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NON-SPECIFIC AGGLUTINATION OF BROMELIN-TREATED RED BLOOD CELLS BY NORMAL HUMAN SERUM : REACTION OF IMMUNOGLOBULINS TO MEMBRANE PROTEINS EXPOSED BY BROMELIN-DIGESTION

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Abstract: Although it is known that agglutinins specific for bromelin-treated red blood cell (BrRBC) exist in normal human serum, their immunological and biological characteristics have not yet been elucidated. We have found two different types of agglutinins specific for BrRBC in the normal serum. The one reacts specifically with red blood cell (RBC) pretreated for 15 minutes with lower concentration of bromelin (Br)(1.25 U/ml to 25 U/ml in terms of casein-digestion activity), and thus, it is responsible for the non-specific agglutination encountered in routine works. The other is detected with RBC pretreated with Br of higher activities (>125 U/ml). The former agglutinin was proved to be IgM and the latter to be IgG on the basis of biochemical and immunological criteria, and they are termed NSA-BrRBC- I (non-specific agglutinin for BrRBC) and NSA-BrRBC-II, respectively. The incidences of NSA-BrRBC- I and -II in normal human sera were 2.3 % and 99.9 %, respectively. The binding site for NSA-BrRBC- I on RBC was specifically elicited by mild Br-treatment but not by other proteinase digestions. From the Br-digestion products of RBC membrane, we partially purified the protein fraction which exhibited a high inhibitory activity against the agglutination by NSA-BrRBC- I without suppressing the agglutination by most of the blood group antibodies examined. This fraction was therefore assumed to contain the target protein of NSA-BrRBC- I. Four fragments with different molecular weights (26,000, 61,000, 74,000 and 160,000 dalton) could be detected by Western blotting of the fractions after immunoprecipitation and electrophoresis in the reduced condition. This fraction successfully inhibited non-specific agglutination by NSA-BrRBC- I in two-stage Br-method but not in one-stage method.

These results indicate that one of the non-specific agglutinations of BrRBC encountered in routine works is caused by the interaction between NSA-BrRBC- I and Br-modified membrane protein(s).

Index Terms

non-specific agglutination, bromelin treated red blood cell, immunoglobulin, membrane protein

INTRODUCTION

It is known that red blood cells (RBCs) treated with proteinases are often agglutinated with normal sera independently of the blood groups of the donors[1~6]. Such a so-called non-specific (NS) agglutination is often encountered in the enzyme digestion process for screening and identifying antibodies in the donor and patient sera or the crossmatching test for the blood

transfusion.

The enzyme methods are highly sensitive for detecting several antibodies, especially Rh antibodies[7~10]. Although the exact mechanism of enhanced sensitivity in these methods are not clear, it is conceivable that the removal of sialopeptides from RBC surface by proteinases would contribute to this enhancement. Two enzyme methods, one-stage and two-stage, are commonly used in the routine tests in clinical laboratories. The former is technically simpler and more quick and the latter is more sensitive[11]. Sulfidryl-proteinases with broad specifity such as bromelin (Br), papain (Pa) and ficin (Fi) are frequently used in the enzyme methods. Br is suitable for both one-stage and two-stage methods and the NS-agglutination can be detected by these two methods.

The presence of an agglutinin specific for trypsin (Tr)-treated RBC in normal human serum is well known[2]. This agglutinin is of IgM type, and its epitope has been suggested to be a glycoprotein present on RBC surface[12]. The presence of NS-agglutinins specific for other proteinase-treated RBCs has also been reported in normal serum[3]. Several workers[5)6)] reported the presence of agglutinins specific for Br-treated RBC (BrRBC) in the serum and the destruction of their agglutinabilities by 2-mercaptoethanol. However, immunological and biochemical properties of the agglutinins and the precise mechanism of NS-agglutination in Br-methods are at present still unclear.

In the present study, we analyzed the NS-agglutinations in the Br-methods and found that these agglutinations were composed of two phases; one is exerted by IgM-type agglutinin and the other by IgG-type one. We also discovered that the reactivities of these agglutinins depend on the degree of RBC surface modification by Br. In addition, a protein fraction which was thought to contain the target of IgM-type agglutinin was obtained from BrRBC membrane. The molecular properties of the target protein(s) are characterized to some extent. A possible mechanism of NS-agglutination by IgM-type agglutinin is discussed on the basis of the reaction of this agglutinin with its target protein(s).

MATERIALS AND METHODS

Blood samples and blood group antibodies. All RBCs used in this study were obtained from blood group O donors. Sera containing NS-agglutinin were from blood donors whose sera showed NS-agglutination in routine tests. Monoclonal anti-glycophorine A (GPA) antibodies (anti-En^{a}TS, anti-En^{a}FS and anti-En^{a}FR)[13] were obtained from Osaka Red Cross Blood Center. Other blood group antibodies were purchased from commercial sources.

Preparation of enzyme solutions. Br, Tr, Pa and Fi were purchased from E. Merck (Darmstadt, Germany). Br, Tr, Pa and Fi solutions were prepared according to the method as previously described[14]. Proteinase K (PrK) (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany) was dissolved at 1 mg/ml in 50 mM Tris buffer (pH 8.0) (TB) containing 1 mM $CaCl_2$. Br solution was freshly prepared in each experiment while the other proteinase solutions were stored below −20℃ until use.

Proteinase activity was determined by casein-digestion method (Hagihara et al[15]). One unit of proteinase activity was defined as the activity giving an increase of absorbance at 275 nm which is equivalent to 1 μg tyrosine liberation per minute.

Serological tests. Agglutination tests, measurement of agglutination titer and dithiothreitol (DTT) treatment of sample serum or sample solution were performed according to the AABB technical manual[16]. The two-stage method was usually used for agglutination tests throughout this study except as otherwise indicated.

The strength of agglutination was expressed in five steps (−to 4+) according to the interpretation of agglutination in the AABB technical manual[16].

Absorption test of NS-agglutinin was performed as follows: RBCs were pretreated with 0.125 % Br (HBrRBC) or 0.0025 % Br (LBrRBC) at 37℃ for 15 minutes. Serum or sample solution was added to an equal volume of packed HBrRBC or LBrRBC and mixed well. After standing at room temperature for one hour, the supernatant solution obtained by centrifugation was used for agglutination test.

Separation of agglutinin. Serum proteins in the pooled plasma with high agglutinability specific for BrRBC were fractionated by sedimentation with ammonium sulfate between 20 % and 50 % saturation. The obtained precipitate was dissolved in PBS and dialyzed against 20 mM TB at 4℃. After centrifugation of the dialysate, the obtained supernatant solution was applied to a Q-Sepharose FF (Pharmacia LKB Biotechnology, Upssala, Sweden) column (2.6×40 cm) equilibrated with 20 mM TB. The column was washed with 300 ml of 20 mM TB and subsequently eluted stepwise with solution of 0.1, 0.16, 0.23, 0.3 and 0.5 M NaCl in 20 mM TB each for 200 or 300 ml. Proteins in effluent were monitored by the absorbance at 280 nm, and the presence of agglutinin activity in the fractions was examined both with HBrRBC and LBrRBC.

