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QUARTERLY

Paul-Bunnell antigen and a possible mechanism of formation of heterophile antibodies in patients with infectious mononucleosis✪

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Sera of patients with infectious mononucleosis contain heterophile anti-Paul-Bunnell (PB) antibodies to erythrocytes of numerous mammalian species. Evidence is presented that the corresponding antigen of bovine erythrocytes is not, as previously described, a single molecule, but a series of glycoproteins with glycans terminated with *N*-glycolylneuraminic acid (Neu5Gc). The latter compound should be an important part of the PB epitope because, in agreement with the results of others, we found that desialylation of the PB antigen abolishes almost completely its activity. We examined three different preparations of GM3 ganglioside for their capacity to bind anti-PB and found that only GM3 from horse erythrocytes containing Neu5Gc exhibited a low although ELISA measurable PB activity. The other two GM3 preparations, from bovine milk and dog erythrocytes, containing *N*-acetylneuraminic acid (Neu5Ac) bound little if any anti-PB antibodies. This finding confirms a previous report that human erythrocyte Neu5Ac containing sialoglycoprotein with similar O-linked glycans as the PB-antigen of bovine erythrocytes exhibits only very low PB activity (Patarca & Fletcher, 1995, *Crit Rev Oncogen.*, 6: 305). In conclusion, we

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Abbreviations: GalNac, *N*-acetylgalactosamine; GlcNac, *N*-acetylglucosamine; Glc, glucose; Gal, galactose; Man, mannose; Sia, sialic acid; Neu5Gc, *N*-glycolylneuraminic acid; Neu5Ac, *N*-acetylneuraminic acid; PB, Paul-Bunnell antigen; HD, Hanganutziu-Deicher antigen; PCA, pyrrolidone carboxylic acid.

present a hypothesis that anti-PB antibodies in patients with infectious mononucleosis are formed against infection-induced cell membrane glycoconjugates containing highly immunogenic Neu5Gc.

Heterophile antibodies that agglutinate or hemolyse erythrocytes of some animals like sheep, ox, horse and goat arise in patients with infectious mononucleosis (for review, see Patarca & Fletcher, 1995). After the names of their discoverers, the respective antigen is referred to as the Paul-Bunnell antigen (PB). The antibodies are highly diagnostic for the disease but raison d'être for their formation in infectious mononucleosis has not been established. The antigen is present in man only in trace amounts but may be increased in certain neoplastic and inflammatory diseases (Malykh *et al.*, 2001). Several researchers isolated PB from bovine erythrocytes and characterized it as a sialoglycoprotein with a molecular mass of 26 kDa (Merrick *et al.*, 1977) or 34 kDa (Fletcher *et al.*, 1982). The PB activity is destroyed by neuraminidase, in large part by pronase, but not by trypsin. The proposed structure of the epitope of PB antigen from bovine erythrocytes is Neu5Gcα2-3Galβ1-3(Neu5Gcα2-6)GalNAcα(PCA)Thr-Pro-Gly-Pro-ProAsx (Patarca & Fletcher, 1995). PCA (pyrrolidone carboxylic acid) is present in a number of animal sialoglycoproteins of red cells but not in human glycophorin A. The above mentioned epitope is the basis for the commercially available kits for anti-PB in the diagnosis of infectious mononucleosis. However, another study found PB activity to depend on two proteins of identical molecular mass of 3.5 kDa but different amino acid composition (Watanabe *et al.*, 1980). The proteins exhibited a high affinity toward gangliosides. In the present report we identified PB of bovine erythrocytes not as a single compound but a series of sialoglycoproteins ranging from 17 to 50 kDa.

We also present evidence that the PB activity depends to a large extent on glycans terminated with Neu5Gc rather than Neu5Ac residues.

MATERIALS AND METHODS

Antisera. Anti-P-B sera from patients with infectious mononucleosis were obtained from the Warsaw Sanitary-Epidemiological Station. Sera with a high agglutinating titer against bovine erythrocytes were selected, absorbed with guinea-pig kidney to remove anti-Forssman antibodies and pooled. Each pool contained sera from at least three patients, and several such pools were used for determination of PB activity throughout this study.

