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Contribution of glycolipids to species-specific antigens on erythrocytes of several animal species as to recognition of antigens with rabbit anti-glycolipids and anti-erythrocyte antisera

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Abstract Because anti-glycolipid antibodies are involved in the onset of several neurological diseases, the reactivity of glycolipids on erythrocytes and the probability of generating the antibodies were determined to clarify the contribution of glycolipids as antigens. Anti-erythrocyte antisera reacted with the following glycolipids in a species-specific manner, *i.e.* blood group A-active glycolipid for man, Forssman glycolipid for sheep, Gg_3Cer for guinea pig, and Gg_4Cer and fucosyl GM1 for rat, and the hemolytic activities of the anti-erythrocyte antisera were attenuated by absorption of the antisera with liposomes prepared from the lipids of erythrocytes to the following levels, 94.5% for man, 24.5% for sheep, 17.5% for guinea pig, and 54.5% for rat. These species-specific glycolipids on erythrocytes reacted well with the respective anti-glycolipid antisera, but Gb_4Cer in man and GM1 in rat were shown to be cryptic on immunization with erythrocytes, indicating that the contribution of glycolipids as erythrocyte antigens differs among animal species.

Keywords Anti-glycolipid antisera · Liposomes · TLC-immunostaining · Hemolytic inhibition · Species-specificity · Glycosphingolipids

The glycolipid nomenclature is based on the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature [1]. The ganglioside nomenclature of Svennerholm is employed throughout [2].

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Abbreviations

SRBC	Sheep red blood cells
GRBC	Guinea pig red blood cells
RRBC	Rat red blood cells
HRBC	Human red blood cells
CF	Complement fixation
ELISA	Enzyme-linked immunosorbent assay
GVB	Gelatin-veronal buffer
FGM1	Fucosyl GM1
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Introduction

Cells including erythrocytes are covered by a glycocalyx layer formed of glycoconjugates, *i.e.* glycolipids, glycoproteins and proteoglycans, among which glycoproteins and proteoglycans are thought to expand on the cell surface and to occupy the majority of the glycocalyx layer, and glycolipids are situated close to the cell surface, and are hidden behind glycoproteins and proteoglycans according to molecular models of them and their proposed organization in the glycocalyx layer [3]. In addition, on the basis of thermodynamic modeling of glycolipids, their carbohydrate chains are supposed to be at right angles to the hydrophobic ceramide chains, lying on the biomembrane [4]. However, the carbohydrate structures of glycolipids dramatically change in species- and cell type-specific manners, and are thought to be present in blood group-, differentiation- and transformation-related antigens, and the receptors for bacteria, bacterial toxins and viruses [5], indicating their strong reactivities with several ligands.

In fact, on immunization of experimental animals with purified glycolipids, cells and plasma membranes, several polyclonal and monoclonal anti-glycolipid antibodies against mono- to octadecahexaosyl ceramides have been successfully generated, and have been applied to basic and clinical research, including for the diagnosis of and therapy for patients with cancers [6–8]. Furthermore, the effectiveness of vaccination with glycolipids has been revealed on immunotherapy for patients with melanomas [9], and anti-glycolipid antibodies detected in sera in several human neurological diseases have been closely implicated in the onset and progress of the diseases [10, 11]. These findings indicate that both purified glycolipids and those on the cell surface are immunologically active, although the immunochemical properties of glycolipids on cells have not been clarified yet.

Recently, glycolipids, cholesterol and sphingomyelin, all of which carry a donor group for hydrogen bonding, were revealed to be enriched together with several proteins, such as caveolin, Src, Rac and Rho in the low density-membrane domains termed rafts, which are linked with the intracellular signal transduction cascade [12, 13]. Accordingly, the reactivity of glycolipids on the cell surface with several ligands including antibodies seems to be affected by the other membrane constituents. To disclose the antigenic properties of glycolipids on the erythrocytes from several animal species, their reactivities with anti-glycolipid antisera and the probability of generating anti-glycolipid antibodies on immunization with erythrocytes were determined by means of several immunochemical procedures.

Materials and methods

Glycolipids

The glycolipids used in this experiment were purified from various sources in our laboratory: GlcCer, LacCer, Gb_3Cer and Gb_4Cer from human erythrocytes, $IV^3GalNAc\alpha$-Gb_4Cer (Forssman antigen) from equine kidney, Gg_3Cer from guinea pig erythrocytes, GM1 and GD1a from bovine brain, and $IV^2Fuc\alpha$, $II^3NeuAc\alpha$-Gg_4Cer (fucosyl GM1, FGM1) from bovine thyroid [14]. Gg_4Cer (asialo GM1) was prepared from GM1 by treatment with *Arthrobacter ureafaciens* sialidase [15] and purified on silica gel (Iatrobeads 8060; Iatron, Tokyo). The purity of the isolated glycolipids was confirmed by thin-layer chromatography (TLC, Silica gel 60; Merck, Darmstadt, Germany) with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) and chloroform/methanol/6 M ammonia (60:40:9, by vol.) as the developing solvents, and orcinol-H_2SO_4 and resorcinol-HCl reagents for detection.

