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Isolation and Partial Characterization of the Heterophile Antigen of Infectious Mononucleosis From Bovine Erythrocytes

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The heterophile antigen (Paul-Bunnell antigen, PBA) of infectious mononucleosis was isolated by extraction of an aqueous suspension of bovine erythrocyte stromata with chloroform-methanol (2:1). The upper aqueous layer contained gangliosides, PBA, and a high-molecular-weight glycoprotein. PBA and gangliosides were separated from the high-molecular-weight glycoprotein by extraction of lyophilized upper layer with chloroform-methanol solvents. Separation of PBA from gangliosides was carried out by chromatography on DEAE-cellulose with chloroform-methanol solvents. PBA appeared to be a minor glycoprotein component of the erythrocyte membrane and had both hydrophobic and hydrophilic properties. It was soluble in either organic or aqueous solvents. On SDS-polyacrylamide gel electrophoresis, it migrated as a single component that stained for protein with Coomassie blue, for carbohydrate with periodic acid-Schiff reagent, and for lipid with oil red 0; it had an apparent molecular weight of 26,000. It was composed of 62% protein with major amino acids: glutamic acid, proline, glycine, isoleucine, leucine, and threonine (158, 116, 98, 90, 85, and 82 residues per 1,000 residues, respectively). Carbohydrate content was 9.2% with major sugar constituents: sialic acid, galactosamine, and galactose. Serologic activity of PBA was destroyed by pronase but not by trypsin.

Key words: bovine erythrocytes, heterophile antigen, infectious mononucleosis, membranes, Paul-Bunnell antigen

Cellular components common to cells of different species have been recognized for many years. Forssman (1), for example, demonstrated in 1911 that the injection of guinea pig kidney suspensions into rabbits induced the formation of antibodies (Forssman antibodies) that reacted with sheep erythrocytes. Forssman antibodies have been termed heterophile antibodies, that is, antibodies which react with cells or tissues of species that are unrelated to the original species that provided the antigenic preparation used for immunization. Heterophile antigens are thus defined as antigens which stimulate the formation of or are capable of combining with heterophile antibodies. The Forssman antigen is

Abbreviations: HD – Hanganutziu-Deicher; IM – infectious mononucleosis; PAS – periodic acid-Schiff reagent; PBA – Paul-Bunnell antigen; PBS – phosphate buffered saline; SDS – sodium dodecyl sulfate.

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found in a variety of mammalian species (e.g., sheep, goat, horse, guinea pig) and has been identified as a glycosphingolipid (2). A ceramide pentasaccharide with similar structures has been isolated from horse spleen and from sheep and goat erythrocytes (2, 3, 4).

Paul and Bunnell in 1932 (5) reported that sera of patients suffering from infectious mononucleosis agglutinated sheep erythrocytes. Subsequently, it was established that these heterophile antibodies which differ from Forssman antibodies, also acted on horse, goat, and bovine erythrocytes. The appearance of Paul-Bunnell antibodies in "normal" sera or in patients suffering from diseases other than IM[1] is apparently extremely rare (6). In fact, the detection of these antibodies forms the basis of a key test in the diagnosis of IM (6). Although the Epstein-Barr virus has been implicated as the causative agent of IM (7, 8), the antigenic stimulus responsible for the formation of Paul-Bunnell antibodies still remains unknown.

The heterophile antigen of IM (Paul-Bunnell antigen, PBA) has been isolated from horse, sheep, goat, and bovine erythrocytes by hot 75%- ethanol extraction of stromata (8, 9). Using similar procedures a glycoprotein possessing M and N specificity has been obtained from human erythrocytes (8). In the case of horse, sheep, and goat erythrocytes, the PBA antigenic determinants appeared to reside on a glycoprotein, that is, closely related to the human erythrocyte major sialoglycoprotein (glycophorin) both with regard to amino acid patterns and carbohydrate constituents and content. However, the bovine erythrocyte PBA differed from PBA isolated from other mammalian species in its relatively lower carbohydrate content and its resistance to proteases (8, 10). Although the overall chemical composition of the various glycoproteins appears to be similar, they are clearly distinguished from the human erythrocyte glycoprotein by their antigenic activity. The underlying structural features responsible for these antigenic differences are unknown.

In this communication we describe a fractionation procedure that permits the isolation of the neutral glycolipids, gangliosides, a high-molecular-weight glycoprotein, and PBA from bovine erythrocyte stromata. PBA has been purified and partially characterized. Its properties are of special interest since it appears to have characteristics of a hydrophilic and hydrophobic glycoprotein. In addition, we also report that the ganglioside fraction obtained from bovine erythrocyte membranes reacted with Hanganutziu-Deicher antibodies (11, 12). These antibodies, commonly referred to as "serum sickness antibodies," were initially observed by Hanganutziu and Deicher as agglutinins for sheep erythrocytes in sera of patients who received therapeutic injections of horse antitoxin. They have also been reported to be present in patients who received γ globulin fractions of goat antisera to human thymocytes (13) and in pathologic human sera of patients who had apparently never received injections of foreign species sera (14). The following differences between PBA, Forssman, and HD antigens may be noted: PBA appears on bovine and sheep erythrocytes but not on guinea pig kidney, Forssman antigen appears on sheep erythrocytes as well as guinea pig kidney but not on bovine erythrocytes, and HD antigens are found in all 3 of these tissues.

MATERIALS AND METHODS

Materials

All organic solvents were reagent grade and were redistilled before use. Trypsin treated with L-(tosylamido 2-phenyl) ethyl chloromethyl ketone was purchased from Worthington Biochemical Corp. (Freehold, New Jersey), pronase from Calbiochem (La Jolla, California), and DEAE-cellulose from Brown Co. (Berlin, New Hampshire). All other chemicals were of the highest purity commercially available.