Fractions containing higher agglutination activity with HBrRBC or LBrRBC were concentrated and applied to a Sephacryl S-300 (Pharmacia) column (2.3×93 cm) previously equilibrated with 0.1 M TB containing 0.5 M NaCl. The elution was performed with the same buffer.

Absorption of agglutinins by immunoglobulin-class specific affinity gel.

1) Absorption of IgM-type agglutinin: Rabbit anti-human IgM (Dakopatts, Glostrup, Denmark) was conjugated with Afigel-10 (Bio-Rad, Richmond, CA, USA) according to the instruction manual. Sample serum was mixed with the anti-human IgM-conjugated agarose (2.7 mg protein/ml gel) and incubated at room temperature for 3 hours. After the agarose was washed with PBS, it was incubated with 3 M KSCN and stood for 30 minutes. The obtained supernatant solution was desalted with a Sephadex G-25 (Pharmacia) mini-column and used for agglutination test after addition of a half volume of 5% bovine serum albumin (BSA) in PBS.

2) Absorption of IgG-type agglutinin: The sample solution containing IgG-type agglutinin was dialyzed against 20 mM phosphate buffer (pH 7.0) (PB) and applied to a Protein G Sepharose (Pharmacia) column (1.14×4.3 cm) previously equilibrated with 20 mM PB. Elution was carried out by stepwise decreasing pH value of elution buffer (0.1 M glycine-HCl). The agglutinin activities were detected in the fractions eluted at pH 3.7 by agglutination test after adjusting the pH of the solution to neutral and adding a half volume of 22% BSA to each sample.

Fluorescent staining of agglutinated RBC. BrRBC suspension (2% in 100 μl of saline) was incubated with an equal volume of serum containing NS-agglutinin at 37℃ for 15 minutes.

Then, the BrRBCs were washed three times with cold saline and incubated with 100 μl of fluorescein isothiocyanate (FITC)-conjugated anti-human IgM or anti-human IgG (diluted 1 : 7 with 5% BSA in PBS) (Behringwerke AG, Marburg, Germany). The labeled BrRBCs were again washed with cold saline and the fluorescent images of the BrRBC were observed under a fluorescence microscope.

Electrophoretic procedures. Immunoelectrophoresis was performed according to the method described by Garbar et al[17]. Sodiumdodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Fairbanks et al[18] using a 5.6 % or 8 % polyacrylamide slab gel with 1 mm thickness. Proteins were detected with Silver Stain Plus Kit (Bio-Rad).

Western blotting was carried out by electrophoretic transfer of proteins from the SDS-PAGE gel to Immobilon P transfer membrane (Millipore, Bedford, MA, USA). The protein blots of NS-agglutinins on the membrane were detected with horseradish peroxidase (HRP)-conjugated anti-human IgM and anti-human IgG (Cappel, Organon Teknika Co., West Chester, PA, USA). For the analysis of the target protein(s) of NS-agglutinin, partially purified NS-agglutinin and anti-GPAs were used as primary antibodies. Protein blots reactive with these reagents were visualized by streptavidin biotinylated HRP complex (Amersham, Amersham, UK) or streptavidin-alkaline phosphatase (ALP) conjugate (Amersham) using biotinylated anti-human IgM (Amersham) or biotinylated anti-mouse immunoglobulin (Dakopatts) as secondary antibodies. 4-Chloro-1-naphtol, and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as color-developing reagents for HRP and ALP, respectively.

Isolation of the target protein(s) of NS-agglutinin from BrRBC. The activity of the target protein of NS-agglutinin was defined as the reciprocal of the highest dilution of samples to inhibit the reaction of LBrRBC with NS-agglutinin. The inhibitory activity of a sample solution against the agglutinin was measured as follows : Serial twofold dilutions of a sample were prepared with PBS containing 5% BSA. To each series of diluted samples, an equal volume of diluted agglutinin solution with 8-fold agglutination titer was added and mixed well. After standing for 30 minutes, a half volume of 2% LBrRBC suspension was added to each mixture. The detection of agglutination was performed in the same manner as two-stage method.

Membrane ghost was prepared from washed LBrRBC by the method described by Dodge et al[19]. and stored after lyophilization. The lyophilized ghost was suspended in 5 mM TB to 1/3 of the original RBC volume. An equal volume of 10 mM TB containing 2% Triton X-100 and 1% Amphitol 20 N (lauryl dimethylamine oxide ; Kao Co., Tokyo, Japan) was added to the ghost suspension and stirred for 30 minutes at 4℃. After removing the sediment by centrifugation at 30,000 g for 30 minutes at 4℃, the obtained supernatant solution was incubated with Bio-Beads SM-2 (Bio-Rad) (approximately 1 g/3 ml) for 2 hours, under gentle stirring, and was concentrated by ultrafiltration with Diaflo membrane YM-10 (Amicon, Danvers, MA, USA). After removal of the sediment by centrifugation, the obtained supernatant solution was applied to a Sephacryl S-300 column (2.2×100 cm) equilibrated with 50 mM TB containing 0.1 % Amphitol 20 N, 0.02 % NaN_3 and 0.5 M NaCl. Elution was performed with the same buffer, and the absorbance of effluent was monitored at 280 nm. The fractions with the inhibitory activity were dialyzed against 20 mM TB containing 0.1 % Amphitol 20 N and 0.02 % NaN_3 (TBA), and were applied to a Q-Sepharose FF column (1.6×20 cm) previously equilibrated

with TBA. After washing the column with TBA and TBA containing 0.15 M NaCl, active fractions were eluted with a linear gradient of NaCl in TBA up to 0.4 M. The obtained fractions were rechromatographed by the elution with a linear gradient of NaCl from 0.25 to 0.5 M. The active fractions eluted were collected and concentrated by ultrafiltration as described above after Bio-Beads SM-2 treatment.

Protein concentration was determined with Bio-Rad Protein Assay (Bio-Rad) and Micro BCA Protein Assay Reagent Kit (Pierce, Rockford, IL, USA).

