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Five Monoclonal Antibodies Against Glycophorin A of Human Erythrocyte Recognize Glycoprotein of Bovine Erythrocyte

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ABSTRACT

To study heterophile blood antigens on erythrocytes between human and experimental or domestic animals, we have produced 295 monoclonal antibodies (MAbs) to human erythrocyte membrane protein. According to the affinity, reactivity, and titre of the MAbs, we selected 40 clones to study the heterophile blood antigens between human and bovine, chicken, guinea pig, horse, rabbit, sheep, and swine. Five MAbs commonly reacted with human type A, type B, and type O erythrocytes and reacted with bovine erythrocytes as well but did not react with erythrocytes from other species. Other MAbs did not react with erythrocytes from all the tested animals. These five MAbs reacted with the same erythrocyte membrane protein, 90 KD glycophorin A (GPA) of human or 200 KD major glycoprotein and other two components of bovine by immunoblotting and GPA competitive inhibition assay. Furthermore, by enzyme treatment and monosaccharide competitive inhibition assay, it was confirmed that these five MAbs recognized antigen epitope of glycosylation free amino acid portion but not glycosylation portion of GPA of erythrocyte membrane.

INTRODUCTION

THERE ARE MORE THAN 20 BLOOD GROUP SYSTEMS and 200 distinct blood group antigens on human erythrocyte membrane.[1,2] The specificity of blood antigens on erythrocytes has been studied using human sera and immunized animal sera. There are some common blood antigens between human and animals and called heterophile antigen systems. The representative heterophile antigen systems are Rh blood systems,[3] Paul-Bunnell (P-B) antibodies,[4] Hanganutziu-Deicher (H-D) antibodies (5,6) and Forssman (F) antibodies.[7] The target specificity of the heterophile antigen has been studied by several immunological and biochemical techniques. However, the detailed specificity and molecular structures of heterophile antigens are not fully understood yet. To study the specificity and molecular structure of heterophile antigens, MAb technique is useful. In this article, we established five MAbs that reacted with human and bovine erythrocytes and studied the chemical nature of target antigens recognized with these MAbs.

MATERIALS AND METHODS

Erythrocytes

Human red blood cells (RBC) were kindly provided by the Kitakyushu Red Cross Transfusion Service, Kitakyushu, Japan. RBC of bovine, chicken, horse, sheep, and swine were purchased from Nippon Bio-Supply Center (Tokyo, Japan). RBC of guinea pig and rabbit were drawn from normal animals.

Preparation of hemoglobin-free stroma of erythrocytes

RBC stroma was prepared by the method of Dodge *et al.*[8] Washed RBC with isotonic (310 imOsm) phosphate buffer (PB, pH7.4) was hemolyzed by pipetting in 20 imOsm PB. The stroma was washed three times with fresh buffer of same ion strength. The washed stroma, hemoglobin-free ghost of RBC, was resuspended in phosphate-buffered saline (PBS, pH7.4) (this procedure gave a resealed ghost of RBC) and the concentration was adjusted to the volume equivalent to 50% RBC.

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Immunization and production of MAbs

BALB/c mice were immunized with three IP injections of human type A, type B, or type O RBC stroma (equivalent to 2×10^7 RBC) at 2-week intervals according to the procedure of Harlow *et al.*[9] Three days after the last injection, spleen cell suspension was prepared and fused with P3U1 mouse myeloma cells (p3 × 63/ag8) at a ratio of 10:1 using 50% polyethylene glycol (MW. 4000, Behringer Mannheim Gmbh, Mannheim, Germany). The fused cells were selected in hypoxanthine, aminopterin, and thymidine medium (Dainippon Pharmaceutical Co., Osaka, Japan). Subsequent cloning was performed by the limiting dilution technique and hybridoma cells producing relevant MAbs were screened by enzyme-linked immunosorbent assay (ELISA) using human type A, type B, and type O RBC stroma-coated plate. The immunoglobulin class was determined by mouse MAb isotyping Kit (Amersham, Buckinghamshire, UK).