Analytic Methods

For the determination of neutral sugars, samples were hydrolyzed in 1 N HCl for 6 h at 100°C in sealed tubes and the hydrolysates were passed through coupled columns of Dowex 50 and Dowex 1 (15). Neutral sugars were determined by means of automated borate complex anion exchange chromatography (Technicon system) at a column temperature of 45°C with the elution gradient described by Lee et al. (16). Sialic acid was estimated by the thiobarbituric acid reaction following hydrolysis in 0.1 N H_2SO_4 at 80°C for 1 h (17). Total hexose was measured by the phenol-H_2SO_4 method (18). For the determination of amino acids and amino sugars, samples were hydrolyzed in constant boiling HCl under nitrogen at 105°C for 28 h. Analyses were carried out on a Beckman 120-C analyzer. Total half-cystine was analyzed as cysteic acid and methionine as methionine sulfone after performic acid oxidation according to the method of Hirs (19). Protein was determined by the method of Lowry et al. (20); phosphate by the procedure of Bartlett (21); and sphingosine by the spectrophotometric method of Lauter and Trams (22). Fatty acids were determined after hydrolysis of 5 mg samples in 1.0 ml of 2 N KOH-methanol at 100°C for 24 h. Prior to hydrolysis, heneicosanoic acid was added as internal standard. After acidification with HCl, the hydrolysate was extracted 3 times with 1.0 ml of n-hexane. The hexane extracts were concentrated to dryness and esterification was carried out with 0.5 ml of 14% boron trifluoride in methanol in sealed tubes at 100°C for 30 min. After esterification, 0.5 ml of water was added to each tube and the contents extracted 3 times with 1.0 ml of n-hexane. The hexane extracts were concentrated to a small volume, and analysis of the fatty acid methyl esters was carried out by gas liquid chromatography (23).

Hemagglutination Inhibition Tests

These tests were carried out in plastic microtiter plates. In studies to detect PBA activity, antigen and IM serum were diluted with diluent, normal rabbit serum 1:60 in PBS (116 mM NaCl-13.6 mM sodium potassium phosphate, pH 7.0). Antigen (0.025 ml) and diluent (0.025 ml) were added to the first well and serial twofold dilutions prepared. IM serum (0.025 ml) at 4 hemagglutinating doses was added to each well and after 1 h of incubation at room temperature, 0.025 ml of a 1% suspension of sheep red blood cells in PBS was added to each well. The tubes were incubated for 2 more hours and examined for hemagglutination. HD antigenic activity was measured in the same way except HD sera were used and the diluent was PBS. One unit of hemagglutination inhibiting activity was defined as the minimum amount of material necessary to completely inhibit the agglutination of sheep erythrocytes by 4 hemagglutinating doses of IM serum (or HD serum) under standard assay conditions.

Double Diffusion Gel Precipitation Tests

These tests were performed in plastic petri dishes containing 1% agarose gel according to the procedure described by Milgrom et al. (24).

Sera

Sera of patients with IM were obtained from the Student Health Service of the State University of New York at Buffalo. Sera from patients who received γ globulin fractions of goat antisera to human thymocytes were our source of HD antibodies and were kindly supplied by Dr. B. Pirofsky of the Division of Immunology and Allergy, University of Oregon, Portland, Oregon.

SDS-Polyacrylamide Gel Electrophoresis

Samples were treated with 1% SDS in 0.005 M sodium phosphate buffer, pH 7.2, containing 2.5 M urea, 0.5% 2-mercaptoethanol, and 0.005% EDTA for 15 min at 37° and 45 min at room temperature (25). SDS-gel electrophoresis was performed in 10% gels by the method of Weber and Osborn (26). Protein was stained with Coomassie blue and carbohydrate with PAS reagent according to the procedures described by Segrest and Jackson (27). For the detection of lipid containing components, gels were fixed overnight in methanol-acetic acid-water (50:5:45), followed by staining for 24 h with a solution of oil red O prepared by mixing equal volumes of 20% trichloroacetic acid and a saturated solution of oil red O in methanol (28). Destaining was carried out with the methanol-acetic acid-water fixative solution. To compare oil red O and PAS stained gels, the latter were treated with the fixative solution until the size of the gel was similar to the gel previously treated with oil red O.

Preparation of Bovine Erythrocyte Membrane Extracts

Fresh pooled blood of cattle was obtained from a local slaughter house and collected into a sodium citrate solution (4.8% sodium citrate – 0.9% NaCl; 1.0 ml per 9.0 ml blood). The erythrocytes were washed 3 times with 3 volumes of PBS. Stromata were prepared by hemolyzing washed erythrocytes with 20–30 volumes of distilled water usually containing from 0.8 to 1.4 ml of 5% acetic acid per liter. The erythrocyte ghosts were allowed to sediment overnight at 4°C and were harvested after centrifugation at 20,000 × g for 15 min at 4°C. The ghosts were washed repeatedly with water followed by 0.9% NaCl until either the water or saline washes were colorless.

Erythrocyte glycoproteins were extracted from stromata suspensions by a modification of the method described by Kornfeld and Kornfeld (29) and Hamaguchi and Cleve (30). Aqueous suspensions of mechanically homogenized stromata (15–20 mg/ml) were stirred vigorously for 2 h with 6 volumes of a chloroform-methanol (2:1) mixture. Unless stated otherwise, all procedures were carried out at room temperature. Preparations with a final volume of less than 1.0 liter were centrifuged at 500 × g for 15 min and the aqueous layer removed and treated as described below. Preparations with a final volume greater than 1.0 liter were allowed to stand overnight. Solid debris was removed by filtration through Whatman No. 1 solvent-washed filter paper. The mixture was added to a separatory funnel and the upper aqueous layer and lower organic layer were separated. The upper layer was concentrated in vacuo in a rotary evaporator at 37°C with frequent additions of n-proponal to prevent foaming, followed by dialysis against distilled water overnight at 4°C. Further concentration to approximately the volume of the original membrane suspension was achieved by ultrafiltration with a PM-10 membrane (Amicon Company, Lexington, Massachusetts). The opalescent material was centrifuged at 38,000 × g for 40 min and the pellets were discarded. The clear supernatant fluid which contained the erythrocyte glycoproteins and gangliosides was lyophilized. The lower layer contained neutral glycolipids and was not further studied.