Immunoprecipitation of target protein. The partially purified NS-agglutinin with IgM properties was conjugated to Afigel-10 (850 μg protein/100 μl gel). After washing three times with a sufficient volume of the coupling buffer containing 0.05 % Amphitol 20 N, the conjugated gel was suspended in an equal volume of 50 mM TB containing 0.05 % Amphitol 20 N and 0.15 M NaCl (TBAS). The pH of the finally obtained solution containing target protein (1.1 mg/ml, 300 μl) was adjusted to 8.3 with 50 μl of 0.5 M sodium bicarbonate buffer (pH 8.3). To this solution, 50 μl PBS containing NHS-LC-Biotin (Pierce) (1 mg/ml) was added and after standing for 2 hours at 4℃, the solution was applied to a Sephadex G-25 mini-column equilibrated with 50 mM TBAS to remove unreacted biotin and change the buffer solution. The eluted biotinylated protein fraction was concentrated to 200 μl by ultrafiltration and added into the micro centrifuge tube containing 100 μl of the agglutinin-conjugated Afigel-10 suspension. After 2 hours of incubation on a rotary mixer at room temperature, the gel was washed three times with 50 mM TBAS and once with 5 mM TB and then mixed with 100 μl of the sample buffer for SDS-PAGE containing 2-mercaptoethanol. After heating at 100℃ for 5 minutes, the supernatant solution was applied to SDS-PAGE. Procedures for SDS-PAGE and Western blotting were the same as described above. The biotinylated proteins on the blotting membrane were detected by incubating with streptavidin biotinylated HRP complex (Amersham) diluted to 1 : 5,000 for an hour at room temperature followed by chemiluminescence detection (ECL Western blotting detection reagents ; Amersham). The membrane was exposed to autoradiography film (Hyperfilm-ECL ; Amersham) for 2 hours. The proteins transferred to the blotting membrane were stained with 0.1 % amido black 10 B in 45 % methanol and 10 % acetic acid.

RESULTS

The presence of two different and sequential NS-agglutinations of BrRBC in normal serum. The NS-agglutinability of normal serum was examined with RBC premodified by different concentrations of Br. As shown in Fig. 1, the NS-agglutination of BrRBC consists of two phases : One is observed by the 15-minute pre-modification of RBC with Br of activity between 1.25 U/ml and 25 U/ml (LBrRBC) and the other is seen above 125 U/ml (HBrRBC). These two types of NS-agglutination were assumed to be caused by the presence of at least two different agglutinins in the normal sera, because the agglutination of LBrRBC was diminished by the preincubation of serum with LBrRBC but not with HBrRBC and *vice versa.*

To substantiate the above assumption, NS-agglutinability of the serum was further analyzed by changing both Br concentration and duration time of Br-treatment. As shown in Table 1, NS-agglutinability was completely separated into two distinct activity areas on the 2-dimensional matrix composed of these two parameters. Thus, the results indicate the presence of two

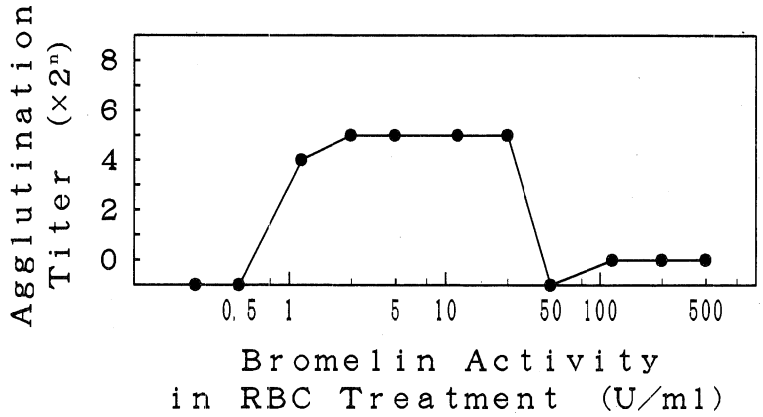

Fig. 1. Agglutination of RBCs treated with increasing concentration of Br by the serum with NS-agglutinins. Intensity of agglutination is expressed as the reciprocal of the highest dilution of the serum which gives detectable agglutination.

types of NS-agglutination which depends on the degree of modification of RBC by Br, i. e., one is induced by mildly Br-modified RBC and the other by drastically modified RBC. Treatment with 0.0025 % Br for 5 seconds was the mildest condition for obtaining LBrRBCs. In such a mild condition, MN antigens on RBCs still remained intact, though Br removes MN antigens from RBC surfaces in the normal condition of pretreatment. The maximum extent of Br-treatment for mildly modified RBC was attained at a value slightly lower than 1,000 U•min/ml, which is expressed as the product of the effective Br activity (U/ml) and the incubation time (minute). On the other hand, the minimum extent for drastically modified RBC was slightly higher than 1,000 U•min/ml. Thus, these results indicate that two physically distinct entities are involved in these two types of agglutination.

Hereafter, NS-agglutinins for mildly modified RBC and drastically modified RBC are defined as NSA-BrRBC-I and NSA-BrRBC-II (NS-agglutinin I and II for BrRBC).

Separation and Characterization of two types of NS-agglutinins. To clarify the molecular properties of NS-agglutinins, an attempt to separate NS-agglutinins from sera was made. Both activities of NSA-BrRBC-I and -II were successfully collected in a fraction obtained from a pooled serum with NS-agglutinability by sedimentation with ammonium sulfate. From this fraction, five fractions (PT, I, II, III and IV) having NS-agglutinability for BrRBC were separated by Q-Sepharose ion-exchange chromatography (Fig. 2). Fractions PT and I contained NSA-BrRBC-II while fractions III and IV exhibited the activity of NSA-BrRBC-I. Fractions I and IV with peak activities of NSA-BrRBC-II and -I, respectively, were further purified by gel filtration (Fig. 3). The activity of NSA-BrRBC-II was finally recovered in fraction I-2 whereas NSA-BrRBC-I was found in fraction IV-2. Since the elution positions of the NSA-BrRBC-I and -II fractions on the chromatographies used in the purification corresponded with those of IgM and IgG, respectively, each immunoglobulin type of NSA-BrRBC-I and -II was assumed to be IgM and IgG. This assumption was supported by the results of DTT treatment, immunoelectrophoresis, SDS-PAGE, Western blotting and immuno-

Table 1. Effects of Br concentration and incubation time of RBC on NS-agglutination

Br Concentration (%)	Br Activity* (U/ml)	Time of Br-Treatment of RBC														Extent† of Br-Treatment of RBC for Positive Agglutination	
		0″	5″	10″	20″	30″	1′	2′	3′	5′	10′	15′	20′	30′	45′	60′	▒	▓
0.5	900	−	2+	2+	2+	2+	±	−	±	±	±	1+	1+	1+	1+	1+	<1,800	>1,800
0.25	450	−	2+	2+	2+	2+	1+	−	−	±	±	1+	1+	1+	1+	1+	<900	>1,350
0.1	180	−	2+	2+	2+	2+	2+	1+	−	−	±	1+	1+	1+	1+	1+	<675	>1,125
0.05	90	−	2+	2+	2+	2+	2+	2+	2+	±	−	±	±	±	1+	1+	<900	>900
0.025	45	−	2+	2+	2+	2+	2+	2+	2+	2+	±	−	−	−	±	±	<675	>1,350
0.01	18	−	2+	2+	2+	2+	2+	2+	2+	2+	2+	1+	1+	−	−	−	<675	n. c.
0.005	9	−	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2−	2+	2+	n. c.	n. c.
0.0025	4.5	−	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	2+	n. c.	n. c.

