solubilized by 2 M NaI, and 15–20 % by 4 M NaSCN. Solubilized and insoluble fractions were tested for M-antibody-binding ability. Components solubilized by NaI showed no M-antigenic activity, and NaSCN supernatants had only slight activity. In both cases, however, the insoluble residues were highly active.

Guanidine–HCl

At high concentrations guanidine–HCl is capable of disrupting non-covalent bonds and has proved effective in solubilizing several components of the human red cell membrane[30]. We therefore tested to see if the M antigen could survive similar treatment. HK membranes were suspended in 6 M guanidine–HCl, 70 mM Tris–HCl, pH 7.6, both with and without addition of 75 mM β-mercaptoethanol and 4 mM EDTA, and were allowed to stand for 2 h at room temperature. Samples were then dialyzed exhaustively against buffer at 4 °C and were tested for M-antibody adsorption (Fig. 3). The M antigen survived exposure to this strong denaturant, including addition of reducing and chelating compounds. In order to guard against the remote

possibility that M-antibody-adsorption sites were uncovered by this procedure, M-negative LK membranes were treated with guanidine–HCl in a parallel manner (Fig. 3). No M-antibody binding was found. Other samples were similarly tested in 6 M urea, 70 mM Tris–maleate, pH 6 7. Results were identical to those in guanidine–HCl.

Membrane protein–lipid interactions are not completely disrupted in high concentrations of guanidine–HCl[31]. The possibility remained, therefore, that the M antigen remained selectively insoluble in this denaturant. A suspension of HK membranes in 7 M guanidine–HCl was spun at 39000 rev./min for 1 h in a Spinco SW39 rotor. As found by Gwynne and Tanford[30] and Maddy and Kelly[31] in cells of other species, a thin, milky band appeared at the top of the tube, and the bottom phase was clear to the eye. Top and bottom phases were collected, dialyzed, and tested as above The top phase contained roughly twice the protein concentration of the lower phase. However, both showed high antigenic activity and antibody adsorption per mg protein was similar for both phases.

We further examined the conformation of the M antigen within the membrane by investigating reactivity of sulfhydryl groups under varying conditions. HK membranes were reduced and alkylated with β-mercaptoethanol and iodoacetamide, in the presence and absence of 6 M guanidine–HCl (see Methods) and results of a typical experiment are illustrated in Fig. 4. The M antigen retained approximately 25 % of its original activity following reduction–alkylation in Tris–HCl alone. However, less than 1 % of its activity remained if the reaction took place in 6 M guanidine–HCl. As noted earlier, any effects of guanidine–HCl and β-mercaptoethanol alone were reversed by dialysis.

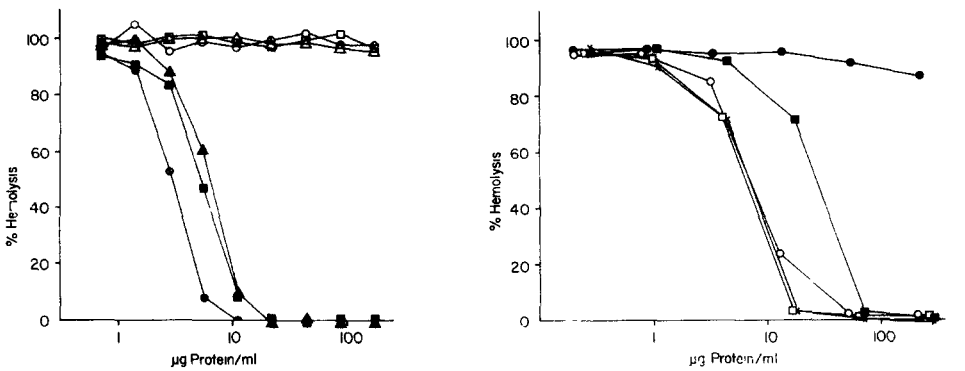

Fig. 3 Effects of guanidine–HCl on the M antigen HK and homozygous LK membranes were suspended in the solutions noted below, dialyzed, and tested for M-antibody binding Filled symbols denote HK membranes, open symbols homozygous LK membranes ● and ○, control, buffer alone; ■ and □, 6 M guanidine–HCl; ▲ and △, 6 M guanidine–HCl, 75 mM β-mercaptoethanol and 4 mM Na_4 EDTA.

