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# Comparative Immunochemical Study of Human Erythrocyte Glycoproteins[1] (40240)

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Integral glycoproteins bearing blood group MN alloantigens and virus receptor activity have been obtained by dissociation of erythrocyte membranes with a variety of lipid solvents and detergents (1–10), and constitute major components in the protein profile of the membrane delineated by polyacrylamide gel electrophoresis (PAGE) (5, 11–16). We describe here the results of immunochemical as well as PAGE analyses of glycoproteins rendered water soluble by treatment of erythrocyte ghosts with chloroform–methanol (CM) or lithium diiodosalicylate–phenol (LIS).

*Materials and methods. Preparation of hemoglobin-free erythrocyte membranes.* Hemoglobin-free membranes were prepared by the method of Dodge *et al.* (17) as modified by Howe and Lee (3). Group O (H) human erythrocytes were separated from single donor units of blood obtained from the New Orleans Blood Bank. All procedures were performed at 2–4°.

*Extraction of glycoprotein from hemoglobin-free membranes.* Chloroform–methanol (CM) extraction was performed according to Hamaguchi and Cleve (5) and Howe *et al.* (1). Hemoglobin-free membranes were dialyzed against deionized water for 36–48 hr at 4° and subjected to three cycles of rapid freezing and thawing. Nine volumes of chloroform–methanol (2:1) were added and the mixture shaken mechanically for 30 min at 25° and centrifuged at 900*g* for 1 hr at the same temperature. The upper, aqueous phase was dialyzed against deionized water for 48 hr at 4° and clarified by centrifugation at 110,000*g* for 1 hr prior to lyophilization. One unit of blood (500 ml) yielded 80–100 mg of CM glycoprotein. Extraction of glycoprotein with lithium diiodosalicylate–phenol (LIS) was done by the method of Marchesi and Andrews (10) with modifications. The yield of LIS glycoprotein was 30–35 mg from one unit of blood and the material was not further purified by column filtration. The major glycoprotein band, designated PAS-1 by Fairbanks et al. (11) was isolated by elution of the single band from preparative sodium dodecyl sulfate (SDS) polyacrylamide gels of CM glycoprotein.

Antisera to preparations of CM and LIS erythrocyte membrane glycoproteins were prepared in rabbits by footpad or intramuscular injection. Antigens were coupled with methylated bovine serum albumin and incorporated in complete Freund's adjuvant to a final concentration of 1.0 mg/ml. Each animal received a total of 1.2 mg of antigen, divided among 4 weekly injections. All animals were bled out 10 days after the last injection.

Rabbit antisera were titered for hemagglutination of cells of groups $A_1$, B and O (H) by conventional twofold serial dilution in phosphate buffered saline, pH 7.0 at 25°, agglutination patterns being read after centrifugation at 1000*g* for 30 sec. Preparations of CM and LIS glycoproteins were tested for specific inhibition of human anti-A and anti-B and *Ulex europeus* lectin with A,B and O (H) cells, respectively. Purified human blood group substances were used as positive controls in the same assay systems.

CM and LIS preparations were tested for inhibition of influenza virus agglutination of chicken erythrocytes according to the procedures previously described (3).

Protein determinations were done according to the method of Lowry *et al.* (18). Sialic acid was quantitated by the thiobarbituric acid method of Warren (19) following hydrolysis in 0.1 *N* $H_2SO_4$, 30 min at 80°. Reducing sugar was measured by the method of Hagedorn and Jensen (20) and hexosamine by the method of Elson and Morgan (21) after hydrolysis in 2 *N* HCl 2 hr at 100° and neutralization.

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*Immunochemical methods.* Quantitative precipitin analysis was carried out according to the procedure of Mage and Dray (22), the total protein precipitated being determined by the Lowry method. The presence of antibody and/or antigen remaining in supernatant fluids after removal of primary specific precipitates was detected by counterimmunoelectrophoresis (CIE) (23). Two dimensional immunoelectrophoresis was done according to the methods of Ollier and Hartmann (24) and Laurell (25).