▒, NS-agglutination of mildly treated RBC. ▓, NS-agglutination of drastically treated RBC.

NS-agglutination was determined by the agglutination tests of sera with RBCs treated with varying concentration of Br and duration time at 37 ℃. Br-treatment of RBC was terminated by the addition of E-64 (100 μM, 1/2～2 volume of reacting solution). The strength of the agglutination was expressed in five steps (− to 4+)[16]. ±' indicated the intermediate strength between − and 1+.

Abbreviation: Br, bromelin; RBC, red blood cell; NSA-BrRBC, non-specific agglutinin for Br-treated RBC; n. c., The value couldn't be calculated from the data.

* Br activity was calculated from the concentration of Br on the basis of 45 U/ml at 0.025%.

† This was calculated from the following equation:

Enzyme activity (U/ml) × Treatment time (min) = Extent of treatment (U•min/ml)

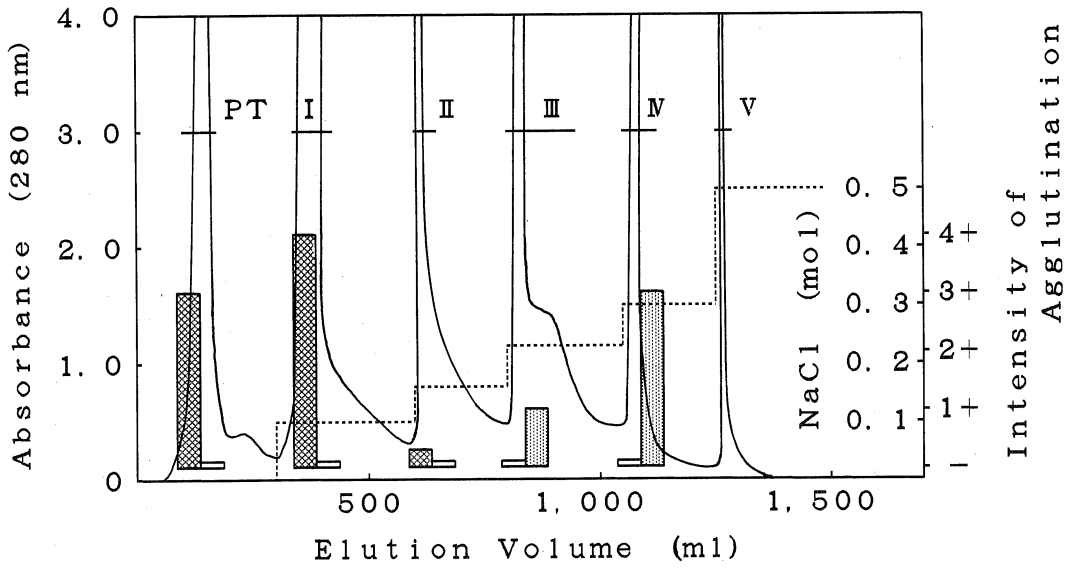

Fig. 2. Q-Sepharose ion-exchange chromatography of serum proteins. Serum proteins in the pooled human plasma with NS-agglutinability were fractionated by sedimentation with ammonium sulfate, dialized and subjected to Q-Sepharose ion-exchange chromatography as described in "MATERIALS AND METHODS". Elution was first carried out with 20 mM TB, and then by stepwise increase of NaCl up to 0.5 M, at a flow rate of 150 ml/hr. Column size was 2.6×40 cm. Agglutination activity in the fractions was expressed as the intensity of agglutination.
———, absorbance at 280 nm; ·····, concentration of NaCl; ▩, agglutination activity with HBrRBC; ▨, agglutination activity with LBrRBC.

detection with these fractions. In addition, NSA-BrRBC-I was found to be specifically absorbed with anti-human IgM-conjugated agarose while NSA-BrRBC-II with Protein G agarose, and they were eluted from each absorbent by the corresponding elution reagents. Furthermore, BrRBCs which were agglutinable by NSA-BrRBC-II were found to be stained specifically with FITC-conjugated anti-IgG but not with anti-IgM, while those by NSA-BrRBC-I were fluoresced only with anti-IgM (Fig. 4). These results are summarized in Table 2.

When sera without alloantibodies from 1,000 normal donors were examined, the incidences of the presence of NSA-BrRBC-I and -II in these sera were 2.3 % and 99.9 %, respectively.

Effects of treatment with other proteinases on the reactivity of NSA-BrRBC-I. The agglutinability of NSA-BrRBC-I for RBCs treated by various proteinases was investigated. When RBCs were treated with several proteinases (Tr, Pa, Fi and PrK) other than Br for different periods of time, they could not be agglutinated by NSA-BrRBC-I even though their MN antigens still remained to be expressed.

To investigate whether the binding site of NSA-BrRBC-I is susceptible to these proteinases, treatment with these proteinases was carried out before and after Br-treatment (Table 3). The agglutination by NSA-BrRBC-I was positive with RBC treated with Tr or PrK before or after Br-treatment. On the other hand, RBC treated with Pa or Fi before and after Br-treatment lost the ability to be agglutinated by NSA-BrRBC-I.