Fig. 4 Chemical modification of HK membranes by reduction–alkylation Membranes in 0.55 M Tris–HCl, pH 8 1 were treated with □, 0.2 M β-mercaptoethanol, ○, 0 2 M β-mercaptoethanol, 6 0 M guanidine–HCl; ■, 0.2 M β-mercaptoethanol, 0 3 M iodoacetamide; ●, 0 2 M β-mercaptoethanol, 6 0 M guanidine–HCl, 0 3 M iodoacetamide; ×, control, buffer only When iodoacetamide was added, pH was maintained at 8 1 with Tris base Membranes were dialyzed into veronal-buffered saline and tested for M-antiserum binding as in Fig 1

Attempts were made to fractionate the guanidine–HCl-solubilized membrane protein by gel filtration on Sepharose 4B in the presence of denaturant. However, in the absence of prior reduction–alkylation[30], resolution was poor, and no fractions enriched in the M antigen were obtained.

Solubilization in anionic detergents

Anionic detergents have been particularly effective in disrupting and solubilizing membrane proteins in a number of systems[1]. We first compared results of solubilization by sodium dodecyl sulfate or sodium deoxycholate. HK membranes were suspended in detergent concentrations ranging from 1 to 9 mg detergent per mg membrane protein. After incubation in detergent, samples were divided, and aliquots dialyzed into 8 mM NaCl, 2.5 mM Tris–HCl, pH 7.6, both with and without addition of 20 mM $MgCl_2$ or 20 mM $CaCl_2$[11,32–34]. The M antigen retained full activity after disruption in deoxycholate, even at the highest detergent concentrations tested, while the antigen was destroyed by even the lowest concentration of sodium dodecyl sulfate used. Further, divalent cations were not required in the initial dialysis media and, in fact, hindered removal of detergent due to the relative insolubility of complexes of these ions with deoxycholate.

Procedures were then developed aimed at separation of membrane protein and lipid components in deoxycholate. Lyophilized HK or homozygous LK membranes were suspended at a concentration of 10 mg protein per ml in a solution of deoxycholate, 90 mg/ml, and stirred for 3 h at room temperature. The resulting slightly cloudy samples were spun at 35000 rev./min for 2 h in a Spinco SW39 rotor. The clear supernatant contained 98 % of the original membrane protein. A very small brownish pellet was not resuspendable, and was not analyzed further.

The supernatant was applied to a Sephadex G-100 column and eluted with solutions of deoxycholate, as described in Methods. Fractions were analyzed for protein and lipid phosphorous, and the elution profile is illustrated in Fig. 5. Separation of membrane proteins and phospholipids was complete. The single protein peak was eluted at the void volume. Fractions were pooled into Peaks A and B as shown. Patterns were identical for HK and homozygous LK membranes Aliquots of Peaks A and B from HK and LK membranes were diluted with the eluting solution or were recombined in various combinations. A volume ratio of 2 vol. of B to 1 vol. of A was maintained, corresponding to the approximate ratio of eluted volumes. Samples were then dialyzed as described in Methods and tested for M-antigenic activity (Fig. 6) Also shown are results for portions of the solubilized membranes as applied to the columns.

HK Peak A adsorbed M antibody, but M activity was only 25 % of that of solubilized but unfractionated HK membranes. HK Peak B was devoid of activity. Run as controls, neither LK Peak A nor LK Peak B showed any M-antibody adsorption Recombination of HK Peak A and HK Peak B resulted in restoration of antigenic activity to original levels Recombination of HK Peak A with LK Peak B gave results identical to those for recombination of HK Peak A with HK Peak B. LK Peak A *plus* HK Peak B was inactive. M-antigenic activity resided, therefore, only in the protein fraction from HK membranes, but recombination with lipid from either HK or LK membranes was requisite for full activity.