Glycoproteins and glycosphingolipids. Human glycophorin A was a gift from Dr. W Dahr, and preparations of GM3 gangliosides were kindly donated by Dr. T Pacuszka. Human transferrin and calf fetuin were from Sigma.

Isolation of P-B. Bovine blood, collected on Acid-Citrate-Dextrose, was obtained from a local abattoir immediately after sacrificing the animals. Erythrocytes were isolated, washed with saline and the cell membranes were isolated (Dodge *et al.*, 1963). The membranes were subsequently treated with 6 vol. of chloroform/methanol (2:1, v/v) and thoroughly mixed (Merrick *et al.*, 1977). The upper phase containing water soluble glycosphingolipids and glycoproteins was collected and dialysed at 4°C for 48 h against frequently changed deionized water, constant stirring. The dialysate was subsequently centrifuged at 40 000 $\times$ ***g*** for 40 min at 4°C and lyophilized. The dry material (crude PB, 720 mg) was dissolved in 20 ml of water, loaded onto a 55 $\times$ 1.5 cm column packed with DEAE/acetate Sephadex in methanol/chloroform/water (60:30:8, by vol.) (Yu & Ledeen, 1972) and left on the column overnight. The next day the column was eluted with methanol/chloforom/0.8 M sodium acetate (60:30:8, by vol.) and fraction of 20 ml were collected. Small aliquots of eluate

were spotted on TLC plates and stained the spots with the orcinol reagent. Orcinol positive fractions were collected, pooled, and freeze-dried. Subsequently, this material was purified on Biosil A according to Watanabe *et al.* (1980) in order to separate glycoproteins from gangliosides.

Analytical methods. Paul-Bunnell activity was assessed by: hemagglutination inhibition tests with bovine erythrocytes and 4 hemagglutination units of the pooled anti-PB serum, by direct ELISA and ELISA inhibition tests performed on erythrocyte membrane or crude PB coated plates, and by immunodiffusion (Morito *et al.*, 1982; Trafny & Grzybowski, 1988). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS/PAGE) was performed by the method of Laemmli (1970). Transfer of proteins from gels onto nitrocellulose membranes and subsequent immunostaining of glycoproteins with anti-PB antibodies was carried out according to Towbin *et al.* (1979), employing a secondary anti-IgM antibody conjugated to peroxidase or alkaline phosphatase, with 4-chloro-1-naphtol and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT), respectively, as substrates (Sigma). Sialic acid from erythrocyte membranes and serum glycoproteins was removed prior to SDS/PAGE by digestion with 16.6 mU (units) of *Vibria cholerae* neuraminidase (Sigma) per 1 nmol of sialic acid for 24 h at 37°C in 40 mM acetate buffer, pH 5.5. Linkage of sialic acid (Sia) to galactose was determined by reacting the SDS/PAGE separated glycoproteins on nitrocellulose blots with Siaα2-3Gal and Siaα2-6Gal specific biotinylated lectins from *Maackia amurensis* (Lectin II) and *Sambucus nigra*, respectively (Vector Laboratories, Burlingame, CA, U.S.A.) according to Duk *et al.* (1997). The bound lectins were subsequently detected with ExtrAvidin-alkaline phosphatase conjugate using BCIP/NBT as substrate. Sialic acids in 0.2 M TFA hydrolyzates of glycoproteins (80°C, 1 h) and neutral sugars with hexosamines in 4 M TFA hydrolyzates of glycoproteins (100°C, 4 h) were determined by High pH Anion Exchange Chromatography with Pulsed Amperometric detection using a Dionex Series 4500i system, a Carbopac PA-1 column (4 × 250 mm), and a Carbopac PA-1 guard column (4 × 40 mm) (Zdebska & Kościelak, 1999). Protein was quantitated by the method of Lowry *et al.* (1951). Sphingosine was determined according to Undenfriend *et al.* (1972) while fatty acids were quantitated as methyl esters on a column of Silar 10 C employing a Hewlett-Packard 9995A GLC-MS mass spectrometer.