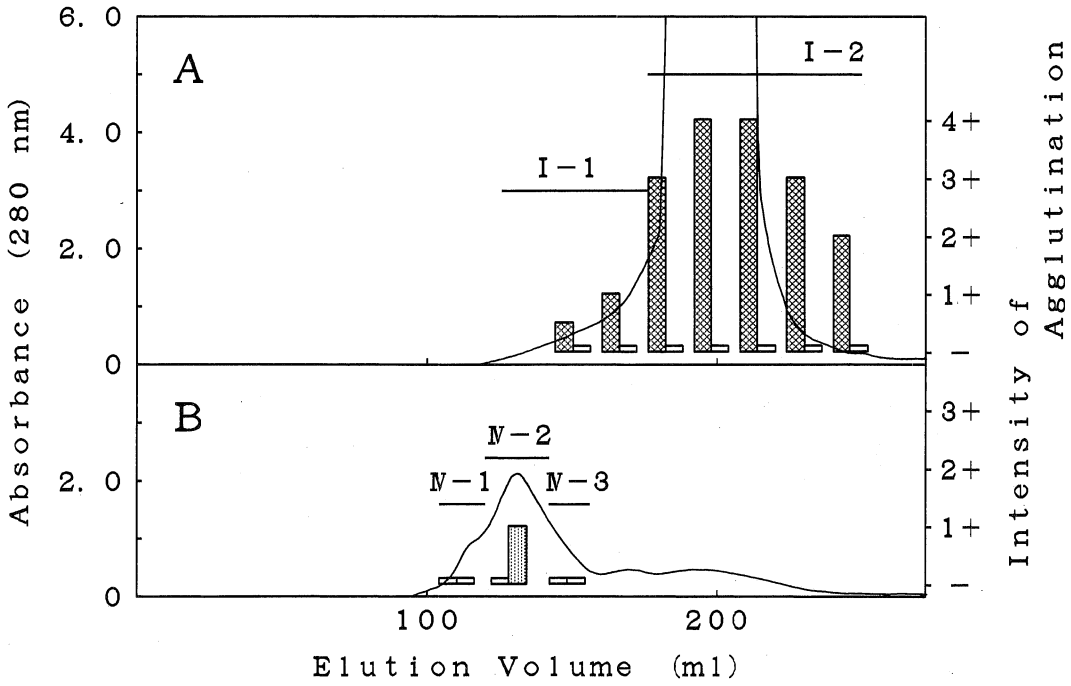

Fig. 3. Gel filtration of the agglutinins. Proteins in the fractions I and IV obtained in Q-Sepharose step (Fig. 1) were collected by sedimentation with ammonium sulfate and subjected to gel filtration through a Sephacryl S-300 column as described in "MATERIALS AND METHODS". Elution was carried out with 0.1 M TB containing 0.5 M NaCl at a flow rate of 48 ml/hr. Column size was 2.3×93 cm. Agglutinin activity in the fractions was expressed as the intensity of agglutination. A ; elution profile of fraction I. B ; elution profile of fraction IV.
———, absorbance at 280 nm ; ▩, agglutinin activity with HBrRBC ; ▨, agglutinin activity with LBrPBC.

Isolation of target protein(s) of BrNSA- I. Since the preceding results suggest that the target of NSA-BrRBC- I is membrane protein, we tried to isolate the relevant membrane protein(s) from BrRBC ghost on the assumption that the target protein might exhibit an inhibitory activity against NSA-BrRBC- I agglutination. In fact, such an activity could be detected in a crude membrane extract of BrRBC, and as shown in Fig. 5 A, the gel filtration of the extract gave two large protein peaks and the inhibitory activity was detected in the second peak (Fig. 5 A). The membrane extracts from RBC without Br-digestion and any fractions of this extract eluted by gel filtration did not show inhibitory activity (Fig. 5 B).

An anion-exchange chromatography of the obtained fraction separated a peak of the inhibitory activity (Fig. 6 A). The main fractions exhibiting the activity were rechromatographed (Fig.6 B) and the resultant active fractions were combined and termed NSA-BrRBC- I -RP (receptor protein for NSA-BrRBC- I), and were used for further experiments. The final yield of protein in this fraction was 2.4 mg from 100 ml of packed RBC. The final specific activity of this fraction was 80-fold higher than that of the initial extract (Table 4). A marked reduction of the inhibitory activity of the RP found at the gel filtration step seems to be due to the presence of Triton X-100 remaining in this fraction, because the activity of the RP was

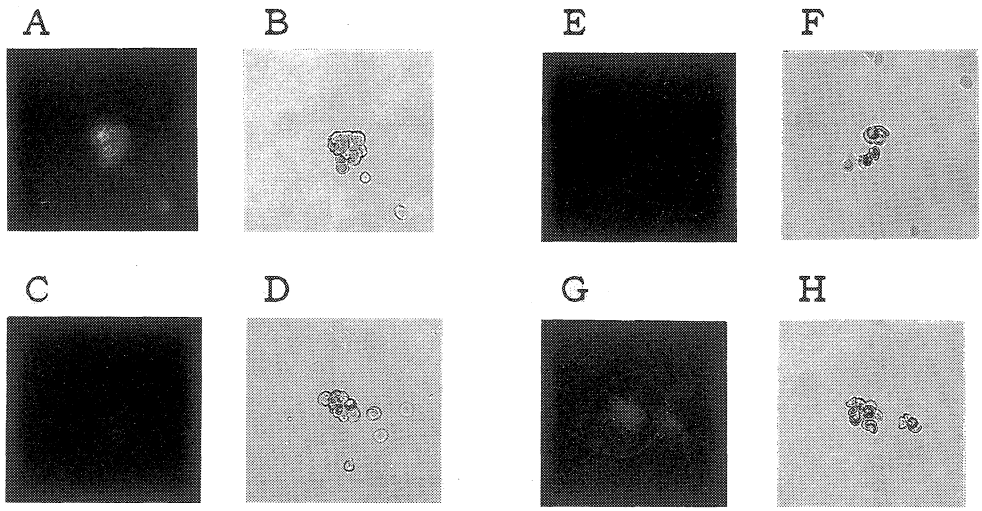

Fig. 4. Immuno-fluorescent staining of HBrPBC (A~D) and LBrRBC (E~H) by NS-agglutinins of IgG and IgM type, respectively. Both HBrRBC and LBrRBC were stained with FITC-conjugated anti-human IgG (A and E) and anti-human IgM (C and G). B, D, F and H are the light microscopic photographs of the agglutinated BrRBCs corresponding to A, C, E and G, respectively.

Table 2. Characteristics of non-specific agglutinins for BrRBC

Item	Non-Specific Agglutinin	
	NSA-BrRBC-I	NSA-BrRBC-II
Incidence in Normal Serum (%)	2.3	99.9
Extent of Br-pretreatment for Agglutinable RBC (Br Activity, U/ml)*	Mild (1.25 to 25)	Drastic (>125)
Absorption with		
LBrRBC	+	−
HBrRBC	−	+
Sensitivity to DTT	Sensitive	Resistant
Behavior in Immunoelectrophoresis	IgM	IgG
Adsorption with		
Anti-IgM-Conjugated Agarose	+	N. T.
Protein G Agarose	−	+
Molecular weight		
Analyzed by SDS-PAGE	Very Large†	150K, 180K, 300K
Analyzed by Western Blotting	Very Large†	150K, 180K, 300K
Detected on the Membrane with		
HRP-Anti-IgM	+	−
HRP-Anti-IgG	−	+
Immunofluorescent Staining with		
FITC-Anti-IgM	+	−
FITC-Anti-IgG	−	+

Procedures of all the investigations cited in this Table were described in "MATERIALS AND METHODS".

* The range of Br activity required to induce NS-agglutination of RBC for 15-minute incubation.

† The band detected on the gel or membrane showed very slow migration rate.

Abbreviation : LBrRBC, RBC treated with low-active Br ; HBrRBC, RBC treated with high-active Br ; N. T., not tested.