Activation of the M antigen by lipid was studied in more detail. Lipids were

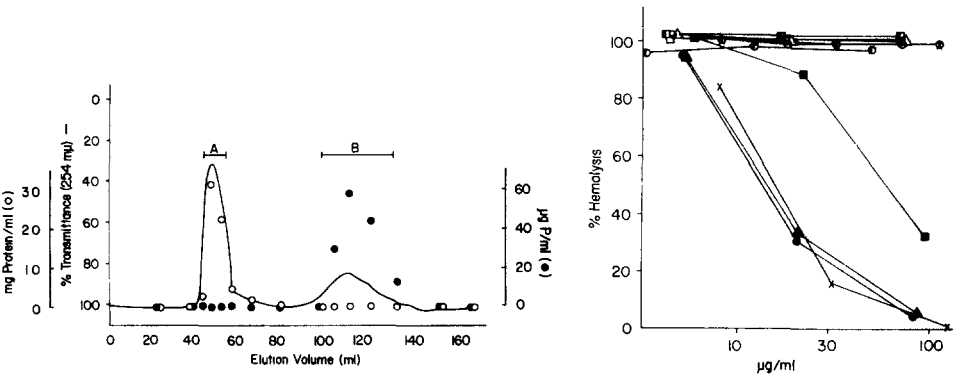

Fig. 5. Elution profile of sheep erythrocyte membranes on Sephadex G-100 in sodium deoxycholate. Solid line: % transmittance at 254 nm. ○, mg protein/ml; ●, μg phosphorus/ml

Fig 6 Hemolysis remaining following M-antibody binding by fractions eluted from Sephadex G-100 in sodium deoxycholate 1 vol of Peak A (or buffer) was recombined with 2 vol of Peak B (or buffer), dialyzed, and tested. Buffer refers to the eluting buffer containing sodium deoxycholate ●, HK Peak A + HK Peak B, ■, HK Peak A + buffer, ▲, HK Peak A + LK Peak B; ○, LK Peak A + LK Peak B; □, LK Peak A + buffer; △, LK Peak A + HK Peak B; ◐, buffer + LK Peak B, ◧, buffer + HK Peak B; ×, HK material applied to column + buffer, ⊗, LK material applied to column + buffer Abcissa refers to μg protein/ml except for (◐) and (◧) when it refers to μg phospholipid/ml

extracted from sheep erythrocyte membranes in organic solvents (see Methods). As needed, aliquots of lipids were dried and dissolved in deoxycholate solutions. HK or LK membranes were solubilized in deoxycholate and run through Sephadex G-100 in deoxycholate, as described above. Peak B was discarded, and Peak A recombined with organic solvent-extracted lipids in deoxycholate. Samples were then dialyzed and tested as before. Results of a typical experiment are shown in Fig. 7. Recombination with membrane phospholipids alone, in their normal amount relative to membrane protein, resulted in only partial activation of the M antigen. Cholesterol was

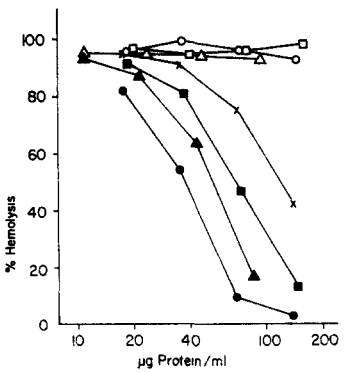

Fig. 7 Hemolysis following M-antibody adsorption by samples after recombination of HK Peak A and LK Peak A with chloroform: methanol-extracted lipids and cholesterol in sodium deoxycholate. Solutions were mixed, dialyzed, and tested. ×, HK Peak A; ■, HK Peak A + phospholipids (0 7 mg phospholipid per mg protein), ▲, HK Peak A + cholesterol (0 5 mg cholesterol per mg protein); ●, HK Peak A + total lipid extract (1 mg lipids per mg protein); □, LK Peak A + phospholipids (0 7 mg phospholipids per mg protein); △, LK Peak A + cholesterol (0 5 mg cholesterol per mg protein); ○, LK Peak A + total lipid extract (1 mg lipids per mg protein)

particularly effective in restoring activity, although full strength was not achieved even at higher cholesterol–protein ratios. Excessively high cholesterol concentrations produced a slight inhibition of complement, and were avoided. Recombination with total lipid extract resulted in the approximately 4-fold stimulation of activity that was obtained by activation with Peak B from the column. Controls, run with LK Peak A, did not adsorb M antibody.