RESULTS AND DISCUSSION

Sixteen milligram of purified PB was obtained from 22.5 l of bovine blood. According to inhibition of ELISA assays, PB specific activity was increased 1500-fold relative to the crude extract. In contrast to the findings of Watanabe *et al.* (1980), we have not observed any increase in PB activity when the crude PB was presented in hemagglutination inhibition assays as a part of liposome. The crude PB contained protein and carbohydrates in the following molar proportions: Neu5Gc, 2.0; Neu5Ac, 0.2; GalNAc, 1.0; GlcNAc, 3.1; Gal, 2.4; Man, 0.9; Glc, 0.4. Sphingosine and fatty acids were not detected. The purified antigen was inhomogenous as indicated by multiple bands in the SDS/PAGE electrophoretogram (Fig. 1e, f). A similar though more complex pattern was seen in electrophoretograms of the crude extract of bovine erythrocyte membranes (Fig. 1b, c). The bands were glycoproteins and most of them exhibited PB activity as shown by Western-blotting. A low molecular mass protein of about 3.5 kDa, possibly identical with that described previously by Watanabe *et al.* (1980), stained with Coomassie Blue (Fig. 1, lane e) but did not bind anti-PB (lane f). In agreement with the results of other researchers, we found that the activity of PB was to a large extent destroyed by pronase but not by trypsin. Since numerous

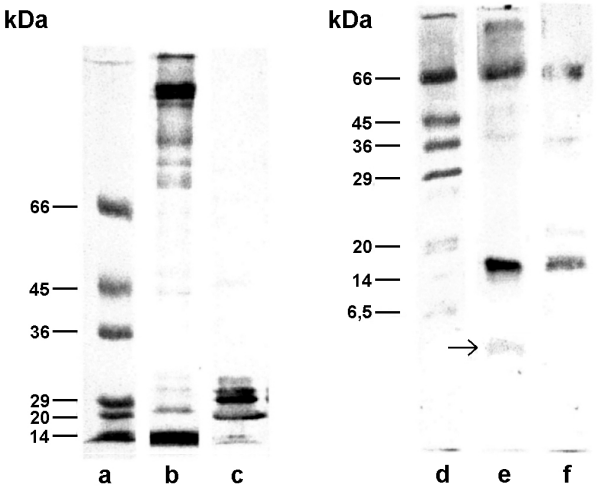

Figure 1. SDS/PAGE of crude extracts of bovine erythrocyte membranes lanes (b, c) on 10% polyacrylamide gel and of the purified antigen on 15% polyacrylamide gel (e, f). Protein standards (a, d).

Gels stained with Coomassie Blue, (a, b, d, e); gels reacted with anti-PB followed by staining with goat, peroxidase linked, anti-human IgM (c, f); Band marked with asterisk represent probably a low molecular mass gangliophilic protein described by Watanabe *et al.* (1980).

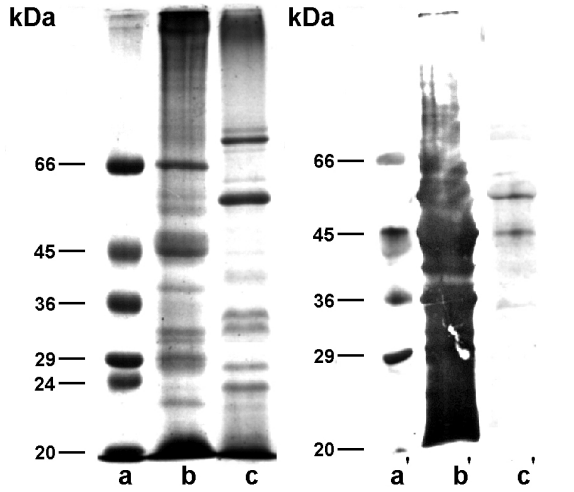

Figure 2. SDS/PAGE of intact and neuraminidase treated bovine erythrocyte membranes.