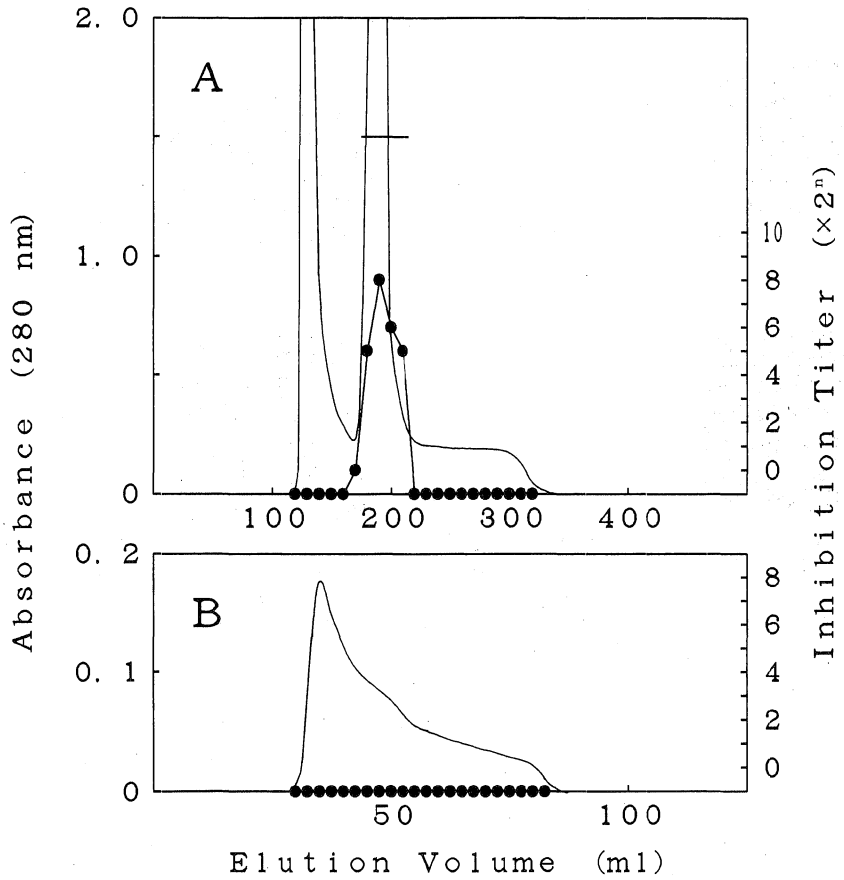

Fig. 5. Gel filtration of the extract from RBC ghost. Each of the extract from LBrRBC ghost (A) and unmodified RBC ghost (B) was treated with Bio-Beads SM-2, concentrated by ultrafiltration and subjected to gel filtration through Sephacryl S-300 column as described in "MATERIALS AND METHODS". Inhibitory activites against NSA-BrRBC-I in effluents were expressed as the reciprocal of the highest dilution of samples to inhibit the agglutination of LBrRBC with NSA-BrRBC-I. Elution was carried out with 50 mM TBA containing 0.5 M NaCl at an elution rate of 37.2 ml/hr (A) or 41.9 ml/hr (B). Column sizes were 2.2×100 cm (A) and 1.6×40 cm (B). ———, absorbance at 280 nm; —●—, inhibitory activity against NSA-BrRBC-I.

Table 3. Susceptibility of the binding sites of NSA-BrRBC-I on RBCs to various Proteinases

Proteinase treatment												
Br	Pa	Fi	Tr	PrK	Pa ↓ Br	Fi ↓ Br	Tr ↓ Br	PrK ↓ Br	Br ↓ Pa	Br ↓ Fi	Br ↓ Tr	Br ↓ PrK
+	−	−	−	−	±	−	2+	1+	±	−	2+	1+

RBCs were incubated with proteinase at 37 ℃ for 15 minutes and, after washing 3 times with saline, were subjected to agglutination tests as described in "MATERIALS AND METHODS". The proteinase activities used in RBC treatments were approximately 10 U/ml for Br, Pa and Fi and 80 U/ml for PrK. The arrow (↓) indicates the order of proteinase-treatments of RBC from 1st treatment to 2nd treatment. The strength of the agglutination was expressed as in Table 1.

Abbreviation: Br, bromelin; Pa, papain; Fi, ficin; Tr, trypsin; PrK, proteinase K.

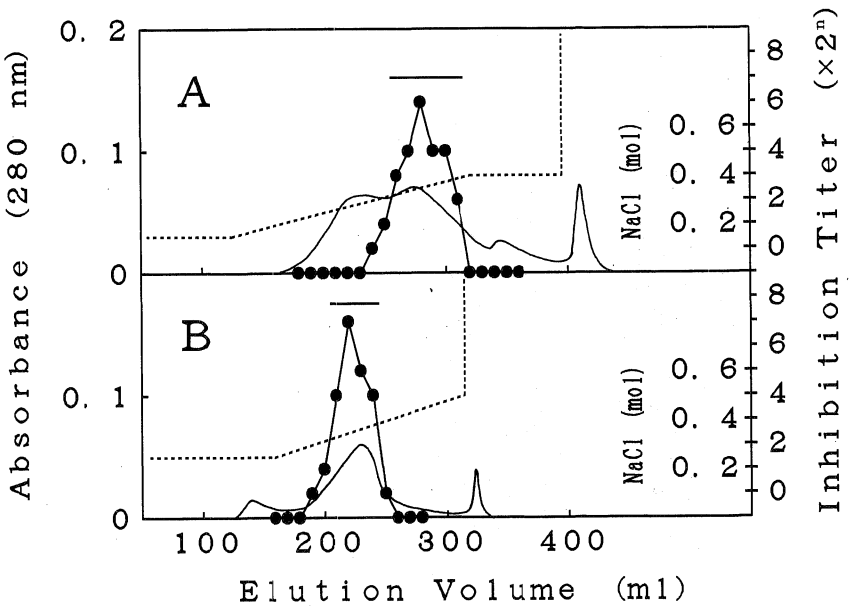

Fig. 6. Q-Sepharose ion-exhcange chromatography of the target protein(s) of NSA-BrRBC-I. The obtained fractions with inhibitory activity against NSA-BrRBC-I in the previous gel filtration (Fig. 5A) were combined, dialized against 20 mM TBA and subjected to Q-Sepharose ion-exchange chromatography as described in "MATERIALS AND METHODS". Elution was first carried out with 20 mM TBA, and then by a gradient of NaCl at a rate of 102 ml/hr. Column size was 1.6×20 cm.
A: The initial chromatography. B: The rechromatography of the fraction marked with the bar in A.
———, absorbance at 280 nm; ·····, concentration of NaCl; —●—, inhibitory activity against NSA-BrRBC-I.