Antisera

Anti-erythrocyte antisera against human, sheep, guinea pig and rat erythrocytes, which were prepared by immunizing rabbits with successive intravenous administration of erythrocytes, were purchased from Cappel Lab. (Cochranville, PA, USA), and their titers on complement-dependent hemolysis were proven to be more than 1:5,000. Rabbit polyclonal antibodies toward Forssman antigen, Gb_4Cer, Gg_3Cer, Gg_4Cer, GM1 and FGM1 were generated by immunizing rabbits (Japanese White; Japanese Biological Materials, Tokyo) intradermally with a water-in-oil emulsion prepared by mixing 1 mg of each purified glycolipid and 0.5 mg of methylated bovine serum albumin in 1 ml of phosphate-buffered saline (PBS) with 1 ml of Freund's complete adjuvant (Sigma, St. Louis, MO, USA), and the antibody titers were subsequently determined by enzyme-linked immunosorbent assaying (ELISA) with the respective glycolipid as the antigen, being more than 1:5,000 for all glycolipids. Also, for determination of antibody titers in the IgM and IgG fractions, anti-Forssman glycolipid antisera were separated by gel permeation chromatography (Sephacryl S-300; GE Healthcare, Uppsala, Sweden) with PBS as the elution buffer.

Separation and quantitation of glycolipids

Sheep red blood cells (SRBC), guinea pig red blood cells (GRBC), and rat red blood cells (RRBC) were purchased from Japanese Biological Materials, and human red blood cells (HRBC) were obtained from the Japanese Red Cross (Tokyo). After hemolysis of erythrocytes with 1% acetic acid in water, their membrane fractions were collected by centrifugation at 4,000×*g* for 20 min, washed with distilled water until the color of the supernatants became clear, and then lyophilized. Total lipids were extracted from the lyophilized membranes with chloroform/methanol/water (20:10:1, 10:20:1 and 1:1, by vol.), and fractionated into neutral and acidic lipids on a DEAE-Sephadex column (A-25, acetate form; GE Healthcare). Then, the neutral glycolipids were separated from the unabsorbed neutral lipid fraction by acetylation, separation of the acetylated derivatives, deacetylation and desalting, whereas the gangliosides were prepared from the absorbed acidic lipid fraction by cleavage of the ester-containing lipids, followed by dialysis [16, 17]. The gangliosides and neutral glycolipids thus obtained were examined by TLC as above. For the quantitation of glycolipids, the density of spots on TLC plates was determined at 580 nm for resorcinol HCl-positive spots and at 420 nm for orcinol H_2SO_4-positive spots, respectively, using a dual wavelength TLC densitometer (CS-9000; Shimadzu, Kyoto). Standard glycolipids, *N*-stearoyl derivatives of GalCer, LacCer, Gb_3Cer and

GM3, and Gb_4Cer, Forssman glycolipid, Gg_4Cer, GM1, FGM1 and GD1a (0.1 to 1.5 μg), were developed on the same TLC plates for the preparation of standard curves.

TLC-immunostaining

Lipids were developed on plastic-coated TLC plates (Macherey-Nagel, Düren, Germany), which were then blocked with a blocking buffer (PBS containing 1% polyvinylpyrrolidone and 1% ovalbumin), and the spots were visualized by immunostaining with the above anti-glycolipid and anti-erythrocyte antisera, followed by immunostaining with peroxidase-conjugated anti-rabbit IgG and IgM antibodies (1:1,000; Jackson Immunoresearch Lab., PA, USA), and substrates of peroxidase, 4-chloro-1-naphthol and H_2O_2, according to the procedure reported previously [18].

SDS-PAGE and Western blotting

Erythrocyte membranes with and without chloroform/methanol-extraction were suspended in PBS by sonication, the solution was treated with the sample buffer at 95°C for 5 min, and SDS-PAGE was carried out according the method of Laemmli [19]. Proteins on the gel were stained with Coomassie Brilliant Blue, and also were transferred electrophoretically to a nitrocellulose membrane, which was stained as described for TLC-immunostaining.