Purification of GPA

The crude sialoglycoprotein was obtained from human type B RBC by the method of Lisowska *et al.*[10] Purification of GPA was performed by the method of Wasniowska *et al.*[11] The crude sialoglycoprotein was fractionated on a Sephacryl S-200 column (Pharmacia Biotec, Uppsala, Sweden) equilibrated with 0.05 M borate buffered saline (BBS, pH8.0) containing 1% sodium dodecyl sulphate (SDS; Sigma, St Louis, MO). The column fractions were monitored for protein content by the absorption at 280 nm and for sialic acid content by the periodate-resorcinol method.[12] By SDS-polyacryl amide gel electrophoresis (PAGE) analysis, the fractions containing periodic acid-schiff stain band 1 (PAS-1), and band 2 (PAS-2) corresponding to dimeric and monomeric GPA were collected and used for the experiment.[13]

ELISA

ELISA was performed in flat-bottomed microtitre plate (Nalge Nunc International Roskilde, Denmark). Plate was first coated with poly-L-lysine solution (20 μg/ml in PBS, Sigma). After two washings with PBS, the plate was incubated with RBC stroma suspension (at a concentration equivalent to 2×10^7 RBC/well in PBS) for 15 min at room temperature. The plate was centrifuged at 490 g for 10 min and the RBC stroma was fixed by the addition of 0.25% glutaraldehyde (Sigma). Nonspecific binding was blocked with 0.1M glycine buffer (Sigma). Primary antibody appropriately diluted in Tween 20/BBS containing 0.05% bovine serum albumin (BSA, Sigma) was added and incubated at room temperature for 2 h. After washing with Tween 20/BBS, horseradish-peroxidase conjugated goat anti-mouse IgG (γ-chain and L-chain-specific) antibody (MBL, Tokyo, Japan) was added and incubated at room temperature for 2 h. The plate was washed with Tween 20/BBS. For the enzyme reaction, 100 μl of substrate, o-phenylenediamine (Nakalai Tesque, Kyoto, Japan) was added and incubated at 37°C for 30 min. The reaction was stopped with 8N H_2SO_4. The optical density of each well was measured by the absorption at 492-nm wavelength by a microplate reader (MPR A4, Toyo Soda, Tokyo, Japan).

For monosaccharide or GPA inhibition assay, antibody was preincubated with an equal volume of different concentrations of each monosaccharide or GPA for 30 min at room temperature and then was applied into RBC stroma-coated plate.[13] The subsequent ELISA procedure was the same as described earlier.

SDS–PAGE and immunoblotting

RBC stroma dissolved in 2% Triton X-100 (Sigma) at 37°C for 1 h. The solubilized membrane proteins were separated with SDS–PAGE[14] using 10% polyacrylamide gel (Funakoshi Yakuhin, Tokyo, Japan). The gel was stained with Coomassie brilliant blue (CBB, Sigma) for proteins and with PAS for carbohydrates[13] (Wako Chemicals, Kyoto, Japan). The samples in the gel of SDS–PAGE were transferred to PVDF membrane (Millipore, Tokyo, Japan). The membrane was blocked with 1% BSA in PBS at 4°C overnight. After three washings with PBS, the membrane was treated with MAb at room temperature for 2 h. After washing, the membrane was incubated with horseradish-peroxidase conjugated goat anti-mouse IgG antibody (MBL). A solution of 4-chloro-1-naphthol and hydrogen peroxide (Sigma) was used as a substrate to develop the reaction.

Enzyme treatment of GPA of RBC stroma

RBC stroma was treated with 5 μg/ml of trypsin or chymotrypsin (Merck, Rahway, NJ) in PBS or with neuraminidase (Sigma) (5 U/ml in PBS containing 0.1M $CaCl_2$ and 0.1M $MgCl_2$) at 37°C for 30 min. Treated RBC stroma was washed with PBS four times and was coated to the plate (see method of ELISA). The control sample of RBC stroma was incubated under the same conditions but the enzymes were omitted.