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (7.5% gels) was done as described by Maizel (26) in 0.1% SDS/0.01 *M* sodium phosphate, pH 7.1 with samples heated to 100° for 1 min, or in 0.1% SDS in 0.05 *M* Tris/glycine, pH 8.6 with samples heated for 5 min at 100°. Gels were stained with Coomassie brilliant blue (CBB) or periodic acid Schiff (PAS) reagent. For the latter, gels were fixed with 25% isopropyl alcohol/10% acetic acid for 14–16 hr at room temperature with several changes, the rest of the PAS staining procedure being carried out at 2–4°. Gels were treated with 0.4% periodic acid for 1 hr and rinsed with several changes of cold 10% acetic acid before staining with Schiff reagent. All stained gels could be stored at 4° for an indefinite period, without diminution in the intensity of the stained bands.

Individual PAS-positive components in CM glycoprotein were separated in preparative electrophoresis (Ortec Inc.) and eluted into water and lyophilized. Unbound SDS was removed by electrophoresis through Triton X-100 [27], or precipitation with 9 vol of absolute ethanol (5) at 25°.

*Results. Chemical analyses and inhibition assays.* As shown in Table I, there were no striking differences between CM and LIS glycoprotein preparations with respect to either inhibition of viral HA or overall chemical composition. Accordingly, it may also be inferred that the complement of neutral sugars in CM preparations, while not yet directly examined, is the same as that of the glycoproteins recovered by phenol extraction of membranes (7, 28). PAS-1 preparations isolated from CM glycoprotein were less active, and contained one third the amount of NANA found in the total CM glycoprotein.

*Polyacrylamide gel electrophoresis.* Preparations of erythrocyte membrane run in Tris/glycine buffer pH 8.6 preheated at 100° for 5 min gave patterns of multiple protein bands, including the spectrin (S) doublet (11, 29), in gels stained with Coomassie brilliant blue (Fig. 1a). Corresponding gels stained with periodic acid Schiff reagent showed four major glycoprotein bands (Fig. 1b). PAGE profiles of LIS glycoprotein in paired gels (CBB and PAS) (Fig. 1c,d) were essentially similar to those of CM glycoprotein (Figs. 1e,f; 4). CM preparations included the spec-

TABLE I. CHEMICAL AND BIOLOGICAL ANALYSIS OF GLYCOPROTEINS FROM ERYTHROCYTE MEMBRANES.

| Glycoprotein preparation | % Protein[a] | %NANA[b] | NANA protein | % Reducing sugar[c] | %Hexosamine[c] | μg[d] Inhibiting 4 HAU[e] influenza A virus (FM1)[f] |
|---|---|---|---|---|---|---|
| CM[g] | | | | | | |
| 1 | 32 | 14 | 0.4 | 24 | 12 | 0.1 |
| 2 | 40 | 15 | 0.4 | 29 | 12 | 0.1 |
| 3 | 50 | 14 | 0.3 | 19 | 10 | 0.1 |
| 4 | 50 | 17 | 0.3 | 28 | 12 | 0.2 |
| LIS[h] | | | | | | |
| 1[i] | 48 | 17 | 0.3 | 26 | 15 | 2.5 |
| 2[i] | 51 | 18 | 0.3 | 24 | 16 | 5.0 |
| 3 | 42 | 15 | 0.3 | 26 | 12 | 0.1 |

[a] Lowry method (18).

[b] After hydrolysis in 0.1 *N* $H_2SO_4$ for 30 min at 80° (19). Represents total NANA of intact membranes.

[c] After hydrolysis in 2 *N* HCL for 2 hr at 100° and neutralization (20, 21).

[d] μg protein.

[e] Hemagglutinating units.

[f] "Indicator" virus (1).

[g] Chloroform-methanol.

[h] Lithium diiodosalicylate–phenol. Neutral sugars (13–16%), galactose: mannose, 5:1; no glucose (7).

[i] Reference 28.