Table 4. Summary of purification of NSA-BrRBC-I-RP

Isolation Step (from 100 ml RBC)	Total Protein (mg) [A]	Protein Recovery (%)	Total Activity (fold) [B]	Recovery of Activity (%)	Specific Activity (fold/mg) [B/A]
Extract	88.0	100	64,000	100	727
Gel Filtration	17.2	20	2,560	4	149
Ion-Exchange Chromatography	2.4	3	140,000	220	58,182

RP activity was defined as the highest rate of dilution of the preparation by which the hemagglutinating activity of NSA-BrRBC-I with 4 fold titer was completely inhibited.

restored or rather increased after complete removal of Triton X-100 by subsequent ion-exchange chromatography.

SDS-PAGE and Western blotting of the partially purified NSA-BrRBC-I-RP. To examine the purity of the obtained NSA-BrRBC-I-RP fraction, SDS-PAGE analysis was performed. It revealed two major and several minor bands by silver staining (Fig. 7 A).

Since the molecular size of RP could not be estimated by SDS-PAGE, we tried to analyze it by Western blotting technique. The chemiluminescent detection of the Western blots of the

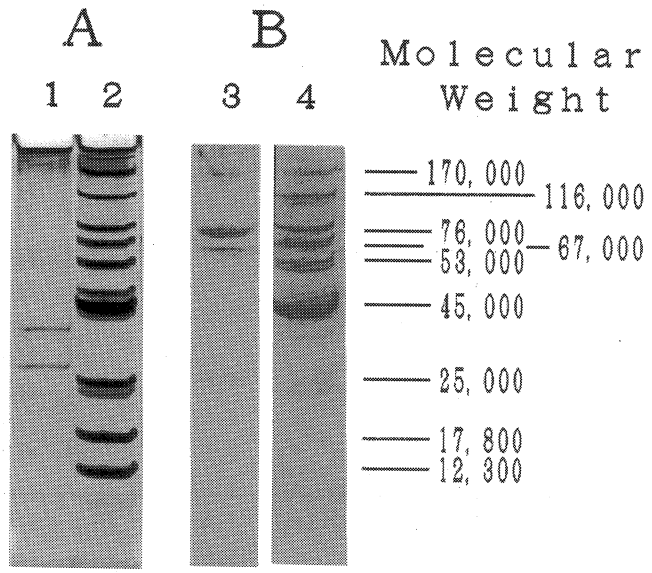

Fig. 7. SDS-PAGE (A) and Western blotting (B) of the target protein of NSA-BrRBC-I (NSA-BrRBC-I-RP). NSA-BrRBC-I-RP fraction finally obtained from the extract of LBrRBC ghost in the ion-exchange chromatography (Fig. 6B) was subjected to SDS-PAGE and Western blotting, SDS-PAGE was performed according to Fairbanks et al[18]. Proteins in the gel were detected by a silver staining method. Western blotting was performed by the method as described in detail in "MATERIALS AND METHODS". The RPs were biotinylated, concentrated by immunoprecipitation and subjected to SDS-PAGE. After transferring the biotinylated RPs from the gel onto blotting membrane, the blots of RPs were detected by the chemiluminescence detection method using streptavidin biotinylated HRP complex. The marker proteins on the membrane were stained with amidoblack 10B. Lanes 1 and 3; the partially purified NSA-BrRBC-I-RP. Lanes 2 and 4; molecular weight marker.

biotinylated RP obtained from the membrane extract by immunoprecipitation showed a main band with the molecular weight of 74,000 dalton and three minor bands with the molecular weights of 26,000, 61,000 and 160,000 dalton (Fig. 7 B).

No significant band was detected from the partially purified RP by Western blotting using anti-GPAs as primary antibodies or by immunoprecipitation using them as ligands.

Specificity and applicability of NSA-BrRBC-I-RP to inhibit NS-agglutination. To confirm that the inhibitory activity of NSA-BrRBC-I-RP is specific to the agglutination of BrRBC with NSA-BrRBC-I, we examined whether NSA-BrRBC-I-RP inhibits the agglutination of RBC with various blood group antibodies (Table 5). The RP weakly inhibited the agglutination with anti-U, but its inhibitory activity was below 1/1,000 of that for NSA-BrRBC-I. The agglutination with other antibodies examined so far in this study was not inhibited by NSA-BrRBC-I-RP.

Table 5. Inhibitory effects of the partially purified NSA-BrRBC-I-RP on agglutination of RBC by blood group antibodies

Antibody	Inhibition	Antibody	Inhibition	Antibody	Inhibition
Anti-A	−	Anti-K	−	Anti-Lu^a	−
Anti-B	−	Anti-k	−	Anti-Lu^b	−
Anti-H*	−	Anti-Kp^a	−	Anti-M	−
Anti-P_1	−	Anti-Kp^b	−	Anti-N†	−
Anti-Le^a	−	Anti-Js^b	−	Anti-S	−
Anti-Le^b	−	Anti-Fy^a	−	Anti-s	−
Anti-D	−	Anti-Fy^b	−	Anti-U	+※
Anti-C	−	Anti-Jk^a	−	Anti-En^aTS	−
Anti-c	−	Anti-Jk^b	−	Anti-En^aFS	−
Anti-E	−	Anti-Xg^a	−	Anti-En^aFR	−
Anti-e	−	Anti-Di^a	−	NSA-BrNSA-I	+′
				NSA-BrNSA-II	−

Each agglutination of RBC by antibodies cited in the Table was investigated in the optimum conditions as described in the instruction manuals.

*, † Lectin.

※ Inhibition titer was 4.

′ Inhibition titer was above 5, 120.

Table 6. Inhibitory effects of the partially purified NSA-BrRBC-I-RP on non-specific agglutination with NSA-BrRBC-I in two different Br-methods

Serum No.	Addition of RP	Two-stage Method				One-stage Method			
		Incubation Time of RP with Serum				Incubation Time of RP with Serum			
		5 min.	15 min.	30 min.	60 min.	5 min.	15 min.	30 min.	60 min.
1	+	−	−	−	−	2+	3+	3+	3+
	−	3+	3+	3+	3+	2+	3+	3+	3+
2*	+	3+	3+	2+	2+	4+	4+	3+	3+
	−	3+	4+	4+	3+	4+	4+	3+	3+
3	+	±	−	−	−	3+	3+	3+	3+
	−	3+	4−	3+	3+	3+	3+	3+	3+
4	+	+	+	±	±	3+	3+	3+	3+
	−	3+	3+	3+	3+	3+	3+	3+	3+
5	+	−	−	−	−	3+w	3+w	3+w	3+w
	−	3+w	3+w	3+w	3+	3+w	3+w	3+w	3+w
6	+	−	−	−	−	1+	1+	1+	1+
	−	2+	2+	1+s	2+	1+	1+	1+	1+

Sera containing NSA-BrRBC-I with agglutinin titer of 32 to 128 were diluted four times with AB serum without NSA-BrRBC-I. The partially purified NSA-BrRBC-I-RP with inhibition titer of 1, 024 was diluted 20 times with the same AB serum as above. An equal volume of these diluted serum and RP was mixed and incubated at room temperature for the indicated times. After the incubation, the samples were subjected to the two Br-methods as described in the text.