RESULTS

Initial screening and characterization of MAbs reacting with human RBC stroma

Using human RBC stroma for screening by ELISA, 295 MAbs against human type A, type B, and type O RBC were produced. According to the affinity, reactivity, and titre of the MAbs, we selected 40 clones from them in this experiment (Table 1). In 9 MAbs from mice immunized with type A RBC stroma, 7 were specific for type A RBC which were entirely inhibited by the addition of 25 nm N-acetylgalactosamine (NAcGal). Two clones reacted with type A, type B, and type O RBC commonly. In 18 MAbs from mice immunized with type B RBC stroma, 13 were specific for type B RBC which were entirely inhibited by the addition of 25 nm galactose (Gal). Five clones reacted with type A, type B, and type O RBC commonly. All 13 MAbs from mice immunized with type O RBC commonly reacted with type A, type B, and type O RBC.

Reactivity of MAbs with RBC of a series of animals

The reactivity of these 40 MAbs against RBC stroma of a series of animals including bovine, chicken, guinea pig, horse, rabbit, sheep, and swine was studied (Table 2). Five MAbs (that were named as 2H11, 1B2, 2H9, 1D1, and 1F6, respectively) from mice immunized with type B RBC that reacted with hu-

TABLE 1. A PANEL OF MABS AGAINST HUMAN ERYTHROCYTES

MAbs*	Number of clone	Reactivity with human RBC†		
		Type A	Type B	Type O
Anti-type A	9			
Specific	7	+	−	−
Common	2	+	+	+
Anti-type B	18			
Specific	13	−	+	−
Common	5	+	+	+
Anti-type O	13			
Specific	0			
Common	13	+	+	+

*MAbs against human type A, type B, and type O RBC were established from BALB/c mice immunized with type A, type B, or type O RBC stroma, respectively.

†Reactivity with MAbs was determined by ELISA using coated plates with human type A, type B, or type O RBC stroma, respectively.

man types A, B, and O RBC commonly, showed heterophile reactivity with bovine RBC. All showed the same pattern of reactivity with bovine RBC, human type A, type B, and type O RBC (Fig. 1). However, these MAbs did not react with RBC stroma from any other animals. The classes of the MAbs are IgG1 κ for 1D1, 1F6, and 2H9, and IgG1 λ for 2H11 and 1B2.

Five MAbs recognize same epitope of RBC stroma

To study the epitope specificity on RBC stroma of five MAbs, we performed the mixing experiment of five MAbs and detected the bound MAbs on RBC stroma using ELISA method. Figure 2A shows the maximum binding of each MAb in which $\times 10$ dilution of the MAbs was used. When each MAb was mixed together, no additive effect was observed (Fig. 2B). This means that the epitope was saturated by one MAb and there is no space that other MAbs can bind. Figure 2C shows about 40% binding of MAbs in which $\times 100$ dilution of the MAbs was used. When each MAb was mixed together at this dilution, the additive effect of MAbs was observed (Fig. 2D). In this experiment, we mixed 1D1 MAb with four other MAbs as a representative experiment. When other combinations of MAbs were used, similar results were obtained (data not shown). These results suggest that five MAbs recognize a single epitope on RBC stroma.