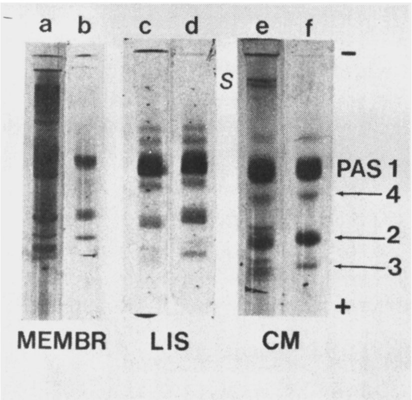

FIG. 1. Polyacrylamide gel electrophoresis of human erythrocyte membranes (MEMBR, 100–125 μg) LIS and CM glycoproteins (25–30 μg) heated 100° for 5 min. In 0.1% SDS/0.05 *M* Tris–glycine buffer (pH 8.6), 7.5% gel. (a,c,e) Coomassie brilliant blue (CBB). (b,d,f,) periodic acid Schiff (PAS). (S) Spectrin.

trin doublet and some minor protein components above the PAS-2 and below the PAS-3 positions, respectively (Fig. 1e). In SDS-PAGE in phosphate buffer pH 7.1, both CM and LIS revealed one and the same major band, and a minor component which stained with both CBB (Fig. 2a,c) and with PAS (Fig. 2b,d).

PAS-1 eluted from SDS-PAGE and analyzed without preheating showed a single band (Fig. 3c,d) corresponding to the position of PAS-1 in the unfractionated CM glycoprotein (Fig. 3a,b). The same preparation of PAGE-purified PAS-1 heated at 100° for 5 min showed bands corresponding to both PAS-1 and PAS-2 (Fig. 3e,f), each staining with both CBB and PAS.

Six individual PAS bands (Fig. 4a-f), from highest to lowest molecular weights in gels of CM glycoprotein, and were eluted and rerun in the same buffer system. As shown in Fig. 4, band a reran in the position of PAS-1, bands b–e in positions of PAS-1 and 2, and band f in the position of PAS-3.

*Immunochemical analysis.* All rabbits immunized with CM glycoprotein produced high levels of agglutinins to A, B and O (H) erythrocytes. Pre- and postimmunization sera tested concurrently showed from 16- to 512-fold rises in agglutination titer, one rabbit giving a 1,024-fold rise. Those immunized with LIS glycoprotein showed 8 to 64-fold rises in antibody level. Those immunized with PAS-1 responded less well, showing only 4- to 64-fold rises in titer (Table II).

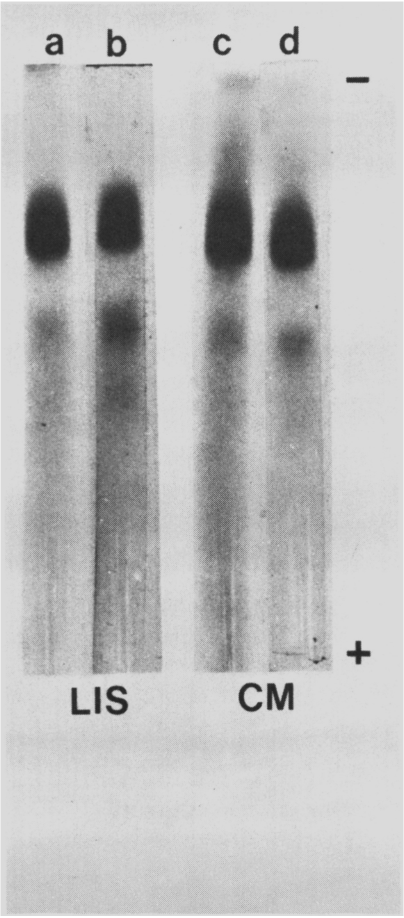

FIG. 2. Polyacrylamide gel electrophoretic profile of CM and LIS glycoproteins (80–100 μg). In 1% SDS/0.01 *M* phosphate buffer (pH 7.1), 7.5% gel. (a,c) CBB. (b,d) PAS.

The ABO (H) agglutinins in representative antisera to CM glycoprotein or to purified PAS-1 were not significantly inhibited by purified soluble blood group A, B, or O (H) substances (results not shown), each of which was a potent inhibitor of the homologous system with human alloantiserum and H lectin (*Ulex europeus*). A higher concentration of CM glycoprotein (12–100 μg), in assays with all three cell types, inhibited antibody to CM glycoprotein but neither human alloantiserum.