Lauf and Tosteson[4] have demonstrated that butanol–water-extracted HK membrane protein[8] has no M-antigenic activity. This was found to be the case for all M antisera tested. Waterphase proteins were lyophilized and solubilized in deoxycholate. Aliquots of this material were recombined with Peak B or cholesterol in deoxycholate, dialyzed, and tested. No M-antigenic activity was detected in any of these samples. Butanol-phase lipids, dried in a rotary evaporator at 29 °C, were taken up in chloroform, then dried under nitrogen and dissolved in deoxycholate. Recombined with HK Peak A, butanol-extracted lipids caused a 4-fold stimulation of M-antigenic activity.

Resolution of membrane protein

Several attempts were made at further purification of the M antigen. HK red cell membranes were solubilized in deoxycholate, applied to a Sephadex G-200 column, and eluted in a manner similar to that used for the G-100 column. The protein fraction emerged in a single peak at the void volume, with a small shoulder of smaller molecular weight material. Attempts were also made to fractionate membrane proteins on Sepharose 4B columns in deoxycholate both with and without addition of 6 M urea. Protein peaks were generally broader than in Sephadex G-200, but no effective resolution of distinct fractions was obtained.

HK Peak A protein, from Sephadex G-100, was dialyzed into 6 M urea, and concentrated in a Schleicher and Schuell collodion bag. 2 ml of this sample were applied to a DEAE-Sephadex column equilibrated with urea, and eluted as described in Methods. Virtually all the protein remained bound at the top of the column, and was not eluted even with 1 M NaCl.

Peak A from membranes of HK red cells was analyzed by polyacrylamide-gel electrophoresis (see Methods) as shown in Fig. 8e. For comparison, the electrophoretic pattern of whole membranes from HK (d), and LK (b) red cells, and the *n*-butanol-extracted[8] membrane proteins of HK (c) and LK (a) red cells are also shown. The highly heterogeneous protein pattern of Peak A (e) resembles that found in whole membranes as well as in the butanol-extracted proteins from membranes derived from either HK or LK sheep red cells. The patterns indicate therefore no major differences among the deoxycholate protein, whole membranes, and butanol-extracted protein, nor does any qualitative difference appear between membranes derived from red cells of either genetic type.

DISCUSSION

Identity of the M-antigen determinant as a membrane protein

Following gel filtration of HK membranes in sodium deoxycholate, M-antigenic activity was found only in the protein peak. Red cell lipids alone were devoid of M-antibody binding ability. Coupled with earlier results demonstrating the antigen's

destruction by heating to 100 °C (Lauf, P.K., unpublished), and survival following treatment with neuraminidase[7], the data strongly suggest that the M-antigenic determinant is of membrane protein nature

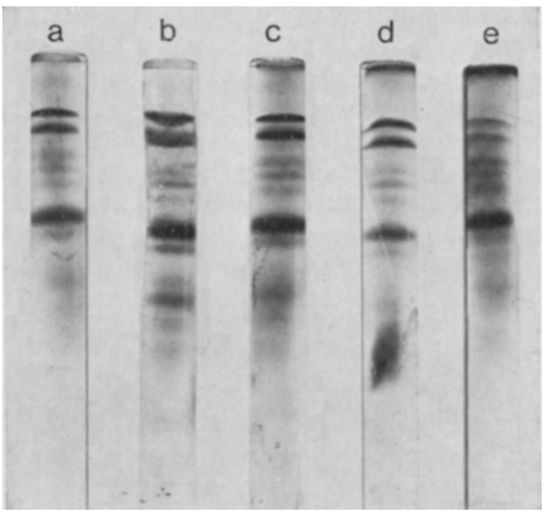

Fig 8 Sodium dodecyl sulfate–urea–polyacrylamide-gel electrophoresis of HK and LK membranes and their lipid-free proteins (a) *n*-Butanol-extracted protein from LK membranes. (b) Whole membranes from LK red cells (c) *n*-Butanol-extracted protein from HK membranes. (d) Whole membranes from HK red cells. (e) Peak A protein from HK membranes