Hemagglutination and hemolysis with SRBC

For hemagglutination, 100 μl aliquots of antisera diluted serially were mixed with 100 μl of 1% SRBC in gelatin-containing veronal buffer (GVB) in a U-bottom microtiter plate, and then allowed to react at 4°C overnight. The antibody titer was defined as the dilution at which aggregated cells were no longer observed. For hemolysis, 100 μl aliquots of antisera diluted serially and 100 μl of 1% SRBC were reacted at 37°C for 30 min, followed by incubation with 500 μl of guinea pig complement diluted 1:1,000 at 37°C for 1 h. After centrifugation of the solution at 250×*g* for 15 min, the optical density of the supernatant was measured at 414 nm.

Liposome aggregation and liposome lysis

Liposomes containing Forssman glycolipid for liposome aggregation were prepared as follows. Forssman glycolipid (2.6 μmol), egg lecithin (10.3 μmol, Sigma), and cholesterol (25.8 μmol, Sigma) were dissolved with 1 ml of ethanol at 60°C, and then the solution was injected into 14 ml of PBS with a hypodermic needle (27 G) to yield single-layered liposomes. The resultant liposome solution (25 μl) and 25 μl aliquots of antisera diluted serially were shaken at room temperature for 1 h, and the flocculant liposomes were observed visually or microscopically. Liposomes containing Forssman glycolipid for liposome lysis were prepared as follows. Forssman glycolipid, dipalmitoyl phosphatidic acid (Sigma), cholesterol and egg lecithin in a molar ratio of 1:1:10:10 (by weight) were dried in a flask to yield a thin film, which was then mixed with 0.2 M carboxyfluorescein (Sigma) in distilled water to yield multi-layered liposomes. After extensive washing of the liposomes with GVB to remove free carboxyfluorescein and their suspension in GVB with 50 μM lipid-bound phosphorus, 5 μl of the liposome solution was incubated with 25 μl aliquots of antisera diluted serially together with 25 μl of guinea pig component (1:64) at room temperature for 30 min, and the reaction was terminated with 2 ml of 10 mM EDTA in GVB. Then the fluorescence intensity released was measured at the excitation wavelength of 490 nm and the emission wavelength of 520 nm [20].

Complement fixation

The complement fixation (CF) assay was carried out according to the method described in the literature [21]. The liposomes used for liposome aggregation and SRBC sensitized by pretreatment with anti-SRBC antisera (1:5,000) were used as the antigen and target cells, respectively.

Enzyme-linked immunosorbent assay (ELISA)

A purified glycolipid (5 μg) including Forssman glycolipid in 50 μl ethanol was put in each well of a microtiter plate, which was left at room temperature until the ethanol had completely evaporated off. The following steps, *i.e.* blocking of the plate with 100 μl of blocking buffer and reaction with 100 μl aliquots of antisera diluted serially, followed by detection of antibodies bound to the plate with 100 μl of peroxidase-conjugated anti-rabbit IgG and IgM antibodies, were similar to those for the above TLC-immunostaining, but the substrates of peroxidase for ELISA were o-phenylenediamine and H_2O_2, as described in the literature [22].

Flow cytometry

Anti-Forssman glycolipid antisera (50 μl) diluted serially with PBS were reacted with SRBC (5×10^6) at 4°C for 20 min. After washing the cells three times with 500 μl of PBS, IgG on the cells was stained with 50 μl of fluorescein-conjugated anti-rabbit IgG antibodies (1:20; Cappel Lab., Cochranville, PA, USA) at 4°C for 45 min, and then the fluorescence intensity was measured with a FACScan

(Becton Dickinson, MountainView, CA, USA) with a logarithmic amplifier [23].

Inhibition of hemolysis with liposomes

Anti-SRBC and anti-Forssman antisera (100 μl), both of which were diluted 1:5,000 with GVB, were reacted with 100 μl of Forssman glycolipid (0.01 to 45 ng)-containing liposomes, which were prepared with the lipid extracts from SRBC by the procedure used for liposome aggregation, at 4°C overnight, and then their hemolytic activity was determined as above. In a similar way, the hemolytic activities of anti-HRBC, anti-GRBC and anti-RRBC antisera with the respective erythrocytes as the target cells were inhibited by absorption of anti-erythrocyte antisera with liposomes prepared with the lipid extracts from HRBC, GRBC and RRBC, respectively.