Immunoelectrophoretic analysis (results not shown) of CM and LIS antigens developed with antisera to CM glycoprotein demonstrated a single heavy precipitin line with antigen of the same mobility in every prepa-

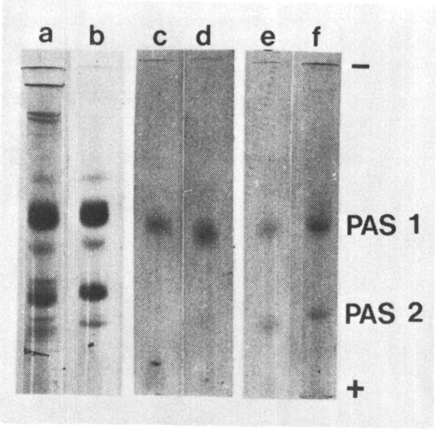

FIG. 3. Polyacrylamide gel electrophoresis of PAS-1 isolated from preparative gels, reelectrophoresed in 0.1% SDS/0.5 *M* Tris/glycine buffer (pH 8.6), 7.5% gel. (a,b) unfractionated CM glycoprotein (25–30 μg), 100° 5 min. (c,d) PAS-1,13 μg unheated. (e,f) PAS-1,13 μg 100° 5 min. (a,c,e) CBB. (b,d,f) PAS.

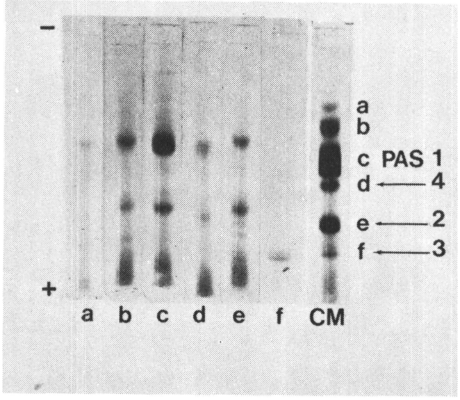

FIG. 4. Polyacrylamide gel electrophoretic pattern of PAS-positive bands (10–15 μg protein) separated from CM glycoprotein (50–60 μg), preheated 100° 5 min. In 0.1% SDS/0.05 *M* Tris-glycine buffer (pH 8.6), 7.5% gel. (a) to (f), highest to lowest molecular weight. PAS stain.

ration. Patterns developed with antisera to LIS antigen, as well as with antiserum to PAS-1, showed the same single major component in both CM and LIS glycoproteins. PAS-1 which had been isolated from CM extract by elution from SDS gels also gave a well defined single precipitin line with homologous serum as well as with antibody to CM glycoprotein (serum 26).

Two dimensional immunoelectrophoresis of CM glycoprotein developed with antiserum to PAS-1 (serum 94) showed a single arc of precipitation (Fig. 5A). The same pattern was seen when either CM or LIS glycoprotein was electrophoresed against antibody to CM glycoprotein (serum 89) and to PAS-1 (serum 93) to form a single precipitin line.

In quantitative precipitin analyses with antiserum to PAS-1 (serum 93) LIS and CM preparations each precipitated approximately the same amount of total protein at the maximum (Fig. 6). The supernatant tests clearly delineated regions of excess antibody and excess antigen for each curve.

*Discussion.* We have attempted to examine immunochemical relationships among integral erythrocyte membrane glycoproteins obtained by two different extraction procedures. Our CM and LIS glycoprotein preparations were comparable in biological activity to products obtained by treatment of membranes with warm phenol (3, 7). The single component (PAS-1) isolated from CM glycoprotein was consistently less active in inhibiting influenza virus hemagglutinin than the whole CM glycoprotein from which it had been derived. This may be explained by aggregation of the active subunits in the CM preparation, in which the larger molecule would be expected to be more reactive with influenza viruses than the single subunit.

Glycoprotein obtained by LIS-phenol treatment of human erythrocytes was first described (10) as a single homogeneous band in SDS-phosphate gel. Subsequently, one major and one or two minor bands were found (5) which stained for both protein and carbohydrate (5, 31). Our gels run in phosphate buffer, pH 7.1 contained one major and one minor band when stained for either protein

TABLE II. HEMAGGLUTININS ELICITED BY GLYCOPROTEINS ISOLATED FROM ERYTHROCYTE MEMBRANES.

| Antigen (No. of rabbits) | Fold rise[a] in agglutinin titer for erythrocytes[b] of group | | |
|---|---|---|---|
| | $A_1$ | B | 0 |
| CM[c,d] (5) | 32—1024 | 32–512 | 16–256 |
| LIS[e,f] (3) | 8–32 | 8–64 | 16–64 |
| PAS-1[g,d] (3) | 16–32 | 4–32 | 4–64 |

[a] Pre- and postimmunization sera tested concurrently.