Separation of Gangliosides, PBA, and High Molecular Weight Glycoproteins

The lyophilized membrane extract was further resolved into a fraction which contained gangliosides, a component tentatively identified as ganglioside and PBA, and into a high-molecular-weight glycoprotein fraction. Lyophilized membrane extract (1 g) was vigorously stirred with 200 ml of chloroform-methanol-water (1:1:0.3) for 1 h. The

mixture was filtered with a Büchner funnel using an aspirator and a Whatman No. 54 solvent-washed filter paper. The residue was reextracted with 200 ml chloroform-methanol-water (1:1:0.3) 3 more times and the combined extracts were concentrated under reduced pressure, dialyzed against distilled water overnight and lyophilized (ganglioside and PBA fraction). The residue remaining was further extracted sequentially, 3 times for 1 h with 100 ml of chloroform-methanol (2:1) containing 4% H_2O and 1% ammonium acetate, 3 times for 1 h with 100 ml of chloroform-methanol-water (1:1:0.3) containing 1% ammonium acetate, and 3 times for 1 h with 100 ml of chloroform-methanol-water (1:1:10) containing 1% ammonium acetate. The latter extract contained the high-molecular-weight glycoprotein. The extraction protocol is summarized in Fig. 1.

Gangliosides and PBA were resolved by chromatography on a DEAE-cellulose column prepared as described by Rouser et al. (31). DEAE-cellulose (9.6 g) in glacial acetic acid was packed in a column of 1.5 cm diameter and the column was washed successively with 6 bed volumes of methanol and 3 bed volumes of chloroform-methanol (1:1). The sample was applied directly to the column or as a suspension in chloroform-

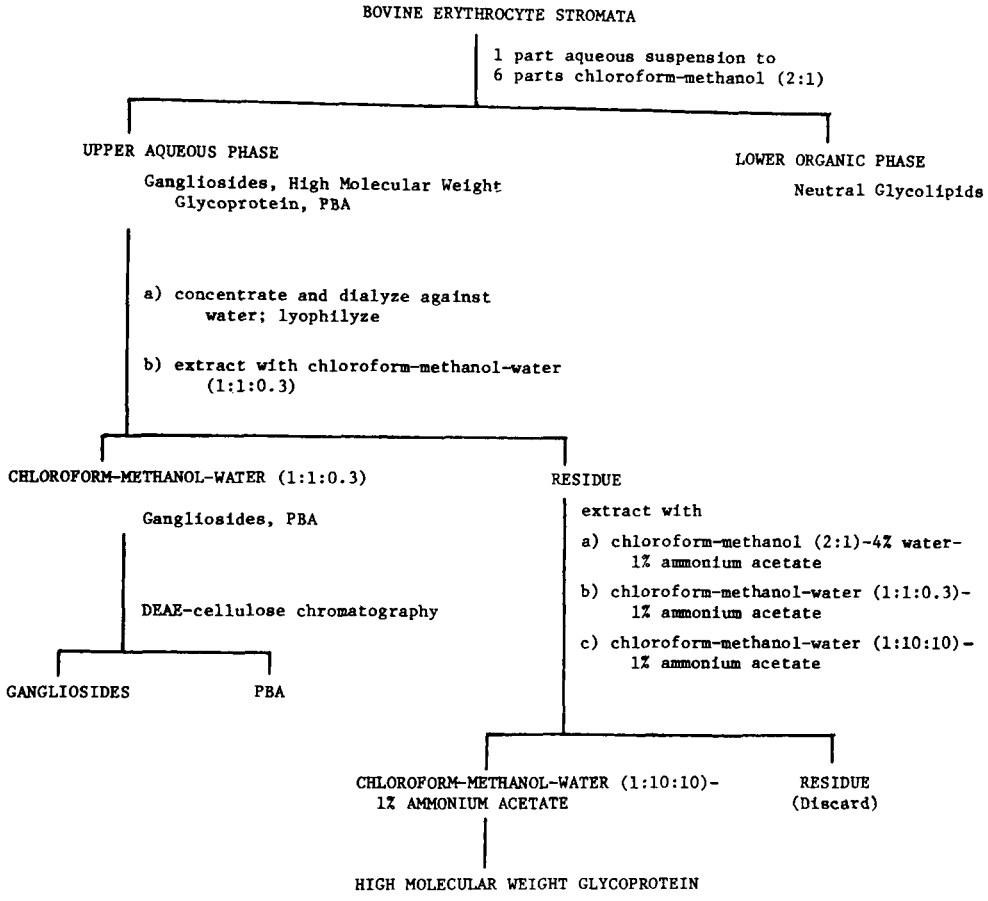

Fig. 1. Summary of fractionation procedures for the isolation of PBA, gangliosides, and high-molecular-weight glycoprotein. Bovine erythrocyte membranes were treated as diagrammed in the figure and described under Materials and Methods.