Results

Glycolipids in erythrocyte membranes

As shown in Fig. 1 and Table 1, the glycolipids in HRBC, SRBC, GRBC and RRBC exhibited distinctly different compositions from each other, and those present in a species-specific manner were as follows: Gb_4Cer and $IV^3NeuAc\alpha$-nLc_4Cer (58% and 30% of the total glycolipids, respectively) in HRBC, Forssman glycolipid (71% of the total glycolipids) in SRBC, Gg_3Cer (93% of the total glycolipids) in GRBC, and GM1, FGM1 and GD1a (25%, 14% and 15% of the total glycolipids, respectively) in RRBC, respectively. Also, the species-specific glycolipid in the erythrocytes of rabbit was reported to be

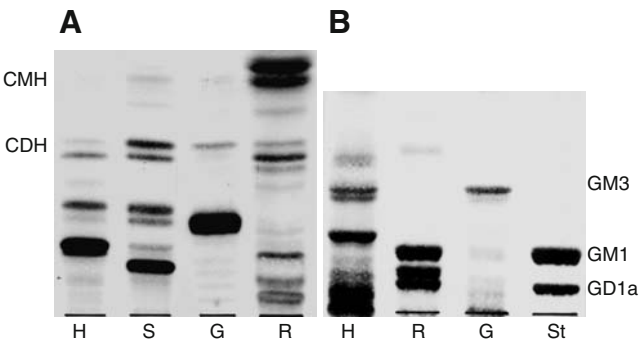

Fig 1 TLC of glycolipids from erythrocytes of man (*H*), sheep (*S*), guinea pig (*G*), and rat (*R*).The glycolipids, corresponding to 1 mg of dried erythrocyte membranes, were developed on TLC plates with chloroform/methanol/water (65:35:8, by vol.) for **A**, and chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.) for **B**, and were detected with orcinol-H_2SO_4 for A and resorcinol–HCl for **B**, respectively. *St* standard glycolipids, GM1 and GD1a; *CMH* ceramide monohexoside; *CDH* ceramide dihexoside

Table 1 Concentrations of glycolipids in the membrane fractions of erythrocytes

	Human	Sheep	Guinea pig	Rat
	(μg/mg of dry weight)			
GlcCer	0.02	0.06	0.04	4.32
LacCer	0.13	0.42	0.08	0.40
Gb_3Cer	0.20	0.38	–	–
Gg_3Cer	–	–	4.25	tr
Gb_4Cer	3.60	0.09	–	–
Forssman glycolipid	–	2.40	0.01	–
Gg_4Cer	–	–	–	0.15
Unknown glycolipid	–	–	–	0.31
GM3	0.40	0.02	0.22	–
$IV^3NeuAc\alpha$–nLc_4Cer	1.84	–	–	–
GM1	–	–	–	2.57
Fucosyl GM1	–	–	–	1.49
GD1a	–	–	–	1.58

$IV^3Gal\alpha$-nLc_4Cer, which was comprised 63% of the total glycolipids [17, 24].

Glycolipids recognized by anti-erythrocyte antisera

As shown in Fig. 2, the neutral glycolipids from the erythrocytes of different species in Fig. 1 were characteristically stained on TLC-immunostaining with the respective anti-erythrocyte antisera. As is well known, Forssman glycolipid and Gg_3Cer, which comprised 71.2% and 92.5% of the total glycolipids in SRBC and GRBC, respectively, were the dominant antigens recognized by anti-SRBC and anti-GRBC antisera, and Forssman glycolipid was found also to be present in GRBC at a level of less than 1.0% of the total glycolipids (Fig. 2). However, antibodies against Gb_4Cer of the major glycolipid in HRBC were not detected in anti-HRBC antisera, the glycolipid antigen in HRBC being characterized as a blood group A-active glycolipid, because it was present in blood group A and AB, but not blood group O and B erythrocytes, and its mobility on a TLC plate and its reactivity with anti-blood group A antibodies were identical with that of the standard glycolipid, $IV^3GalNAc\alpha$, $IV^2Fuc\alpha$-nLc_4Cer (data not shown).

On the other hand, anti-RRBC antisera contained antibodies toward Gg_4Cer, an unknown neutral glycolipid (probably $IV^2Fuc\alpha$, $IV^3Gal\alpha$-Gg_4Cer, judging from the position on TLC [25]), and FGM1, which comprised 3.2%, 2.8%, and 13.5% of the total glycolipids in RRBC, respectively (Figs. 1 and 3), but antibodies toward GM1 and GD1a, which comprised 23.3% and 14.3% of the total glycolipids in RRBC, were not present in anti-RRBC antisera. Identical results were obtained on TLC-immunostaining with anti-RRBC antisera of the lipid extracts without alkaline treatment from RRBC, since the presence of alkali-labile gangliosides, *e.g.* 9-*O*-acetylneuraminic