[b] 1% cells at 25°.

[c] Chloroform-methanol extracted.

[d] Toe-pad injections.

[e] Lithium diiodosalicylate-phenol extracted.

[f] Intramuscular injections.

[g] Single component isolated from CM glycoprotein by preparative electrophoresis.

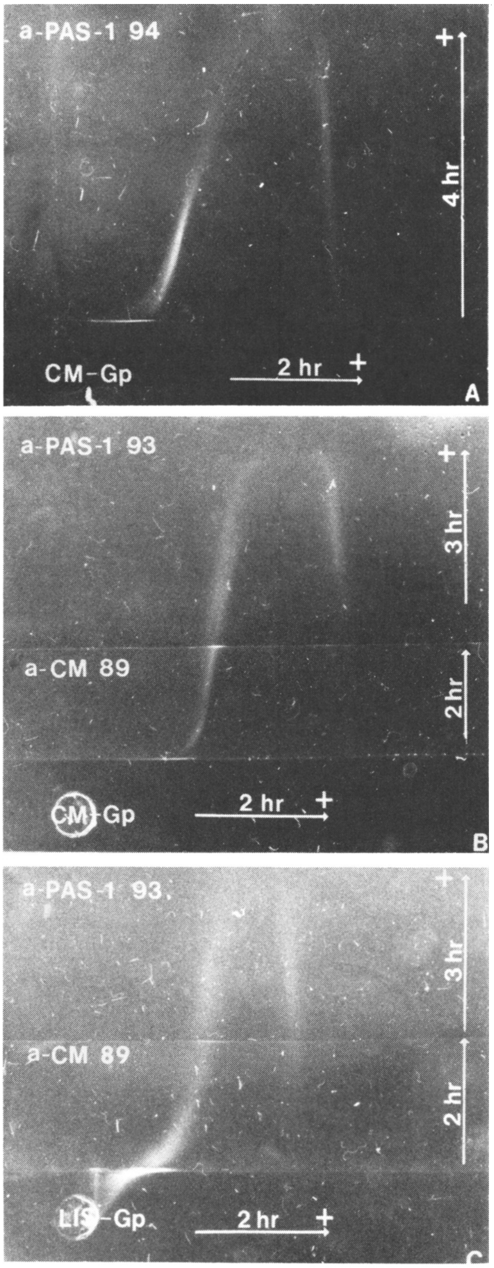

FIG. 5. Two-dimensional immunoelectrophoresis. (A) CM antigen against serum 94 (anti-PAS-1). (B) CM antigen and (C) LIS antigen against serum 89 (anti-CM) and serum 93 (anti-PAS-1).

or carbohydrate (Fig. 2). These discrepancies may be accounted for in part by variations in dissociating conditions and in protein quantities submitted to analysis. In the gel patterns in Tris/glycine buffer, pH 8.6, at least six PAS bands were revealed, including those readily identified as PAS-1,2,3 and 4, in both CM and LIS preparations. Spectrin was present in CM but not in LIS preparations.

PAS-1 appears to be a dimer of PAS-2, the larger molecule dissociating under different conditions and to varying extent (13, 14, 16, 32, 33). In our hands, the dimer converted to the monomeric form only when the whole membrane was preheated to 100°. The conversion of PAS-1 and PAS-2 does not occur in the phosphate buffer system (13).

The results of SDS–PAGE analysis of each of the six PAS bands (Fig. 4) isolated by preparative electrophoresis from CM glycoprotein confirmed that fact that there are but three major glycoproteins, in accord with the findings of other investigators utilizing different methods of fractionation (5, 13, 14, 16, 32). We observed no aggregational conversion of PAS-3 to higher molecular weight components. PAS-1 was converted by heating at 100° from dimeric to monomeric form (Fig. 3).