methanol (1:1) (Solvent 1). After the column was washed with 200 ml of the same solvent, gangliosides were eluted with chloroform-methanol (2:1) saturated with aqueous 28% ammonium hydroxide (Solvent 2); 10-ml fractions were collected and analyzed for hexose by the phenol-sulfuric acid method. The fractions containing hexose were combined, evaporated in vacuo, dialyzed against distilled water overnight, and lyophilized. After no further hexose containing material was detected in the fractions, the column was washed with an additional 100 ml of solvent. About 600 ml of this solvent was poured through the column. The column was then washed successively with 200 ml of chloroform-methanol (2:1) containing 4% water (Solvent 3), 200 ml of chloroform-methanol-water (1:1:0.3) (Solvent 4), and chloroform-methanol-water (1:1:0.3) containing 0.02 M ammonium acetate (Solvent 5). In the latter case 11.5 ml fractions were collected and analyzed for hexose which appeared in fractions 22 through 36. After no further hexose material was detected, the column was washed with an additional 200 ml of the same solvent. Solvent 5 eluted a component tentatively identified as a ganglioside and a small amount of PBA. PBA was next eluted from the column with a linear gradient of ammonium acetate in chloroform-methanol-water (1:1:0.3). The gradient was started with 250 ml of chloroform-methanol-water containing 0.02 M ammonium acetate in the mixing chamber and 250 ml of chloroform-methanol-water (1:1:0.3) containing 0.2 M ammonium acetate in the reservoir (Solvent 6); 10-ml fractions were collected and aliquots of selected fractions were assayed for protein by the Lowry procedure after the solvent was removed by heating at 37°C under a stream of N_2.

RESULTS

SDS-polyacrylamide gel electrophoresis of washed bovine erythrocyte membranes revealed the presence of 3 PAS staining components (Fig. 2A). These components were released into the upper aqueous layer by extraction of aqueous suspension of erythrocyte membranes with chloroform-methanol (2:1) (Fig. 2B). In a typical fractionation 1,100 mg of membrane extract was obtained from 13.18 g of stromata (Table 1). Routinely this procedure released from 60 to 80% of the sialic acid of the membrane. The more mobile PAS staining components were easily resolved from the high-molecular-weight glycoprotein by appropriate solvent extractions (Fig. 1) of lyophilized preparations of the upper aqueous phase (Fig. 2C and D). By hemagglutination inhibition tests it was readily demonstrated that PBA was in the fraction which contained the mobile PAS components (266 mg of this fraction, and 613 mg of the high-molecular-weight glycoprotein fraction were obtained from 1,100 mg of membrane extract, Table I). In addition, this fraction also contained the ganglioside components of the membrane; at least 5 gangliosides were detected on thin layer chromatograms.

The composition of the bovine erythrocyte membrane and the partially resolved components are given in Table I. The high-molecular-weight glycoprotein contained the major carbohydrate components of the erythrocyte membrane and appears to be similar to the bovine erythrocyte glycoprotein isolated by Emerson and Kornfeld (32) by extraction of ghosts with lithium 3,5-diiodosialicylate. There are however differences regarding the relative sugar content of the glycoproteins as well as the reported inability of the protein isolated by the above workers to stain with Coomassie blue after SDS-polyacrylamide gel electrophoresis. These workers also failed to detect the minor glycoprotein component of the bovine erythrocyte membrane.

PBA was resolved from gangliosides and the low-molecular-weight PAS staining

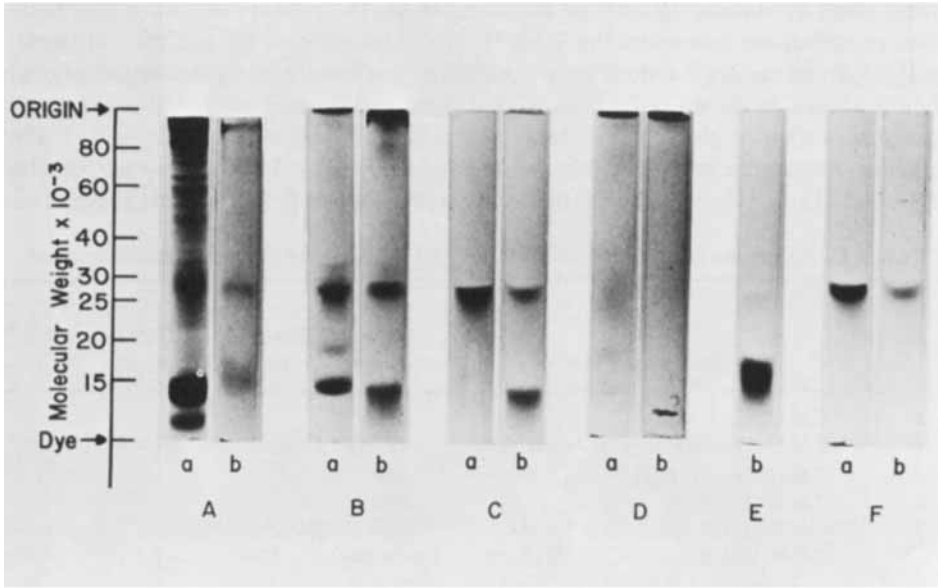

Fig. 2. SDS-polyacrylamide gel electrophoresis of bovine erythrocyte membranes and isolated components. Samples were treated with 1% SDS and subjected to electrophoresis on 10% acrylamide gels in 0.1% SDS and 0.1 M phosphate buffer, pH 7.0; a) samples stained for protein with Coomassie blue; b) samples stained for carbohydrate with periodic acid-Schiff reagent. A) washed erythrocyte membranes [a) 59 μg protein; b) 470 μg protein]; B) upper aqueous phase [a) 28 μg protein; b) 98 μg protein]; C) PBA-ganglioside fraction [a) 14 μg protein; b) 29 μg protein]; D) high-molecular-weight glycoprotein fraction [a) and b) 14 μg protein]; E) ganglioside fraction eluted from DEAE-cellulose column with solvent no. 5 [b) 14 μg protein]; F) PBA eluted from DEAE-cellulose column with solvent no. 6 [a) 13 μg protein; b) 26 μg protein].