SDS–PAGE of RBC stroma and immunoblotting

To study the molecules of RBC of human and bovine recognized with these MAbs, immunoblotting assay was performed. RBC stroma of human and bovine solubilized in 2% Triton X-100 was separated by SDS–PAGE. The electrophoretic pattern of the protein components of human type A, type B, type O, and bovine RBC stroma showed no difference (Fig. 3A). However, carbohydrate staining showed a different pattern between human and bovine RBC (Fig. 3B). The electrophoregram of bovine RBC stroma showed a major carbohydrate component (about 200 KD) and minor component (40 KD). In human type A, type B, and type O RBC, although they have the same pattern [97 KD which correspond to dimeric GPA(GPA_2) and 40 KD which correspond to monomeric GPA and GPB], the GPA_2 of human type B and type O RBC showed a more intense staining reaction than type A RBC. The immunoblotting showed that the MAb reacted with 97 KD and 40 KD proteins of human RBC (Fig. 3C, Lanes 10–12). In bovine RBC, the MAb reacted with 200 KD and 40 KD proteins whose position corresponded to major and minor glycoproteins. Furthermore, the MAb also reacted with 97 KD component of bovine RBC. In this study, we used 2H9 MAb. However, four other MAbs showed the same reactivity (data not shown). These results suggest that the five MAbs react with the antigen epitope located on GPA of human RBC and on glycoproteins of bovine RBC.

Inhibition of MAbs by GPA and carbohydrates

GPA, one of the major membrane proteins of erythrocyte, is heavily glycosylated and contains 60% carbohydrate in humans and much more carbohydrate in bovines. It is reported that N-acetylneuraminic acid (NANA) is a major component of GPA and some epitopes recognized by MAbs are carbohydrates.[15] We studied the competitive inhibition of five MAbs to the stroma of human type B and bovine RBC with GPA or differ-

TABLE 2. REACTIVITY OF MABS WITH RBC FROM A SERIES OF ANIMALS

MAbs	Reactivity with RBC strom						
	Bovine	Chicken	Guinea pig	Horse	Rabbit	Sheep	Swine
Anti-type A							
Specific[7]*	−	−	−	−	−	−	−
Common[2]	−	−	−	−	−	−	−
Anti-type B							
Specific[13]	−	−	−	−	−	−	−
Common[5]	+ (5)†	−	−	−	−	−	−
Anti-type O							
Common[13]	−	−	−	−	−	−	−

The reactivity of MAbs was determined by ELISA using each species RBC stroma as antigen.

*The numbers of MAbs studied. − reactivity can not be detected by ELISA.

†Indicates the number of MAbs reacted.