Structural requirements of the antigen

Antibody-binding properties require the antigenic determinant to be located at the outer surface of the membrane. Results of reduction–alkylation experiments indicate that important portions of the M antigen may lie in the membrane interior. 25 % of the M-antigenic activity remained after reduction–alkylation of native HK membranes. If the reactions were carried out in the presence of 6 M guanidine–HCl, conditions under which proteins unfold to a random coil configuration[35–37], loss of activity was complete. If the alkylation step was omitted, the M antigen was capable of re-assuming its native configuration following denaturation and reduction of disulfide bonds. These results suggest that certain requisite functional groups are "buried" in the native membrane and are accessible to small molecular weight reagents only after loss of conformational integrity. It is, however, impossible to exclude completely the possibility that interaction of the M antigen with a neighboring membrane protein may be essential for antibody-binding ability, and that the observed effects of reduction-alkylation are due to reaction with this other constituent

Only a small percentage of the M antigen was released by salt concentrations of the order of 1 M, suggesting that this substance is not bound to the red cell surface primarily by ionic bonds. Nor was the M antigen solubilized at low ionic strength, in the presence of reducing and chelating compounds, conditions under which the membrane protein spectrin[27] is released The chaotropic anions I^- and SCN^- promote the exposure of apolar groups to the aqueous environment and therefore disrupt

hydrophobic bonds near the surface of membranes[29]. The M antigen remained in the insoluble residue following incubation in high concentrations of these ions. Effective solubilization of the antigen required high concentrations of the anionic detergent sodium deoxycholate, much stronger conditions for disrupting hydrophobic bonds than the use of chaotropic anions. These results suggest, therefore, that the M antigen is bound largely by relatively strong hydrophobic interactions, and that these bonds are localized in the interior of the membrane. A similar situation has recently been reported for a calcium sequestering protein from sarcoplasmic reticulum by MacLennan and Wong[38].

Several authors have demonstrated that membrane proteins and lipids are dissociated in anionic detergents[9,12,32,39,40], and successful separation methods have included gel electrophoresis[12,41], gel filtration[39,42–44] and sucrose density gradient centrifugation[9,32,39]. Investigating deoxycholate solubilization of human red cell stroma, Philippot[44] has chromatographed membrane preparations on Sepharose 6B in deoxycholate concentrations of the order of 10 mM. Proteins and phospholipids were resolved with this technique, although a small amount of phospholipid appeared in the void volume, with the major protein component. We have employed higher deoxycholate concentrations (150 mM) and on Sephadex G-100 separation of proteins and phospholipids of sheep stroma was complete. In gels of higher molecular weight exclusion limit, including Sepharose 4B, the protein component was partially fractionated, in results similar to those of Philippot[44].

Our experiments on the recombination of membrane proteins and lipids gave results consistent with our observations concerning the hydrophobic nature of the binding of the antigenic protein in the membrane. The protein peak of the deoxycholate–G-100 column had only 25 % of the M-antibody-binding activity of unfractionated membranes. Recombination of protein and lipid peaks resulted in restoration of the original activity. Lipid activation was identical for lipids from HK or from M-negative LK membranes, and was also equally effective with deoxycholate-fractionated or organic solvent-extracted lipids. Antigenic specificity thus resided only in the protein fraction, but activity was dependent on the presence of lipid.

Full activation required a total lipid extract. Phospholipids and cholesterol were each able to effect partial activation, with cholesterol proving especially potent. This latter result, together with the fact that both sodium dodecyl sulfate and butanol rendered the M antigen inactive, suggests that the ability of deoxycholate to solubilize the M antigen with retention of activity may have been due to similarities in structure between this detergent and cholesterol. When the protein peak from a G-100 column was dialyzed for 5 days in the usual manner, then dialyzed into distilled water and lyophilized, the resulting material contained 60 ± 5 % protein. It is likely that the remaining 40 % was bound deoxycholate, and this is just the figure given by Kahlenberg *et al.*[45] as the percentage of Lubrol, a non-ionic detergent, remaining bound to solubilized ATPase. Cholesterol is normally present in sheep erythrocyte membranes at a level of 0.3 mg/mg protein[46,47]. Therefore, some, but not all, of the binding of deoxycholate to membrane protein in the absence of lipid may occur as a result of substitution for cholesterol. Noguchi and Freed[48] have recently demonstrated activation of the (Na^+–K^+)-ATPase from rat brain by cholesterol, and this enzyme also survives exposure to deoxycholate[49]. These results suggest, therefore, that preservation of activity of membrane proteins may best be achieved with the use of

solubilizing reagents that are closely similar to the normally associated membrane lipid