TABLE I. Composition of Bovine Erythrocyte Membranes and Extracted Components*

	Erythrocyte membranes	Membrane extract[a]	Extracted components of membrane extract: Gangliosides-PBA	Extracted components of membrane extract: HGP[b]
dry weight (mg)	13,180	1,100	266	613
protein (mg)	6,063	311	85	98
fucose (μmoles)	62.2	25.3	3.3	ND[c]
mannose (μmoles)	70.4	13.2	0.9	11.8
galactose (μmoles)	1,685.7	1,122.0	116.5	1,075.2
glucose (μmoles)	179.5	55.0	33.0	18.1
sialic acid (μmoles)	361.1	217.8	38.5	159.4
N-acetylglucosamine (μmoles)	1,229.5	901.6	60.2	720.3
N-acetylgalactosamine (μmoles)	329.9	169.5	13.2	133.2
HIU[d] (IM serum)	3.0×10^7	1.66×10^7	2.55×10^7	9.8×10^4

*Extraction procedures are described in the text and summarized in Fig. 1.
[a]Membrane extract, upper aqueous phase
[b]HGP – high molecular weight glycoprotein
[c]ND – none detected
[d]HIU – hemagglutination inhibiting units

component by chromatography on DEAE-cellulose. The solvents utilized in this fractionation procedure are summarized in Table II. The distribution of HD and PBA antigenic activity in the various fractions from the column was monitored by hemagglutination inhibition tests. As shown in Table II, HD antigenic activity was principally found in the ganglioside fraction eluted with solvent system No. 2. The low-molecular-weight PAS staining component was eluted with solvent system No. 5 and did not contain significant PBA or HD antigenic acitivty. On SDS-polyacrylamide gels (Fig. 2E) this sialic acid con-

TABLE II. Separation of Gangliosides and PBA by DEAE-Cellulose Column Chromatography*

Solvent no.	Elution solvents[a]	Elution volumes ml	Substance eluted	HIU[b]-IM serum	HIU[b]-HD serum
1	C-M (1:1)	200			
2	C-M (2:1) saturated with 28% NH_4OH	600	Gangliosides	5.6×10^5	1.3×10^6
3	C-M (2:1) + 4% H_2O	200			
4	C-M-W (1:1:0.3)	200			
5	C-M-W (1:1:0.3) + 0.02 M NH_4Ac	600	Ganglioside (?)	8.0×10^4	1.1×10^4
6	C-M-W (1:1:0.3) + 0.02 M NH_4Ac to C-M-W (1:1:0.3) + 0.2 M NH_4Ac	Linear gradient 250 ml of each	PBA	1.2×10^7	3.2×10^3

*The PBA-ganglioside fraction (204 mg; 1.96×10^7 HIU-IM serum and 1.67×10^6 HIU-HD serum) was placed on a column of DEAE-cellulose (acetate) (9.6g) and the column washed with solvents in the sequence shown above.

[a]C-M-W – chloroform, methanol, water, respectively

[b]HIU – hemagglutination inhibiting units

taining component migrated in the glycolipid region and has for this reason been tentatively identified as a ganglioside. Approximately 10 mg of this component was isolated from the column. PBA antigenic activity was eluted from the DEAE-cellulose column with a linear gradient of ammonium acetate (0.02 M to 0.2 M) in chloroform-methanol-water (1:1:0.3) (Table II, Fig. 3). Approximately 42 mg of material (3.4 ng contained 1 unit of hemagglutination inhibitory activity) was obtained in this fraction from 204 mg of starting material. Chloroform was an obligatory requirement in the solvent system used for elution of PBA; methanol or aqueous ammonium acetate solutions were totally ineffective. Occasionally in some fractionations a small peak of PBA activity (about 15% of the PBA activity found in the major peak) was eluted just prior to the major peak. No difference in the components eluted in either peak were detected by serological analysis, by SDS-polyacrylamide gel electrophoresis, or in sugar or amino acid content. Thus the reasons for this elution pattern were not readily apparent. Residual PBA containing about 10% of the activity put on the column could be eluted after the gradient was completed with 200 ml of chloroform-methanol-water (1:1:0.3) containing 0.2 M ammonium acetate and 11.3 mM glacial acetic acid. Again, this material appeared identical to material eluted in the major fraction by the criteria indicated above.

To establish unequivocally the nature of the isolated antigens, double diffusion gel precipitation tests were carried out. As can be seen on Fig. 4, PBA formed a strong precipitation line with IM serum and reacted weakly with a serum containing HD antibodies. Complete removal of HD antigenic activity from PBA preparations was difficult and

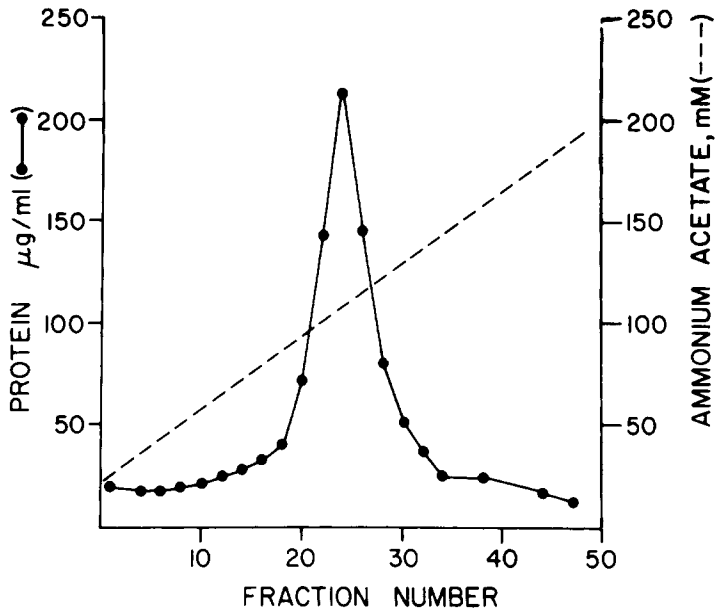

Fig. 3. DEAE-cellulose chromatography of PBA. After removal of gangliosides as described in Table II, PBA was eluted from the DEAE-cellulose column with a linear gradient of ammonium acetate (0.02–0.2 M, 250 ml each) in chloroform-methanol-water (1:1:0.3). Fractions (10 ml) were collected and selected tubes analyzed for protein.