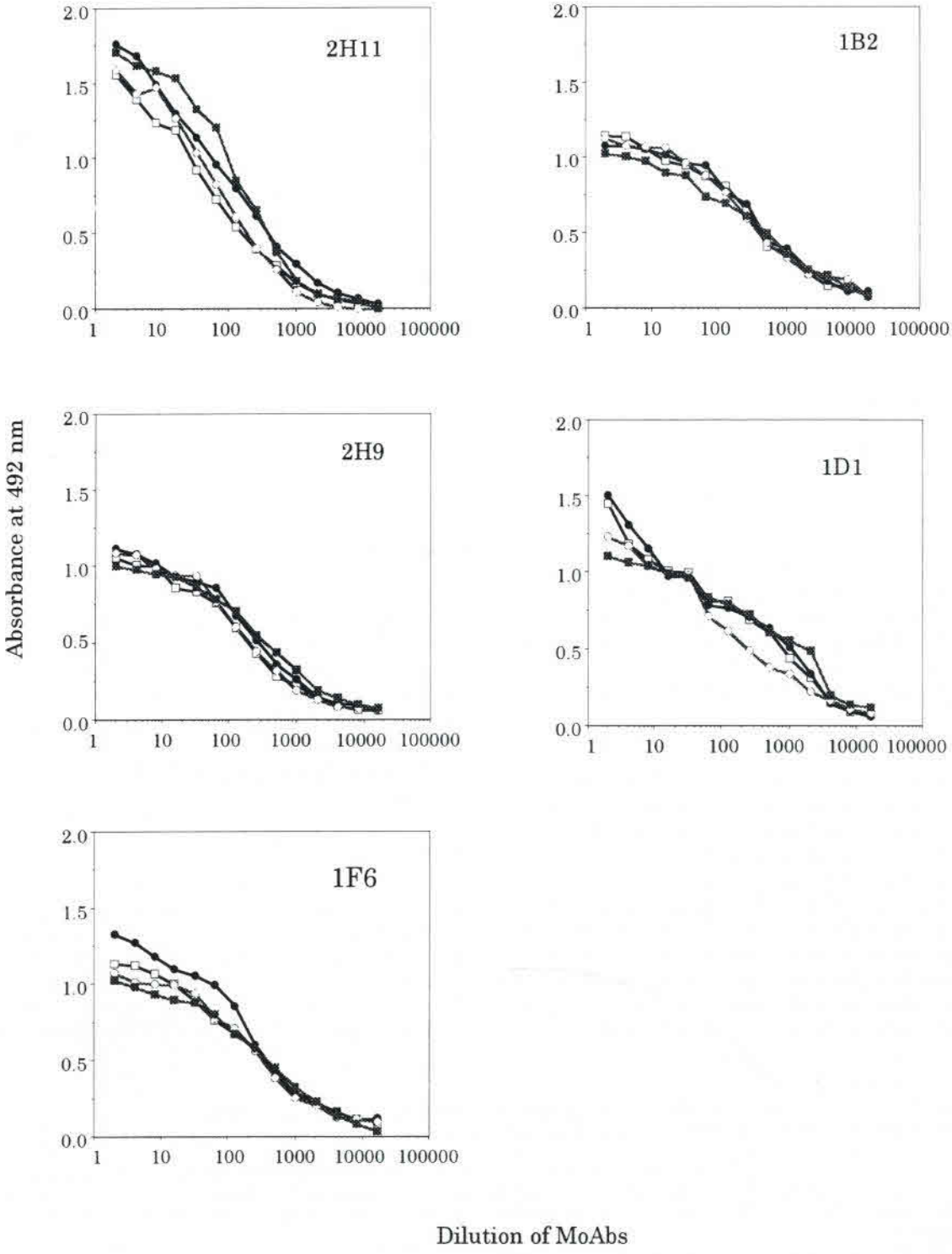

FIG. 1. Binding curve of MAbs to human and bovine RBC stroma. Microtiter plates were coated with human type A (□), type B (●), type O (○), and bovine RBC stroma (■) and analyzed by ELISA. The results with five MAbs are shown.

ent sugars such as Gal, NAcGal, glucose (Glu), fucose (Fuc), and NANA (all from Nacalai Tesque, Kyoto, Japan). No inhibition was observed with any sugars for the reactivity of five MAbs to both human and bovine RBC. But GPA significantly inhibited their reactivity (Table 3). This indicates that the five MAbs are specific for GPA of human erythrocytes and GPA-like glycoprotein of bovine erythocytes. Furthermore, their reactivity to the epitope was independent of sialic acid and other sugars.