All the PAS positive components of CM glycoprotein reacted in gels with resorcinol reagent (34) for sialic acid, and there was close correspondence between protein and PAS stains (Fig. 3), as already noted by other investigators (5, 30–32). From our own findings (Fig. 4), we conclude that (i) PAS-1 and 2 are interconvertible and include two glycoproteins (bands a and b in Fig. 4) not initially migrating as PAS-1; (ii) what appears to be PAS-4 preceding PAS-1 in the complete profile of CM protein (band d in Fig. 4) is disaggregated, and hence changed in size, as

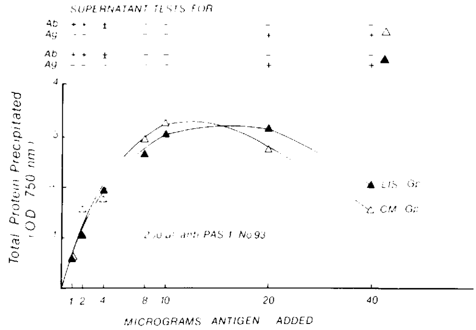

FIG. 6. Quantitative precipitin analyses of CM (△) and LIS (▲) glycoproteins with serum 93 (anti-Pas-1). Supernatants from both curves tested by counterimmunoelectrophoresis (+ or −) for antigen with serum 93, and for Ab with CM and LIS antigens, respectively.

a result of repeated electrophoresis, and ends up as part of PAS-1 and 2 (16); (iii) PAS-3, the smallest glycoprotein, remains unchanged.

The interconversion of PAS-1 and 2 may depend on the distribution of certain groupings among the individual molecules which render them able to "self-assemble" into two classes of macromolecules, i.e. PAS-1 and 2.

Our preparations of glycoproteins, (CM,LIS,PAS-1) evoked agglutinins in rabbits for human erythrocytes of each of the three major blood groups (A,B,O(H)). These hemagglutinins were not inhibited by pure blood group substances, but were inhibited by whole unfractionated CM glycoprotein and the homologous dimeric antigen (PAS-1). Although some reports have indicated that blood group ABO (H) activity is associated with glycoproteins in the membrane (35), recent evidence supports the contention that ABO (H) blood group activity resides in glycolipids which may become occluded to glycoproteins during their separation from the membrane (36). Only MN blood group activity has been regularly associated with the glycoprotein fraction (37), in agreement also with our own earlier findings (3). From the foregoing, it appears that antibody elicited by either CM or LIS glycoproteins (Table II) displays a specificity distinctly broader than, and independent of, the ABO (4) system. As shown in previous quantitative studies (3), agglutinins for all three cell types were completely removed from antiserum to membranes by precipitation with purified glycoprotein at equivalence, regardless of blood group of the erythrocytes from which the soluble antigen had been derived. The several glycoprotein components which are separable on the basis of molecular weight (PAS 1-4) have an overall antigenic identity demonstrated by the results of two dimensional immunoelectrophoretic analysis (Fig. 5). Accordingly, it appears that our antisera do not discriminate between the physical "subunits" or its various aggregated forms. This conclusion is also consistent with the results of quantitative precipitin analyses (Fig. 6) with antiserum to purified PAS-1 in which the curves with LIS and CM glycoproteins were virtually superimposable.

The distribution of receptor activity among the several physical components is uncertain. However, sialic acid, a necessary component for interaction with myxoviruses, was present in all components. Dimeric PAS-1, even after purification by elution from preparative gels and ethanol precipitation, was active. These findings, taken together with the evidence for immunochemical homogeneity, suggest that all the PAS components may be involved in myxovirus inhibitory activity in proportion to their molecular size.

*Summary.* Human erythrocyte membrane glycoproteins were extracted by the chloroform-methanol (CM) and the lithium diiodosalicylate-phenol (LIS) methods. Each preparation comprised up to 50% protein and 30–40% carbohydrate (reducing sugar and *N*-acetylneuraminic acid). Both types of preparations contained receptors for influenza virus.

Electrophoretic analysis in SDS polyacrylamide gel in a phosphate buffer system revealed one major and one minor band in both preparations. In contrast, numerous interconvertible bands, including PAS 1,2,3 and 4 were found in gels using a Tris/glycine buffer system. The major glycoprotein (PAS-1) was separated from other CM glycoprotein components by preparative electrophoresis.

Antisera made to both CM and LIS glycoproteins elicited agglutinins which were equally reactive with cells of all three ABO(H) blood groups and unrelated to the corresponding blood group determinants. PAS-1 also evoked similar broadly specific antibodies which, in quantitative precipitin analyses and two dimensional immunoelectrophoresis, served to establish antigenic identity among CM and LIS glycoproteins, probably based on common determinants in the protein moiety.