residual activity was usually observed even in preparations that had been subjected to a second fractionation on DEAE-cellulose by the procedures described previously. With some IM sera we also observed the formation of 2 well-separated lines (Fig. 4). In earlier studies, Milgrom et al. (24) reported similar results with ultrasonic extracts of stromata prepared from trypsinized erythrocytes. Ganglioside fractions eluted with solvent No. 2 and partially purified on silica gel columns gave a strong reaction with serum containing HD antibodies and a negligible reaction with IM serum (Fig. 4). As expected, absorption of the sera with guinea pig kidney removed HD but not Paul-Bunnell antibodies.

On SDS-polyacrylamide gel electrophoresis, PBA appeared as a single glycoprotein component that stained with PAS or Coomassie blue (Fig. 2G). PBA is readily soluble in water or chloroform-methanol-water (1:1:0.3) and thus has both hydrophobic and hydrophilic properties. Its hydrophobic characteristics were also indicated by its behavior on DEAE-cellulose columns. Elution of PBA under our fractionation conditions was completely dependent on the presence of chloroform in the solvent system. These extreme hydrophobic properties suggested that PBA may be closely associated with lipid. To explore this possibility, gels were also stained with oil red O, a dye that had previously been used to stain for lipoproteins (33). Yamamoto and Lampen (33) for example, stained the membrane penicillinase phospholipoprotein of Bacillus licheniformis with oil red O. PBA was run on replicate gels, and gels stained with PAS or oil red O were compared. It was, however, necessary to reduce the size of the PAS stained gel (see Materials and Methods) so that comparisons could be made with the gel stained with oil red O. As can be seen on Fig. 5, oil red O staining did indeed detect a single component. The small differences noted be-

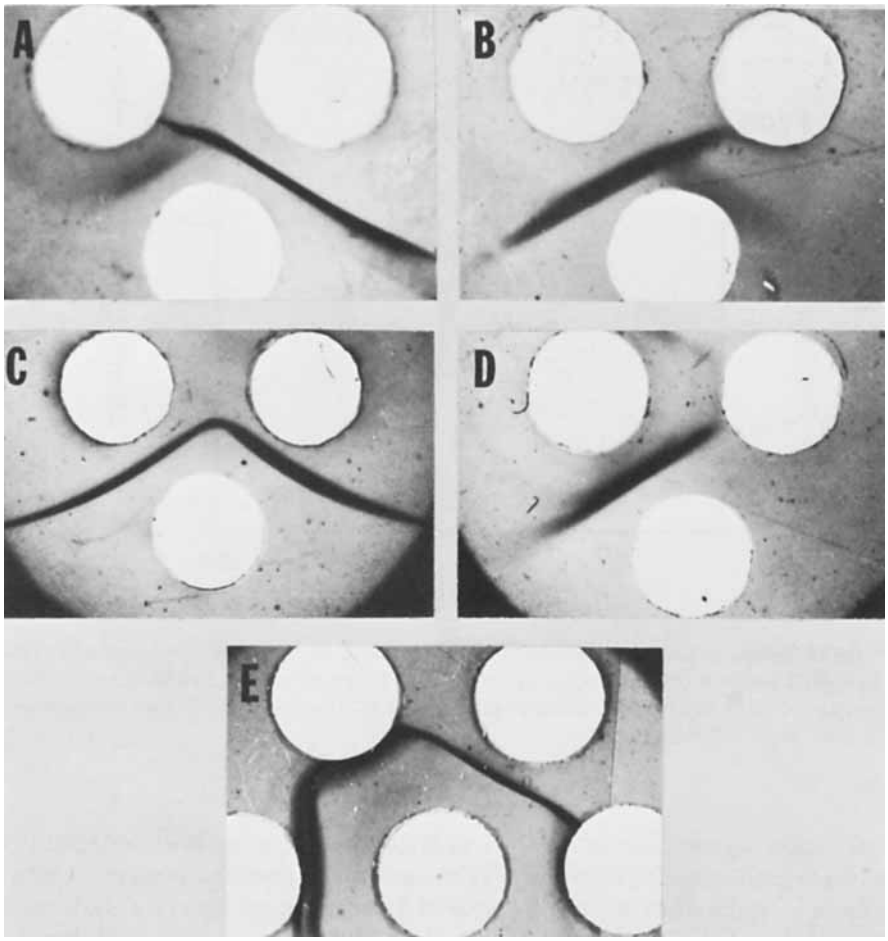

Fig. 4. Double diffusion gel precipitation studies. A) lower well: PBA, B) lower well: gangliosides. A and B) Upper left well: serum containing HD antibodies; upper right well: IM serum. C) lower well: PBA; D) lower well: gangliosides; C) upper left well: IM serum, upper right well: IM serum absorbed with guinea pig kidney; D) upper left well: HD serum, upper right well: HD serum absorbed with guinea pig kidney; E) center well: PBA; outer wells: various IM sera.

tween the position of the PAS and oil red O staining components is presumably a reflection of the differential changes in gel length that occurred during the 2 different staining procedures. These differences were also noted between oil red O and Coomassie blue staining of the bacterial membrane and exopenicillinases (33). In our studies it is unknown whether the ability of PBA to stain with oil red O is in fact due to lipid covalently attached to protein. It is also feasible that these results could have been obtained if the PBA fraction were contaminated with a lipid with mobility characteristics on SDS gels that are similar to PBA, or alternatively, staining may simply be due to the hydrophobic amino acid content of the protein.