* This serum contained anti-I.

In order to examine the applicability of NSA-BrRBC-I-RP in routine works, the ability of the RP to inhibit NS-agglutination was tested by using several sera with different NS-agglutination activities (Table 6). In the two-stage method, the RP successfully suprressed the agglutinability of all the sera with different activity levels of NSA-BrRBC-I by pre-incubation for over 30 minutes. Such an inhibition, however, could not be detected in the one-stage method.

DISCUSSION

In this study, we confirmed the existence of two different types of agglutinins specific for BrRBCs in normal sera, showing that their presence is responsible for NS-agglutination in Br-methods. Biochemical and immunological studies revealed that one (NSA-BrRBC-I) is specific for RBCs mildly treated with Br (LBrRBC) and is IgM type and the other (NSA-BrRBC-II) specific for RBCs drastically treated with Br (HBrRBC) and is IgG.

Although NSA-BrRBC-II was detected in human blood at a high incidence (99.9%), this agglutinin does not usually affect the screening and detection of blood group antibodies or the crossmatching tests, because the condition of RBC treatment by Br in conventional tests is not so drastic as to induce the agglutinability by NSA-BrRBC-II. On the other hand, the presence of NSA-BrRBC-I in the sera of some donors or patients, at a low incidence (2.3%), is largely responsible for NS-agglutination encountered in routine works. In fact, we observed that NS-agglutination detected by both two-stage and one-stage Br-methods was abolished by the preincubation of sera with BrRBC possessing the ability to be agglutinated by NSA-BrRBC-I. Therefore we intended to analyze the mechanism of NS-agglutination by NSA-BrRBC-I in more detail and tried to identify and characterize the target protein(s) of NSA-BrRBC-I.

It is noted that NSA-BrRBC-I reacted specifically with the binding site unmasked by the sensitive and specific cleavage of RBC surface protein by Br. Thus the binding sites on RBC surface could be disclosed by a very mild Br-treatment even at the concentration of 0.0025% (approximately equivalent to 4.5 U/ml of proteinase activity) for 5 seconds in spite of broad substrate specificity of Br (Table 1). M and N antigens on the RBC surface still remained intact by such a mild treatment. The appearance of the binding sites is quite specific for Br because other proteinases of broad substrate specificity could not induce the agglutination with NSA-BrRBC-I (Table 3). In addition, the exposed binding sites are destroyed with Br at higher concentrations or for longer incubation time, the condition which induces the agglutination with NSA-BrRBC-II. Therefore, the mechanism of Br action to disclose NSA-BrRBC-I binding sites seems to be quite different from that to enhance reactivity of certain well known antibodies with their antigens, such as Rh system. In those latter cases, Br is not a specific enhancer of agglutination and the excess Br-treatment of RBC dose not affect the agglutinations by the antibodies. It has been supposed that the approach of these antibodies to their antigens is hindered by major sialoglycoproteins such as GPA, which forms a half of all sialyl-residues on RBC and is itself the carrier of M and N antigens[20~22]. It is clear that NS-agglutination by NSA-BrRBC-I is not interrupted by GPA, at least, because this agglutination was positive with very mildly Br-treated RBC in which M antigen still remained intact. Therefore, proteolytic removal of neighboring sialopeptides such as GPA may not be the cause of the exposure of NSA-BrRBC-I binding sites by Br-treatment of RBC. From these considerations, it is reasonable to presume that Br-treatment directly exposes the binding sites of NSA-BrRBC-I on RBC surface.

Judd[5] reported that a bromelin dependent 'pan-agglutinin' was present in the serum of a certain blood donor. This agglutinin exhibited similar serological properties to those of NSA-BrRBC-I but may not correspond to NSA-BrRBC-I, because its agglutinability was inhibited by 0.1 M N-acetyl neuraminic acid in his report, while that of NSA-BrRBC-I was not

(data not shown).

We partially purified protein(s) (NSA-BrRBC-I-RP) from BrRBC membrane which is the target of NSA-BrRBC-I. NSA-BrRBC-I-RP is thought to be a protein not intact but modified by Br-treatment, because the agglutinability of NSA-BrRBC-I was not inhibited by the extract from intact RBC membrane or any fractions from this extract obtained by gel filtration. These results are consistent with the previous assumption that Br-treatment directly exposes the binding site of NSA-BrRBC-I on RBC surfaces. The outer membrane domain of this RP carrying this binding site is accessible to Pa and Fi because RBCs treated with these proteinases before and after Br-treatment were not agglutinated by NSA-BrRBC-I (Table 3).

The molecular size of NSA-BrRBC-I-RP is assumed to be large, because the Western blotting of NSA-BrRBC-I-RP following electrophoresis by SDS-PAGE in the reduced condition showed four bands of molecular weights between 26,000 and 160,000 dalton. These bands could be observed only when, from the biotinylated partially purified RP preparation, the biotinylated RP was concentrated by an immunoprecipitation with NSA-BrRBC-I, subjected to Western blotting and detected with streptavidin biotinylated HRP complex by use of the highly sensitive chemiluminescent staining method (able to detect less than 1 pg of antigen). When the partially purified RP was analyzed by the conventional Western blotting technique, no immuno blots specific for NSA-BrRBC-I could be detected even by the use of the chemiluminescent detection method. The partially purified RP fraction, though not very pure in SDS-PAGE analysis, showed a very high inhibitory activity against NSA-BrRBC-I agglutinability (above 50,000 fold/mg protein). These results suggest that the amount of this RP exposed on RBC is relatively small and that the density is much less than that of RBC membrane proteins usually detected in SDS-PAGE (10^4-10^6/cell). It is obvious that this RP protein is not derived from GPA because no bands were detected with anti-GPAs in the Western blotting analysis, though this fact was also expected from the other observations as described above.

Partially purified NSA-BrRBC-I-RP is expected to be useful for the inhibition of NS-agglutination in routine works. In fact, the addition of this RP to serum with different NSA-BrRBC-I activities was effective for suppressing NS-agglutination in two-stage method and did not affect the agglutination of RBC by usually encountered blood group antibodies except for anti-U. The inhibition of the agglutination with anti-U is thought to result from coextraction of derivatives of GPB which is the carrier of U antigen[23]. The membrane-free RP may be easily inactivated by Br-digestion, because the RP was ineffective in one-stage method.

By using biochemical and immunological methods, the results of the present study provide confirmative evidence that NS-agglutination of BrRBC can be ascribed to the interaction between IgM type agglutinin (NSA-BrRBC-I) and a certain membrane protein extremely sensitive to Br-treatment. A full description of the mechanisms of Br-induced NS-agglutination will require further investigation on the structure and properties of this relevant protein.

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