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1. Howe, C., Rose, H. M., and Schneider, L., Proc. Soc. Exp. Biol. Med. **96,** 88 (1957)
2. Folch, J., Arosove, S., and Meath, J. A., J. Biol. Chem. **191,** 819 (1951).
3. Howe, C., and Lee, L. T., J. Immunol. **102,** 573 (1969)
4. Kornfeld, R., and Kornfeld, S., J. Biol. Chem. **245,** 2536 (1970)
5. Hamaguchi, H., and Cleve, H., Biochim. Biophys.

Acta **278,** 271 (1972)
6. Maddy, A. H., Biochim. Biophys. Acta **88,** 448 (1964)
7. Lloyd, K. O., Howe, C., and Lee, L. T., Methods Enzymol. **28,** 236 (1972)
8. Rosenberg, S. A., and Guidotti, G., J. Biol. Chem. **244,** 5118 (1969)
9. Blumenfeld, O. O., Gallop, P. M., Howe, C., and Lee, L. T., Biochim. Biophys. Acta **221,** 109 (1970)
10. Marchesi, V. T., and Andrews, E. P., Science **174,** 1247 (1971)
11. Fairbanks, G., Steck, T. L., and Wallach, D. F. H., Biochemistry **10,** 2606 (1971)
12. Tanner, M. J. A., and Boxer, D. H., Biochem J. **129,** 333 (1972)
13. Teuch, J. K., and Morrison, M., Biochem. Biophys. Res. Commun. **59,** 352 (1974)
14. Slutzky, G. M., and Ji, T. H., Biochim. Biophys. Acta **373,** 337 (1974)
15. Furthmayr, H., and Marchesi, V. T., Biochemistry **15,** 1137 (1976)
16. Potempa, L. A., and Garvin, J. E., Biochem. Biophys. Res. Commun. **72,** 1049 (1976)
17. Dodge, J. T., Mitchell, C., and Hanahan, D. J., Arch. Biochem. Biophys. **100,** 119 (1963)
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193,** 265 (1951)
19. Warren, L. J., Biol. Chem. **234,** 1971 (1959)
20. Hagedorn, H. C., and Jensen, B. N., Biochem. Z. **135,** 46 (1923)
21. Elson, L. A., and Morgan, W. T. J., Biochem. J. **27,** 1824 (1933)
22. Mage, R., and Dray, S., J. Immunol. **95,** 525 (1965)
23. Lee, L. T., Howe, C., Meyer, K., and Choi, H. U., J. Immunol. **102,** 1144 (1969)
24. Ollier, M. P., and Hartmann, L., Biomedicine **25,** 184 (1976)
25. Laurell, C. B., Analyt. Biochem. **10,** 358 (1976)
26. Maizel, J. V., *in* "Methods in Virology" (K. Maramorosch and H. Koprowski, eds.), pp. 179, Academic Press, N.Y. and London (1971)
27. Lee, L. T., Deas, J. E., and Howe, C., J. Immunol. Methods **19,** 69 (1978)
28. Mitchell, T. G., Lee, L. T., and Howe, C., Immunol. Commun. **2,** 35 (1973)
29. Marchesi, V. T., Furthmayr, H., and Tomita, M., Ann. Rev. Biochem. **45,** 667 (1976)
30. Cleve, H., Hamaguchi, H., and Hütteroth, T., J. Exp. Med. **136,** 1140 (1972)
31. Hamaguchi, H., and Cleve, H., Biochem. Biophys. Res. Commun. **47,** 459 (1972)
32. Furthmayr, H., Tomita, M., and Marchesi, V. T., Biochem. Biophys. Res. Commun. **65,** 113 (1975)
33. Marton, L. S. G., and Garvin, J. E., Biochem. Biophys. Res. Commun. **52,** 1457 (1973)
34. Heuser, E., Lipp, L., and Weigandt, H., Anal. Biochem. **50,** 382 (1974)
35. Whittemore, N. B., Trabold, N. C., Reed, C. F., and Weed, R. I., Vox Sang. **17,** 289 (1969)
36. Gardas, A., and Kóscielak, J., Eur. J. Biochem. **32,** 178 (1973)
37. Springer, G. F., and Desai, P. R., Biochem. Biophys. Res. Commun. **61,** 420 (1974).

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