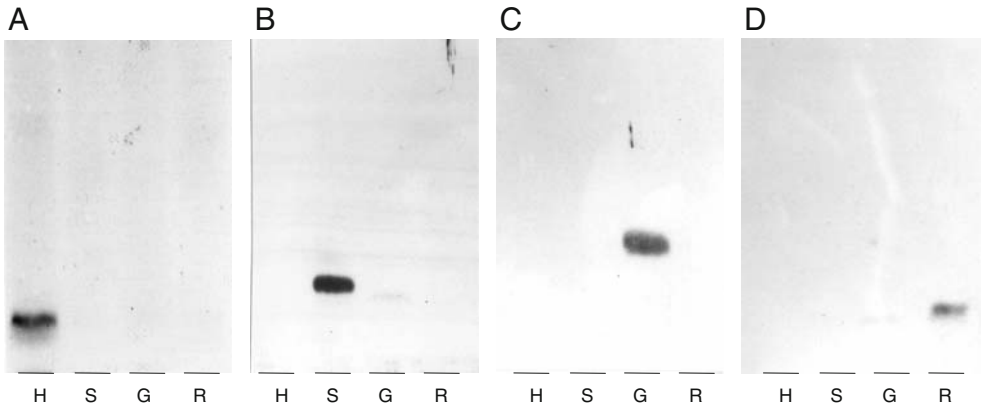

Fig 2 TLC-immunostaining of neutral glycolipids from human (*H*), sheep (*S*), guinea pig (*G*), and rat (*R*) erythrocytes with anti-HRBC (**A**), anti-SRBC (**B**), anti-GRBC (**C**), and anti-RRBC (**D**) antisera. The glycolipids, corresponding to 0.2 mg of dried erythrocyte membranes, were developed on plastic-coated TLC plates with chloroform/methanol/water (65:35:8, by vol.) and then stained with anti-erythrocyte antisera as described in the text. Note: The glycolipid in SRBC detected with anti-SRBC antisera was also present in GRBC, but at a lower concentration than that in SRBC

acid-containing GD1a, in RRBC was reported in the literature [26]. Also, no reaction of anti-HRBC, anti-SRBC and anti-GRBC antisera toward acidic glycolipids of the respective erythrocytes was observed on TLC-immunostaining.

On SDS-PAGE and Western blotting of the membrane fraction of SRBC, the major antigens recognized with anti-SRBC antisera (1:200 dilution) were proteins with molecular weights of 80, 50 and 29 kDa, and Forssman glycolipid that migrated on the gel front was only faintly stained, as shown in Fig. 4B, lane 1. Whereas, anti-Forssman antisera (1:200 dilution) intensively visualized Forssman glycolipid on the gel front (Fig. 4C, lane 1), which disappeared on extraction with chloroform/methanol (1:1, *v*/*v*) (Fig. 4C, lane 2). Also, proteins with molecular weights of 80 and 40 kDa, both of which were not detected on staining with Coomassie Brilliant Blue and anti-SRBC antisera, were stained with anti-Forssman antisera, and the intensities of the bands did not differ after extraction with chloroform/methanol.

Reactivity of glycolipids in erythrocytes with anti-glycolipid antisera

Glycolipids in erythrocytes characteristic of several animals species, *i.e.* Gb_4Cer in HRBC, Forssman glycolipid in SRBC, Gg_3Cer in GBRC, and Gg_4Cer, GM1, FGM1 and GD1a in RRBC, were used as immunogens, among which Gb_4Cer, Forssman glycolipid, Gg_3Cer, Gg_4Cer, GM1 and

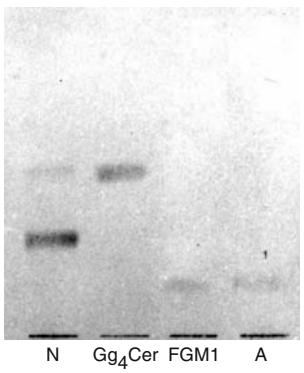

Fig 3 TLC-immunostaining of glycolipids from RRBC with anti-RRBC antisera. Neutral (*N*) and acidic (*A*) glycolipids, corresponding to 0.5 mg of dried membranes of RRBC, and standard glycolipids, Gg_4Cer and fucosyl GM1 (*FGM1*), were developed on plastic-coated TLC plates with chloroform/methanol/0.5% $CaCl_2$ in water (55:45:10, by vol.), and then stained with anti-RRBC antisera as described in the text

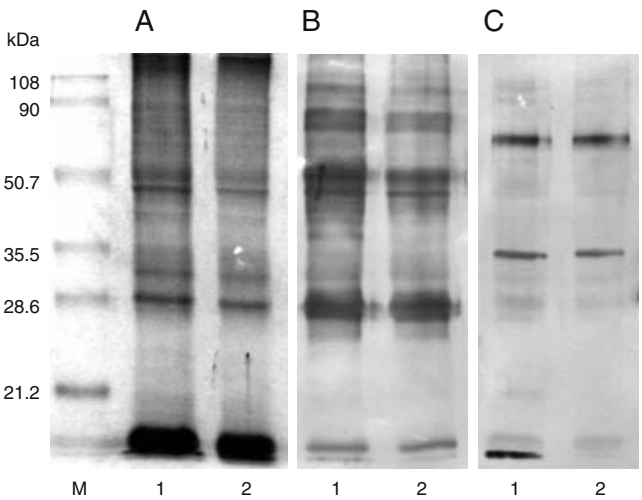

Fig 4 SDS-PAGE and Western blotting of the membrane fraction of SRBC. The membrane of SRBC (*1*) and that after removal of lipids by extraction with chloroform/methanol (1:1, *v*/*v*) (*2*) were separated by SDS-PAGE and the proteins were stained with Coomassie Brilliant Blue (*A*). Also, nitrocellulose membranes, after transfer of proteins from the SDS-PAGE gel, were immunostained with anti-SRBC (*B*) and anti-Forssman (*C*) antisera