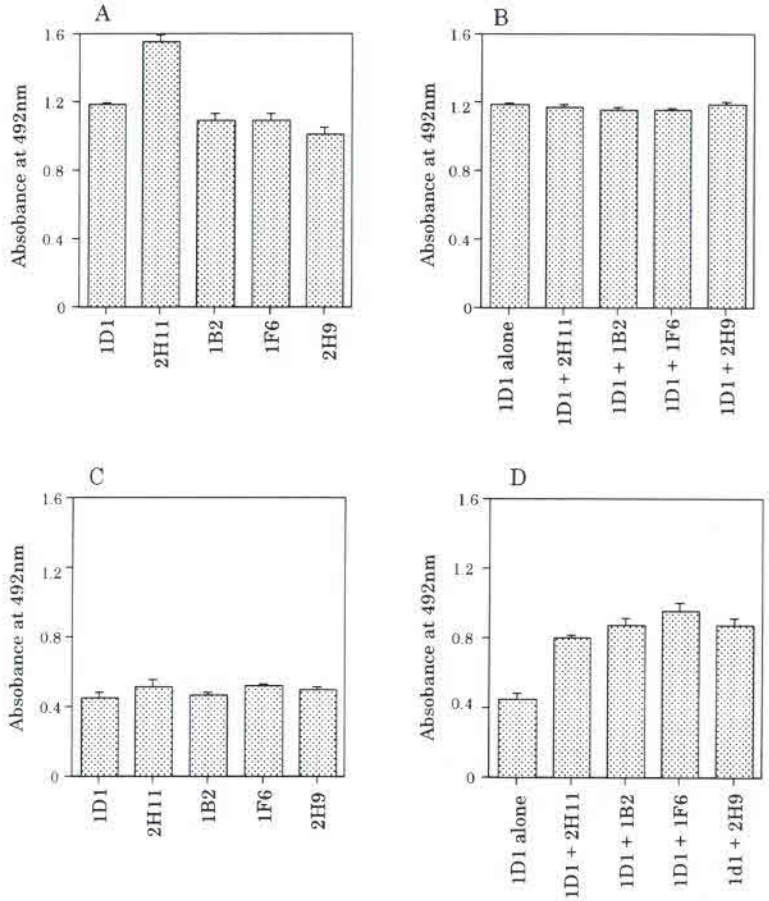

FIG. 2. Mixing experiment of five MAbs. Microtiter plates were coated with human type B RBC stroma. (A) ×10 dilution of each MAb was used. (B) 1D1 was mixed with four other MAbs at ×10 dilution. (C) ×100 dilution of each MAb was used. (D) 1D1 was mixed with four other MAbs at ×100 dilution. The amount of bound antibody was detected by ELISA.

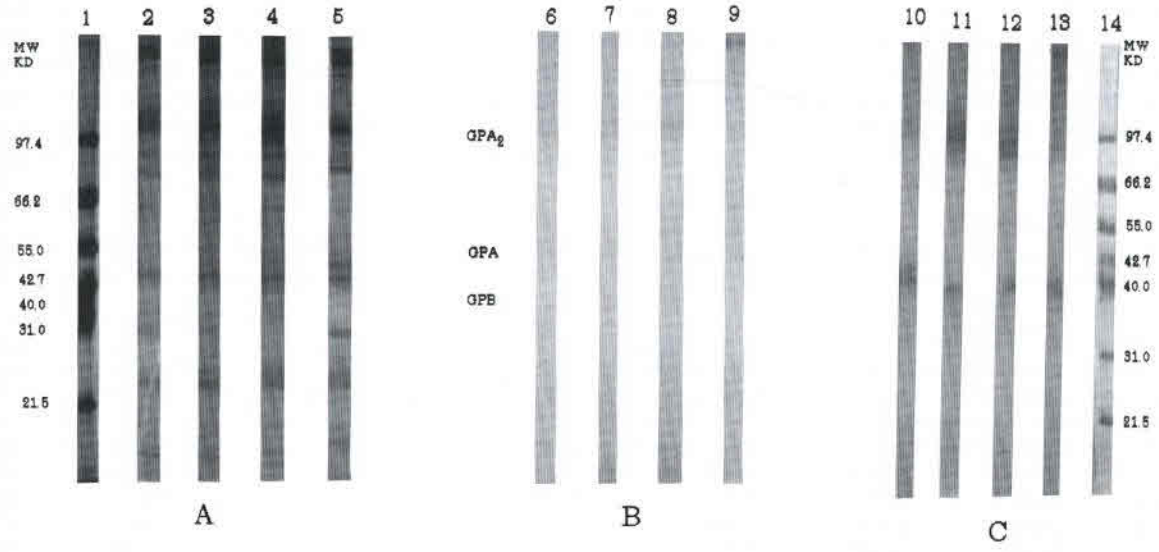

FIG. 3. Electrophoregrams of human and bovine RBC stroma by SDS–PAGE. The electrophoregrams were stained with CBB (Panel A), PAS (Panel B), and immunoblotting with MAb 2H9 (Panel C). Lanes 2, 6, and 10 are human type A RBC stroma; Lanes 3, 7, and 11 are human type B RBC stroma; Lanes 4, 8, and 12 are human type O RBC stroma; Lanes 5, 9, and 13 are bovine RBC stroma. The positive bands correspond to GPA_2 and GPA of human RBC (Lanes 10, 11, and 12) and to 200, 97, and 40 KD components (Lane 13) of bovine RBC. Lane 1 and 14 are marker proteins.