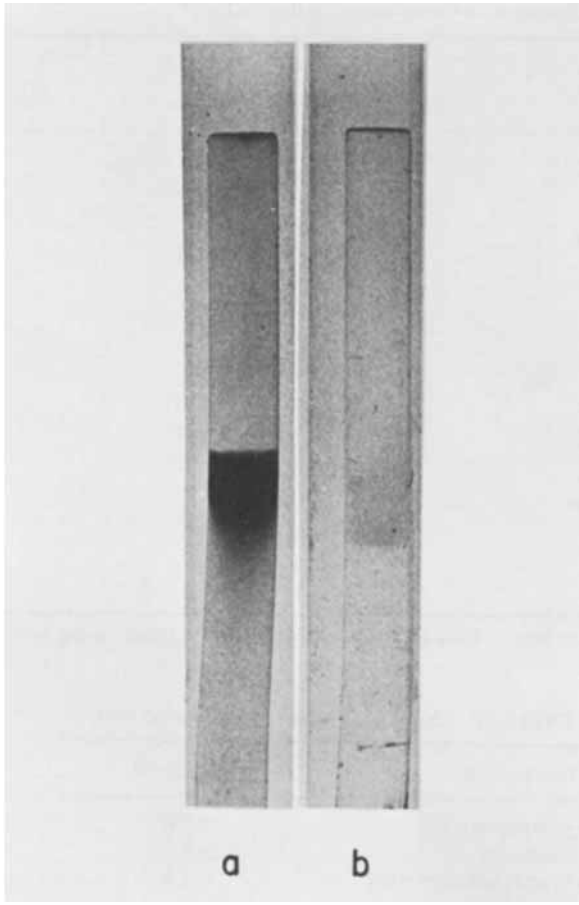

Fig. 5. SDS-polyacrylamide gel electrophoresis of PBA. Conditions as described in Fig. 2. a) Gel was stained with periodic acid-Schiff reagent; b) gel was stained with oil red O. a) 26 μg protein; b) 214 μg protein.

Table III shows the amino acid content of PBA. It can be seen that the protein contains a relatively high content of hydrophobic residues (proline, leucine, isoleucine, valine, alanine, phenylalanine). These results are in agreement with the amino acid composition of the bovine IM heterophile antigen reported by Springer (34, 35) and in somewhat less agreement with the data reported by Fletcher and Woolfolk (36). Both groups of workers extracted PBA from bovine erythrocyte stromata with hot 75% ethanol; however, Springer (34) further purified the ethanol extracted component by ethanol fractionation, gel filtration, and sucrose gradient ultracentrifugation.

The overall chemical composition of PBA is presented in Table IV. Approximately 9.2% of the antigen was analyzed as carbohydrate which differs significantly from the carbohydrate content of bovine PBA reported by Springer (35) and Fletcher and Woolfolk (36). The predominating sugar constituents were sialic acid, galactose, and N-acetylgalactosamine. The solubility of PBA in organic solvents as well as its apparent staining with oil

TABLE III. Amino Acid Composition of Bovine PBA*

Amino acid	Residues 1,000 Amino acid residues	g Amino acid 100 g Antigen
aspartic acid	59.9	4.1
threonine	81.9	4.9
serine	67.5	3.5
glutamic acid	157.7	12.0
proline	115.9	6.6
glycine	97.8	3.3
alanine	58.9	2.5
valine	57.6	3.4
1/2 cystine	–	–
methionine	27.1	2.1
isoleucine	84.5	5.6
leucine	89.9	5.9
tyrosine	–	–
phenylalanine	35.5	3.1
lysine	19.2	1.5
histidine	1.1	0.1
arginine	36.1	3.3

*Average values from 3 different samples, uncorrect for losses during hydrolysis

TABLE IV. Chemical Composition of Bovine PBA

Component	Weight %
polypeptide	62.0
sialic acid	4.3
N-acetylgalactosamine	2.5
N-acetylglucosamine	0.2
galactose	2.1
glucose	0.1
mannose	< 0.1
phosphorous	0.04
sphingosine	ND[a]
fatty acids	
16	0.05
18	0.06
18:1	0.02

[a]ND – none detected; 2.2 mg sample used for analysis

red O suggested that lipid components may be present. However, we could only detect small amounts of phosphorus and fatty acids. It still remains to be determined whether lipid components or the hydrophobic properties of the protein itself resulted in staining by oil red O.

The molecular weight of PBA estimated by SDS-polyacrylamide gel electrophoresis was 26,000 (Fig. 6). This molecular weight can only be considered tentative for the reasons discussed by Tanford and Reynolds (37) who point out that the only reliable determination of molecular weight of membrane proteins is the use of sedimentation equilibrium in SDS.

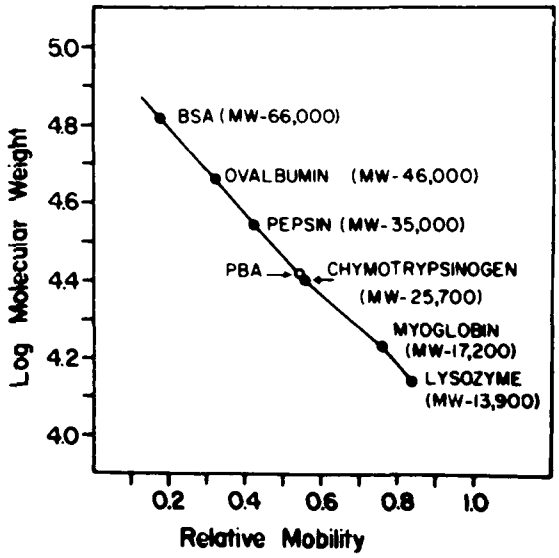

Fig. 6. Molecular weight estimation of PBA by SDS-polyacrylamide gel electrophoresis. Conditions as described in Fig. 2. The relative mobilities were calculated as the ratio of the distance moved by the protein to the distance moved by the marker dye. The protein standards used in the construction of the standard curve and their molecular weights are indicated on the graph.