Table 2 Antibody titers determined by ELISA with glycolipids as the antigen

Antiserum	Antigen for ELISA	Titer
Anti-HRBC antisera	Gb_4Cer	–
Anti-HRBC antisera	Blood group A glycolipid	1:3,200
Anti-Gb_4Cer antisera[a]	Gb_4Cer	1:6,400
Anti-SRBC antisera	Forssman glycolipid	1:102,400
Anti-Forssman antisera[a]	Forssman glycolipid	1:204,800
Anti-GRBC antisera	Gg_3Cer	1:102,400
Anti-Gg_3Cer antisera[a]	Gg_3Cer	1:102,400
Anti-RRBC antisera	Gg_4Cer	1:3,200
Anti-RRBC antisera	GM1	–
Anti-RRBC antisera	FGM1	1:6,400
Anti-RRBC antisera	GD1a	–
Anti-Gg_4Cer antisera[a]	Gg_4Cer	1:102,400
Anti-GM1 antisera[a]	GM1	1:51,200
Anti-FGM1 antisera[a]	FGM1	1:25,600
Anti-GD1a antisera[a]	GD1a	–

Antibodies to GM1 and Gb_4Cer were generated by immunization with the purified glycolipids, but not with RRBC and HRBC

HRBC human red blood cells, *SRBC* sheep red blood cells, *GRBC* guinea pig red blood cells, *RRBC* rat red blood cells, – titers of less than 1:100

[a] Anti-glyclolipid antisera were prepared by immunization of rabbits (three to six) with the purified glycolipids, which were present in the erythrocytes of individual species, according to the method described in the text, and the titers giving the highest values are presented in the table

FGM1 effectively produced antibodies to themselves with ELISA titers of more than 1:6,400, but antibodies against GD1a were not produced in rabbit even with eight inoculations (Table 2).

By means of several immunochemical procedures, the reactivity of Forssman glycolipid on erythrocytes was compared with that on liposomes and on an ELISA plate. As shown in Fig. 5, Forssman glycolipid on SRBC was revealed to react well with the antibodies on flow cytometry, hemagglutination and hemolysis, like that on liposomes on liposome aggregation, liposome lysis and CF. The antibody titers with several procedures were determined to be $1:2^{17}$ for flow cytometry, $1:2^{16}$ for ELISA, $1:2^{14}$ by CF, $1:2^{13}$ for liposome lysis, $1:2^{12}$ for hemolysis, $1:2^{9}$ for liposome aggregation, and $1:2^{7}$ for hemagglutination, and thus flow cytometry and ELISA allowed the detection of antibodies in the most highly diluted antisera among the procedures applied.

In addition, the anti-Forssman activity in the IgM and IgG fractions obtained on gel permeation chromatography of anti-Forssman antisera was estimated to comprise 25% and 75% of the total activities, respectively, by ELISA and CF, based on the binding activities of antibodies toward glycolipids on an ELISA plate and liposomes, respectively (Fig. 6). Whereas the aggregation activity with Forssman glycolipid-containing liposomes at room temperature was preferentially observed in the IgG fraction, but that at 4°C in the IgM fraction. On the other hand, although complement-mediated liposome lysis occurred in both the IgM and IgG fractions, hemolysis occurred preferentially in the IgM fraction, suggesting that complement-regulating factors, such as a decay-accelerating factor, are involved in the complement cascade following the complex formation between immunoglobulin and Forssman glycolipid on erythrocytes.