TABLE 3. INHIBITORY ACTIVITIES OF MONOSACCHARIDES AND GPA ON THE BINDING ACTIVITY OF FIVE MABS TO HUMAN AND BOVINE RBC

	MAbs reacted with human and bovine RBC stroma									
	2H11		*1B2*		*2H9*		*1D1*		*1F6*	
Inhibitors	*Human*	*Bovine*	*Human*	*Bovine*	*Human*	*Bovine*	*Human*	*Bovine*	*Human*	*Bovine*
NAcGal	−	−	−	−	−	−	−	−	−	−
Gal	−	−	−	−	−	−	−	−	−	−
Fuc	−	−	−	−	−	−	−	−	−	−
Glu	−	−	−	−	−	−	−	−	−	−
NANA	−	−	−	−	−	−	−	−	−	−
GPA	+	+	+	+	+	+	+	+	+	+

The microtiter plates were coated with human type B RBC or bovine RBC stroma. MAb 2H11, 1B2, 2H9, 1D1, and 1F6 used at the dilution of 1:64, 1:256, 1:32, 1:128, and 1:128, respectively, were pretreated with each monosaccharide or GPA, added to the plates, and the bound MAbs were detected by ELISA. The results showed that: $^{-}$not inhibited by up to 500 nmol/well of each monosaccharides, $^{+}$more than 50% inhibition at 25 μg/ml of GPA.

Effect of enzyme treatment of human and bovine RBC stroma on the reactivity of MAbs

To further study the chemical nature of RBC epitope recognized with MAbs, the enzyme treatment of RBC stroma was performed. Treatment of human erythrocytes with trypsin or chymotrypsin decreased their reactivity with five MAbs. The treatment of bovine erythrocytes with trypsin or chymotrypsin gave the same pattern but slightly stronger. However, neuraminidase treatment of both species erythrocytes gave almost no effect on their reactivity with the MAbs (Table 4). These results suggest that the five MAbs reacted with protein determinant on both species erythrocytes but not sialic acid residues.

DISCUSSION

In this article, we established five MAbs from immunized mice with human type B RBC which commonly reacted with human type A, type B, and type O RBC (Table 1, Table 2, and Fig. 1). These MAbs also reacted with bovine erythrocyte heterophile antigen. However, these MAbs did not react with RBC of guinea pig, horse, and sheep whose RBCs expressed Forssman heterophile antigen,[16] with RBC of horse, rabbit, and sheep whose RBCs expressed Hanganutziu–Deicher (H–D) heterophile antigen. Accordingly, the antigenic system recognized with these MAbs, seems to belong to Paul–Bunnell (P–B) antigen complex.[17] By SDS–PAGE, immunoblotting, and competitive inhibition assay, we found that the epitope recognized with these MAbs was on GPA of human erythrocytes (Fig. 3 and Table 3) and 200 KD major glycoprotein, 97 KD, and 40 KD minor glycoproteins on bovine erythrocytes.

The study on glycophorin or glycophorin-like glycoprotein from erythrocyte membrane of various species has been reported by several groups.[18–20] However, the detailed structures of bovine glycophorin have not yet been elucidated and the number of glycophorin components in bovine erythrocyte membrane is still ambiguous. Capaldi,[21] Emerson and Kornfeld purified a high molecular weight material (about 200 KD) as bovine glycophorin.[22] Merrich *et al.* demonstrated the presence of a minor glycophorin having a molecular weight of 26 KD besides the high molecular glycophorin which was found to be P–B heterophile antigen that was also expressed on sheep RBC and reacted with the serum of human infectious mononucleosis.[23] The MAbs established in this report seem to react with different antigen from the so-called P–B antigen because