Trypsin treatment of PBA failed to inactivate the antigen as determined by the hemagglutination inhibition test (Table V). SDS-polyacrylamide gel electrophoresis, however, indicated that PBA was altered by trypsin treatment and had an estimated molecular weight of 19,000. The trypsinized component stained normally with PAS but only very faintly with Coomassie blue. PBA antigenic activity was sensitive to pronase treatment (Table V) which suggests that the antigenic determinants are located on the glycoprotein.

TABLE V. Effect of Trypsin or Pronase Treatment of PBA*

Expt.	Treatment	Total HIU[a]	Activity remaining
1	PBA alone	6.4×10^4	100
	PBA + trypsin	6.4×10^4	
2	PBA alone	4.8×10^4	8
	PBA + pronase	4×10^3	

*PBA (0.20 mg) was incubated with trypsin (19.4 μg) in 0.06 8 M phosphate buffer, pH 7.0 (Expt. 1) or with pronase (27.5 μg) in 0.07 M Tris HCl buffer, pH 8.2 containing 1 mM $CaCl_2$ (Expt. 2). Final volume was 0.25 ml. Incubations were carried out for 24 h at 37°C in the presence of a small amount of toluene. Hemagglutination inhibiting activity was measured by the ability of PBA to inhibit the agglutination of sheep erythrocytes by infectious mononucleosis serum.

[a]HIU – hemagglutination inhibiting units

DISCUSSION

Our studies were initially undertaken in an effort to reexamine the chemical nature of the bovine IM heterophile antigen. Earlier investigations carried out by Springer (34, 35) and by Fletcher and Woolfolk (36) suggested that PBA of animal cells were glycoproteins that possessed remarkable similarity in carbohydrate and amino acid composition to the blood group M and N glycoproteins. However, the isolation procedures utilized by these workers did not exclude the possibility that their preparations were contaminated with glycolipids. The difficulty in removing ABO blood group determinants from glycoproteins isolated from human erythrocyte is well known (38).

The fractionation procedures that we have described in this communication permit the separation of glycoprotein and glycolipid constituents of the bovine erythrocyte membrane. PBA appears as a minor glycoprotein component of bovine erythrocyte membranes and has an estimated molecular weight of 26,000. Thus, our results support the conclusions reached by the above workers. The amphipathic nature of PBA is suggested by its hydrophobic and hydrophilic properties. PBA is therefore ideally suited as an integral membrane protein. The carbohydrate content of bovine PBA is significantly lower than the content reported for PBA isolated from horse, sheep, and goat erythrocytes (35, 36, 39). However interestingly, its antigenic activity is greater. At the present time the reasons for this are unknown. Sialic acid has been implicated as an important immunodeterminant since neuraminidase destroys PBA activity of sheep, horse, and goat antigens. In the case of bovine PBA, Fletcher and Woolfolk (36) reported that after neuraminidase treatment only 0.5% of the original sialic acid remained, yet PBA retained 15% of its hemagglutination inhibiting activity. In our studies, Clostridium perfringens neuraminidase (40) released 69% of the sialic even after extensive treatment with the enzyme. This resulted in about a 50% loss of activity in the standard assay. The inability of neuraminidase to completely hydrolyze sialic acid containing polymers has been observed previously (40). Cassidy et al. (40) point out that the position of attachment of sialic acid or the nature of the sugar to which it is attached influences greatly the rate of hydrolysis by neuraminidase. It is of interest to note that Hakomori and Saito (41) have described a neuraminidase resistant glycolipid which contains O-acetyl (N-glycoyl) neuraminic acid. After removal of the O-acetyl group, the sialic acid of the glycolipid was now susceptible to hydrolysis by neuraminidase.

IM is a self-limiting lymphoproliferative disease in which heterophile antibodies are characteristically produced (6). In addition, Epstein-Barr virus associated antibodies directed against viral capsid antigen, membrane antigen, and "early" antigen are also produced (42). Heterophile antibodies of the IM type are highly specific for IM and rarely appear, if at all, in other diseases. However, the role of IM heterophile antibodies in determining the self-limiting course of the disease is unknown. It is of considerable interest that Milgrom et al. (43) have reported that PBA is present on spleen cells of patients suffering from various forms of lymphoma and leukemia. These patients, however, do not produce Paul-Bunnell antibodies. These observations have prompted Milgrom et al. (43) to speculate that lack of IM heterophile antibodies may account for the malignant character of these diseases while their production in high titer accounts for the benign character of IM.

In IM the antigenic stimulus responsible for Paul-Bunnell antibody formation is not known. There are apparently few reports where PBA has been identified on human tissue during the course of the disease (44, 45). It is not known if PBA is a product of the in-

fecting viral genome or a host cellular component that becomes uncovered or slightly modified as a result of viral infection. Our understanding of the chemical structure of PBA may provide further insight into the resolution of these possibilities.

This investigation has also demonstrated that the ganglioside fraction of bovine erythrocyte membranes reacts with HD antibodies. In humans, HD antibodies are primarily formed as a result of injection with foreign proteins, presumably containing antigenic gangliosides. However, they have also been detected in pathological human sera in patients who have not received such injections. In these cases, the antigenic stimulus remains unknown. Further studies on the identity of active ganglioside components and their antigenic determinants are underway.

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