Contribution of glycolipids in erythrocytes to the production of anti-erythrocyte antisera

To estimate the contribution of glycolipids in erythrocytes to the generation of anti-erythrocyte antisera, the hemolytic activities of erythrocytes from several species were determined after absorption of antisera with liposomes, which were prepared from lipid extracts of the respective erythrocytes. As shown in Fig. 7, hemolysis of SRBC by anti-SRBC and anti-Forssman antisera, both of which were

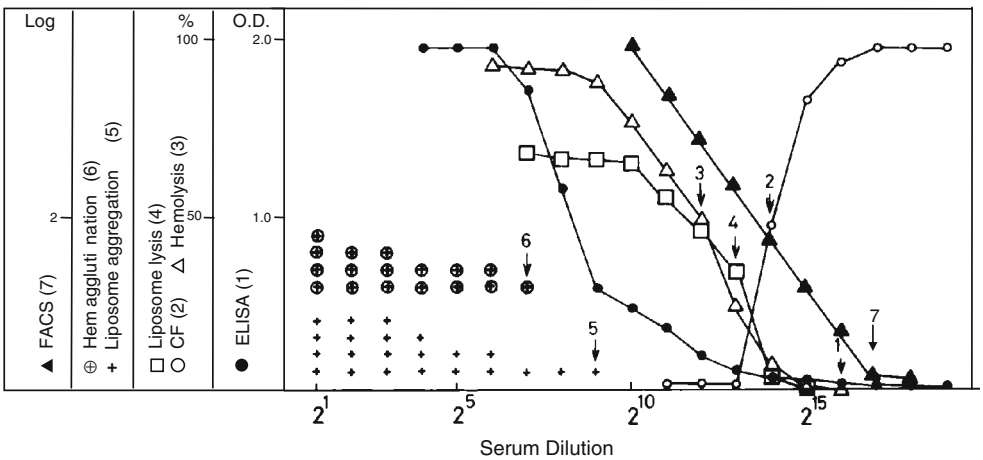

Fig 5 Antibody titers of anti-Forssman glycolipid antisera measured by means of several procedures. Arrows *1* to *7* indicate the titers for antisera on ELISA, CF, hemolysis, liposome lysis, liposome aggregation, hemagglutination and flow cytometry (FACS). The numbers of symbols, ⊕ and +, for hemagglutination and liposome aggregation indicate the intensity of reactions, respectively

diluted 1:5,000 with GVB, was inhibited with liposomes prepared from the lipid extract of SRBC in a dose-dependent manner, the maximum inhibition being reached with liposomes containing 11.25 and 5.63 ng of Forssman glycolipid, respectively. However, in contrast to that hemolysis of SRBC by anti-Forssman antisera was completely inhibited by liposomes, about 24.5% of the hemolytic activity of SRBC with anti-SRBC antisera was no longer inhibited with liposomes, indicating that the activity of anti-Forssman glycolipid antibodies comprises about 75.5% of the total hemolytic activity of anti-SRBC antisera.

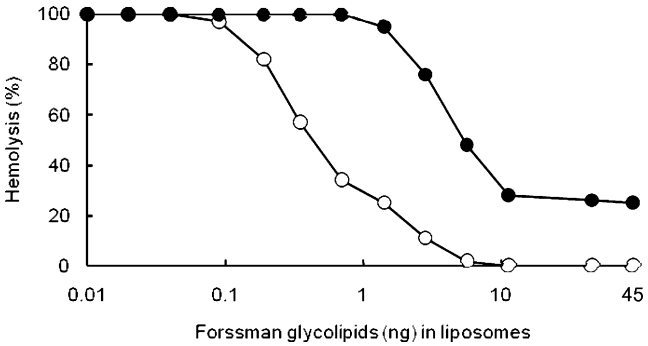

Fig 7 Inhibition of hemolysis of SRBC by anti-SRBC (*filled circles*) and anti-Forssman (*open circles*) antisera with liposomes prepared from the lipid extract of SRBC. Liposomes (100 μl) prepared from the lipid extract of SRBC in GVB, corresponding to 0.01 to 45 ng of Forssman glycolipid, were mixed with antisera (100 μl) diluted 1:5,000 with GVB, and then the solutions were left at 4°C overnight. Then, the hemolytic activities toward SRBC of antisera were measured as described under "Materials and methods", and the relative hemolytic activities as to those with antisera without an inhibitor are presented in the figure