TABLE 4. EFFECT OF ENZYME TREATMENTS OF HUMAN AND BOVINE RBC STROMA ON THEIR REACTIVITY WITH FIVE MABS

	RBC treated with:							
	—		*Trypsin*		*Chymotrypsin*		*Neuraminidase*	
MAbs	*Human*	*Bovine*	*Human*	*Bovine*	*Human*	*Bovine*	*Human*	*Bovine*
2H11	1/1024	1/512	1/256	1/8	1/64	–	1/2048	1/512
1B2	1/4096	1/4096	1/256	1/16	–	–	1/4096	1/4096
2H9	1/2048	1/4096	1/256	1/32	1/32	–	1/4096	1/4096
1D1	1/2048	1/2048	1/256	1/4	1/16	–	1/4096	1/4096
1F6	1/2048	1/2048	1/256	1/8	1/16	–	1/4096	1/4096

Microtiter plates were coated with enzymes (−, trypsin, chymotrypsin, or neuraminidase) -treated human type B RBC or bovine RBC stroma, MAbs were added and were detected by ELISA. The results are shown by the titer of MAbs reactive with RBC stroma.

–indicates lack of reactivity.

these did not react with sheep RBC and 26 KD component of bovine RBC. Murayama *et al.*[20] reported that bovine erythrocyte membrane contained at least three glycophorin components, in which the fraction III_a have a molecular weight of 46 KD and 42 KD; III_b has a molecular weight 26 KD. In this report, 200 KD major component and 97 KD, 40 KD minor glycoproteins were isolated from bovine erythrocytes (Fig. 3). These five MAbs reacted with 200 KD, 97 KD, and 40 KD glycorotein components but not with 26 KD components.

Several investigators have compared the chemical nature of human and bovine glycophorin of erythrocytes and noted that the amino acid component of bovine glycophorin had strict similarity with its human counterpart. Both glycophorins have a high content of serine, threonine, and glutamic acid, but oligosaccharide contents from bovine were very heterogeneous.[24,25] Bovine glycophorin is more rich in carbohydrates (80% by weight) than human (60% by weight). In the present study, we found that the epitope recognized with five MAbs was not on carbohydrates (Table 3). The GPA of human RBC carries MN blood group antigens. The M- and N-type GPA have different amino acid residues at position 1 (Ser in M and Leu in N) and 5 (Gly in M and Glu in N) of the peptide chain. The epitope recognized with these five MAbs is at least not M and N type 1–5 amino acid residues of NH_2-terminal because these MAbs reacted with type M as well as type N erythrocytes (data not shown). This is the same as the report by Jaskiewicz *et al.*[26]

The GPA molecule has three distinct domains: a glycosylated segment composed of 64 residues from NH_2-terminal is located outside of the membrane; a hydrophobic segment is transmembrane and a COOH-terminal segment is located entirely in the cytoplasm. It is reported that trypsin cleaves GPA on human RBC membrane at ARG31 and ARG39 and the chymotrypsin cleaves it at ALA35 and LEU64 from NH_2-terminal.[27] The result from the enzyme treatment study showed that the epitope recognized with these MAbs is on NH_2-terminal domain that is not in the glycosylated portion which may be closed to the membrane domain.

In conclusion, we established five MAbs against human erythrocytes. Unexpectedly, they reacted not only with human erythrocytes but also with bovine erythrocytes. Furthermore, these MAbs reacted with glycoproteins of bovine erythrocytes that showed a same pattern to GPA of human RBC. From these results, it is suggested that the epitope recognized with these MAbs is a new heterophile antigen expressed on GPA of human RBC and on glycoproteins of bovine RBC.

Heterophile antibodies are often detected in sera from patients with infectious mononucleosis,[4] and heterophile antigens are expressed on some malignant cell surfaces such as leukemia, melanoma, lung cancer, and colon cancer.[17] The MAbs established in this article seem to react with a new type of P–B antigens. Thus, these MAbs are useful for the further classification of heterophile-antigen systems and some types of malignant cells. The reactivity of these MAbs with several tumor cell lines is under investigation.

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