Lanes a and a', protein standards; b, crude extract of bovine erythrocyte membranes; c, neuraminidase treated crude extract of bovine erythrocyte membranes. Lanes a–c and a' were stained with Coomassie Blue; lanes b', c' were stained with anti-PB as in Fig. 1.

previous studies suggested an important role of sialic acid in the PB epitope, we subjected the bovine erythrocyte membranes to degradation with either *V. cholerae* neuraminidase or 0.2 M TFA at 80°C. Under these conditions the material lost about 99% of its PB activity, irrespectively of whether examined by hemagglutination inhibition or immunodiffusion (not shown). A similar conclusion may be drawn from an experiment in which desialylated erythrocyte membranes were examined by Western blotting (Fig. 2). Hence, the PB epitope is mostly carbohydrate in nature. The residual PB activity after removal of sialic acid may result from the presence in bovine glycoproteins of PCA linked to α-amino group of N-terminal threonine residue that may also contribute to PB activity, or of other sialic acid independent, presumably protein derived structures, as proposed by Patarca & Fletcher (1995). These authors also claimed that the role of sialic acid in the PB epitope "is not clear", though at the same time noticed a higher PB activity of Neu5Gc containing erythrocyte sialoglycoproteins from bovine and equine erythrocytes over that from sheep erythrocytes containing both Neu5Gc and Neu5Ac at a ratio of 1:1. The same authors reported that Neu5Ac containing the human erythrocyte sialoglycoprotein with otherwise similar glycans as bovine PB antigen exhibited PB activity by three orders of magnitude lower than that from bovine erythrocytes. Admittedly, however, the human sialoglycoprotein lacks PCA. To test a possible role of the nature of sialic acid on PB activity, we examined the binding of anti-PB by protein free GM3 gangliosides that contained either Neu5Gc or Neu5Ac. The glycan of GM3 has the structure: Siaα2-3Galβ1- 4Glcβ1-ceramide. As shown in Fig. 3, all three GM3 glycosphingolipids bound little anti-PB antibodies in comparison to the purified PB antigen from bovine erythrocytes. Nonetheless, the GM3 from horse erythrocytes that contained Neu5Gc bound definitely more antibodies than the two GM3 preparations from either bovine milk or dog erythrocytes that contained Neu5Ac. This finding was confirmed in

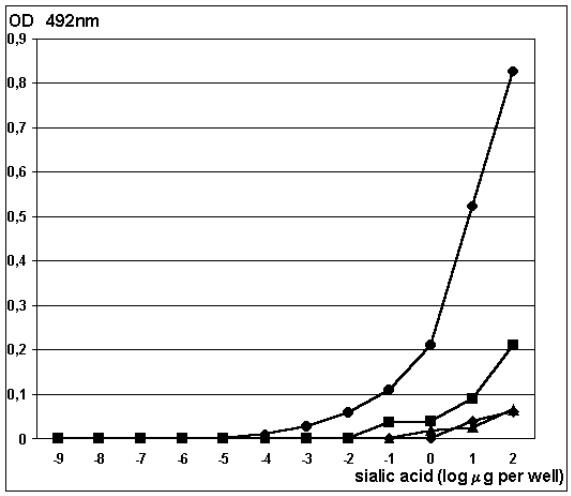

Figure 3. Direct ELISA of the purified PB, filled circles; GM3 from Neu5GC horse erythrocytes, filled rectangles; of GM3 containing Neu5Ac from erythrocytes of dog, filled triangles; and bovine milk, filled rhombs.

three independent experiments. The low activity of GM3 from horse erythrocytes as compared to the activity of the PB antigen from bovine erythrocytes may be due to the fact that the Neu5Gcα2-3Gal structure is only a part of the disialylated PB epitope, as proposed by Patarca & Fletcher (1995). Lastly, we turned our attention to the known lack of PB activity in bovine serum glycoproteins even though they contained Neu5Gc. A possible explanation is the fact that in serum glycoproteins Neu5Gc is usually bound to galactose by an α2-6 linkage, which is not present in glycoproteins of erythrocytes, whereas the reverse is true for the Neu5Gcα2-3Gal structure. This assumption turned out to be only partially true in that Siaα2-6Gal specific *Sambucus nigra* lectin reacted with serum glycoproteins but not with bovine PB-active glycoproteins of erythrocytes (Fig. 4). However, *Maackia amurensis* Siaα2-3Gal specific lectin bound not only to bovine erythrocyte glycoproteins but also to some serum glycoproteins. A probable explanation for the lack of PB-activity in bovine serum glycoproteins is identical to that already presented for the weak PB activity of Neu5Gc containing GM3, i.e., that anti-PB antibodies require a disialylated structure.