It is possible that membrane lipids (or an appropriate substitute) are essential for the native conformation of the M-antigen protein. Lipid activation could also result from inclusion of this protein in lipoprotein complexes in a manner that would allow greater accessibility to antigenic sites than in the absence of lipid. The dependence of the antigen on associated lipid is consistent with the suggestion made earlier regarding the probable role of hydrophobic binding and complex nature of the antigen–membrane interaction. Related phenomena have been reported in other systems. Lipids, including lecithin and cholesterol, have been shown to enhance the immunological activity of several lipid haptens[50] Eagle[51] has found that the stimulating effect of cholesterol on the antigen of the Wasserman reaction may be due to adsorption of the antigen with an attendant increase in accessibility. Finally, Green[52] has shown that the Rh antigen from human erythrocytes requires phosphatidylcholine.

The protein of the sheep red cell membrane was solubilized by deoxycholate, but results of gel filtration experiments in gels of high molecular weight exclusion limits suggest that the membrane protein exists in a partially aggregated state in this detergent. The unusually strong retention of this protein by DEAE-Sephadex may be the result of the high negative charge imparted to the protein by its tightly bound deoxycholate, but may also be due to the presence of sialoglycoproteins in these aggregates.

The marked heterogeneity present in the deoxycholate-solubilized protein is evident from the sodium dodecyl sulfate–urea gel electrophoresis patterns. While the M antigen does not survive treatment with this solvent, other media, perhaps a combination of deoxycholate and urea, which separately do not impair activity, might prove useful in further purification procedures. Anionic detergents have received considerable attention in the solubilization of membrane proteins and in the investigation of protein–lipid interactions Interpretation of much of this work has been difficult due to the lack of functional integrity of solubilized preparations. The M antigen thus provides a useful system for further study of membrane structure.

ACKNOWLEDGEMENTS

We are grateful to Dr A. Ottolenghi, Department of Physiology and Pharmacology, Duke University Medical Center, for numerous invaluable discussions during the course of this work

This research was supported by Grant 5PO1-HE12157, Post-Doctoral Fellowship 5-F2-AI-33,144 (P.S), and Research Career Development Award 1-K-4GM50,194 (P.K.L.), all from the National Institutes of Health.

REFERENCES

1 Chavin, S I (1971) *FEBS Lett* 14, 269
2 Kaplan, D M and Criddle, R S (1971) *Physiol Rev* 51, 249
3 Rasmusen, B A and Hall, J G (1966) *Science* 151, 1551
4 Lauf, P K and Tosteson, D C (1969) *J Membrane Biol* 1, 177
5 Ellory, J C and Tucker, E M (1969) *Nature* 222, 477