Our conclusion that Neu5Gc and not Neu5Ac is essential for PB activity may be crucial for the understanding of the mechanism of formation of heterophile anti-PB antibodies. In contrast to most mammals, including great apes, *N*-glycolylneuraminic acid in human tissues is found only in trace amounts (Malykh *et al.,* 2001; Varki, 2001). In animals this derivative of sialic acid is formed by

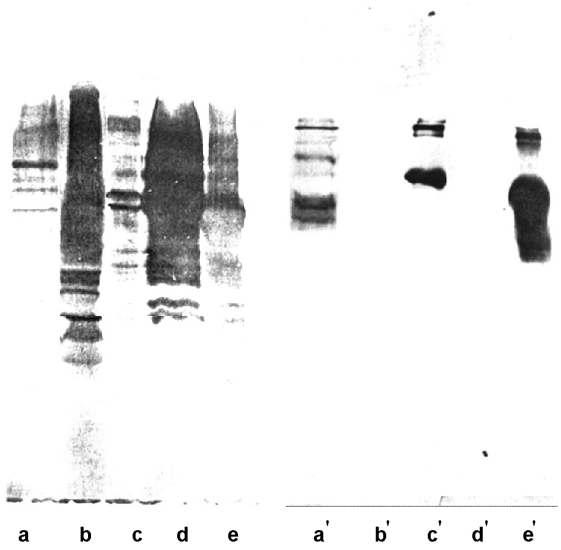

Figure 4. Reactions of SDS/PAGE separated bovine serum (a), bovine erythrocyte glycoproteins (b), human transferrin (c), human glycophorin A (d), and calf fetuin (e) with *Maackia amurensis* Lectin II ((lanes a–e), and *Sambucus nigra* lectin (lanes a'–e').

hydroxylation of the sugar-nucleotide cytidine-5'-monophosphate-*N*-acetylneuraminic acid (Shaw & Schauer, 1988; 1989; Muchmore *et al.* 1989). A gene for the respective hydroxylase is defective in man due to a large deletion within the 5' region (Chou *et al.,* 1998; Irie *et al.*, 1998; Chou *et al.,* 2002). The trace amounts of Neu5Gc found in human tissues are assumed to be of dietary origin (Malykh *et al.,* 2001; Varki 2001; Tangvoranuntakul *et al.,* 2003). Yet human tumors exhibit elevated amounts of Neu5Gc, which they acquire by an unknown mechanism

(Malykh *et al.*, 2001). Consequently antibodies against glycoconjugates containing Neu5Gc, known under the name of Hanganutziu-Deicher, are formed in cancer patients. These antibodies were initially identified in sera of humans vaccinated with animal sera (Hanganutziu, 1924; Deicher, 1926) and later characterized as directed to Neu5Gc containing glycoconjugates (Higashi *et al.*, 1977; Merrick *et al.*, 1978). Thus, our results suggest that the PB is related to the HD antigen. It has been shown that antibodies to the latter also appear in patients with infectious mononucleosis (Morito *et al.*, 1982). Both PB and HD antigens were expressed on human melanoma cell lines (Nakarai *et al.*, 1987). Anti-HD antibodies may be removed from anti-PB sera by adsorption with guinea pig kidney (Kano *et al.*, 1984), procedure employed in the present study. Therefore, the anti-PB sera used in the present work were free of anti-HD antibodies. We propose that in course of infectious mononucleosis the virus infected or virus responsive cells produce or concentrate Neu5Gc, thus initiating the immune response to glycoconjugates comprising this strongly immunogenic carbohydrate. These antibodies would be directed to sialoglycoproteins of cell membranes containing glycans with a Neu5Gcα2- 3Galβ1-3(Neu5Gcα2-6)GalNAcα1-threonine/serine structure rather than to serum glycoproteins containing predominantly the Neu5Gcα2-6-Galβ1-3/4GlcNAcβ1-structure, as in recipients of animal serum vaccines. Nevertheless, the heterophile anti-PB antibodies are related to the HD antibodies in that they both recognize Neu5Gc as an essential part of the immunogenic epitope.

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