6 Lauf, P K, Rasmusen, B A, Hoffman, P G., Dunham, P B, Cook, P. H., Parmelee, M. L. and Tosteson, D C. (1970) *J Membrane Biol.* 3, 1
7 Lauf, P K, Parmelee, M L, Snyder, J. J. and Tosteson, D C (1971) *J Membrane Biol* 4, 52
8 Maddy, A. H. (1964) *Biochim Biophys Acta*, 88, 448
9 Bont, W S., Emmelot, P and Vaz Dias, H (1969) *Biochim. Biophys. Acta* 173, 389
10 Razin, S, Neeman, Z. and Ohad, I (1969) *Biochim Biophys Acta* 193, 277
11 Terry, T. M, Engelman, D M and Morowitz, H J. (1967) *Biochim. Biophys. Acta* 135, 391
12 Rottem, S., Stein, O and Razin, S (1968) *Arch Biochem. Biophys* 125, 46
13 Shrager, P, Tosteson, D C and Lauf, P K (1972) *Biophys Soc Abstr* 12, 259a
14 Dodge, J. T, Mitchell, C. and Hanahan, D J. (1963) *Arch Biochem Biophys.* 100, 119
15 Mayer, M M (1961) in *Experimental Immunochemistry*, p 154, Chas. C Thomas Springfield Ill.
16 Fleischman, J B, Pain, R H and Porter, R R. (1962) *Arch. Biochem Biophys* Suppl. 1, 174
17 Fleischman, J B, Porter, R R and Press, E M (1963) *Biochem J* 88, 220
18 Poulik, M D and Lauf, P K (1965) *Nature* 208, 874
19 Lowry, O H., Rosebrough, N. J, Farr, A. L. and Randall, R J. (1951) *J Biol Chem.* 193, 265
20 King, E J (1932) *Biochem J* 26, 292
21 Glick, D, Fell, B F and Sjolin, K-E (1964) *Anal Chem.* 36, 1119
22 Bligh, E G and Dyer, W J (1959) *Can J. Biochem Physiol* 37, 911
23 Davis, B J (1964) *Ann N.Y Acad Sci* 121, 404
24 Summers, D F, Maizel, Jr, J V and Darnell, Jr, J E (1965) *Proc Natl. Acad. Sci U S.* 54, 505
25 Mitchell, C D and Hanahan, D J (1966) *Biochemistry* 5, 51
26 Rosenberg, S A. and Guidotti, G (1969) *J Biol Chem* 244, 5118
27 Marchesi, V T and Steers, E (1968) *Science* 159, 203
28 Hamaguchi, K and Geiduschek, E P (1962) *J Am Chem Soc* 84, 1329
29 Hatefi, Y and Hanstein, W G (1969) *Proc Natl Acad Sci U S* 62, 1129
30 Gwynne, J T and Tanford, C (1970) *J Biol Chem* 245, 3269
31 Maddy, A H and Kelly, P. G (1971) *Biochim Biophys Acta* 241, 114
32 Engelman, D M, Terry, T M and Morowitz, H. J (1967) *Biochim Biophys. Acta* 135, 381
33 Engelman, D M and Morowitz, H J (1968) *Biochim Biophys Acta* 150, 376
34 Engelman, D M and Morowitz, H J (1968) *Biochim Biophys Acta* 150, 385
35 Tanford, C, Kawahara, K and Lapanje, S (1967) *J Am Chem Soc* 89, 729
36 Tanford, C, Kawahara, K, Lapanje, S, Hooker, Jr, T M, Zarlengo, M H, Salahuddin, A., Aune, K C and Takagi, T (1967) *J Am Chem Soc* 89, 5023
37 Nozaki, Y and Tanford, C (1967) *J Am Chem Soc* 89, 742
38 MacLennan, D H and Wong, P T S (1971) *Proc Natl Acad Sci U S* 68, 1231
39 Rodwell, A W, Razin, S, Rottem, S and Argaman, M (1967) *Arch Biochem Biophys.* 122, 621
40 Emmelot, P. and Vaz Dias, H (1970) *Biochim Biophys Acta* 203, 172
41 Salton, M R J (1967) *Trans N Y Acad Sci* 29, 764
42 Kennedy, E P (1969) *J Gen. Physiol.* 54, 91s
43 Lenard, J (1970) *Biochemistry* 9, 1129
44 Philippot, J (1971) *Biochim Biophys Acta* 225, 201
45 Kahlenberg, A, Dulak, N C, Dixon, J F, Galsworthy, P R. and Hokin, L E. (1969) *Arch. Biochem Biophys* 131, 253
46 Casu, A, Pala, V, Monacelli, R, Ferro, M and Nanni, G (1968) *Ital J Biochem* 17, 77
47 Nelson, G (1967) *Biochim Biophys Acta* 144, 221
48 Noguchi, T. and Freed, S (1971) *Nature, New Biol* 230, 148
49 Jorgensen, P L and Skou, J C (1969) *Biochem Biophys Res Commun* 37, 39
50 Rapport, M M and Graf, L (1969) *Prog Allergy* 13, 273
51 Eagle, H (1930) *J Exp Med* 52, 747
52 Green F A. (1968) *J Biol Chem* 243, 5519