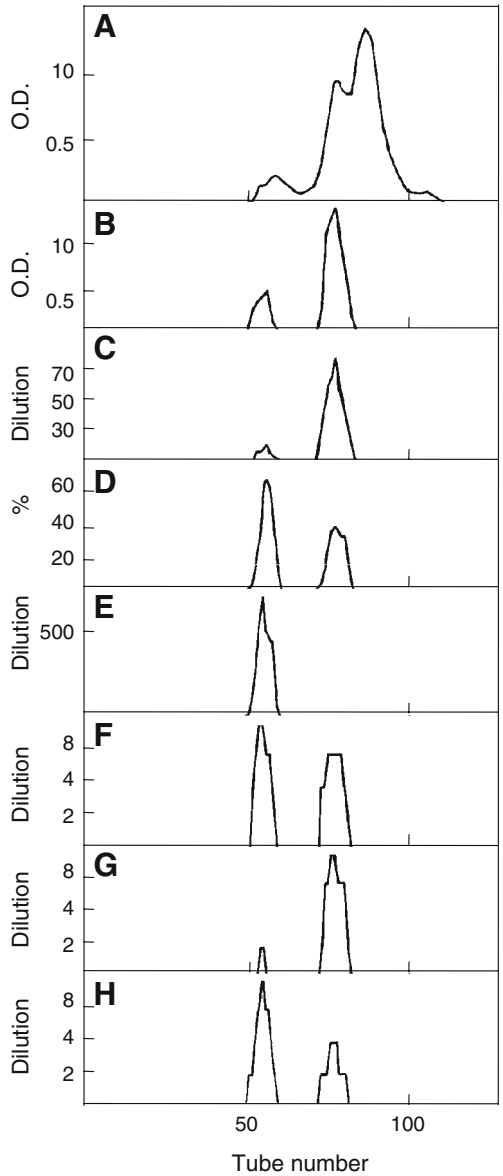

Fig 6 Antibody titer in each fraction of anti-Forssman glycolipid antisera separated by gel chromatography. **A** Optical density at 280 nm; **B** ELISA; **C** CF; **D** liposome lysis; **E** hemolysis; **F** hemagglutination; **G** liposome aggregation at room temperature; and **H** liposome aggregation at 4°C. Fractions 51 to 60 and 72 to 80 were IgM and IgG, respectively

In a similar way, although the hemolytic activities of GRBC and RRBC with anti-Gg_3Cer and anti-fucosyl GM1 antisera were completely absorbed with liposomes prepared from the lipids of GRBC and RRBC, respectively, those with anti-GRBC and anti-RRBC antisera were absorbed by 82.5% and 45.5% with liposomes prepared from the lipid extracts of GRBC and RRBC, respectively, indicating that the hemolytic activities with anti-glycolipid antibodies account for 82.5% and 45.5% of the total hemolytic activities of anti-GRBC and anti-RRBC antisera, respectively (Table 3). However, only 5.5% of the hemolytic activity of anti-HRBC antisera was absorbed by liposomes prepared from the lipid extract of HRBC of blood group A, but not that of blood group O ones, indicating that blood group A-glycolipid is a lipidic antigen and that the majority of antigens for anti-HRBC antisera are against glycoproteins.

Discussion

Commercially available anti-erythrocyte antisera toward HRBC, SRBC, GRBC and RRBC exhibited sufficient and specific abilities to hemolyze the respective erythrocytes, and their lipid antigens were characteristically detected in glycolipids, but not in phospholipids or neutral lipids on TLC-immunostaining. The following glycolipids were detected as species-specific antigens in the erythrocytes of

Table 3 Inhibition of hemolysis by antisera after absorption with liposomes prepared from lipid extracts of erythrocytes

Antiserum	Target cells	Liposomes prepared from lipid extracts of erythrocytes	Hemolysis of antisera at maximum inhibition as to that of antisera without an inhibitor (%)
Anti-HRBC antisera	HRBC	HRBC (blood group A)	94.5±2.5
Anti-HRBC antisera	HRBC	HRBC (blood group O)	100.0
Anti-SRBC antisera	SRBC	SRBC	24.5±1.5
Anti-Forssman antisera	SRBC	SRBC	0.0
Anti-GRBC antisera	GRBC	GRBC	17.5±2.0
Anti-Gg_3Cer antisera	GRBC	GRBC	0.0
Anti-RRBC antisera	RRBC	RRBC	54.5±1.5
Anti-FGM1 antisera	RRBC	RRBC	0.0

Hemolysis of the respective erythrocytes as target cells was measured with antisera after absorption with liposomes prepared from lipid extracts of erythrocytes as in Fig. 6, and the hemolytic activities of antisera at maximum inhibition compared to those of antisera without an inhibitor are presented in the table

individual species, *i.e.* blood group A glycolipid for HRBC, Forssman glycolipid for SRBC, Gg_3Cer for GRBC, and Gg_4Cer, an unknown glycolipid (probably, $IV^2Fuc\alpha$, $IV^3Gal\alpha$-Gg_4Cer), and FGM1 for RRBC. Then, to estimate the contribution of glycolipids to the production of anti-erythrocyte antisera as antigens, the relative proportions of hemolytic activitis of anti-glycolipid antisera as to those of anti-erythrocyte antisera were measured by means of hemolytic inhibition with liposomes, which were prepared from lipid extracts of the respective erythrocytes, and were found to be 5.5% for HRBC, 75.5% for SRBC, 82.5% for GRBC and 45.5% for RRBC. Thus, Forssman glycolipid and Gg_3Cer were preferentially found to generate anti-erythrocyte antisera on immunization of rabbits with SRBC and GRBC, respectively.

Also, SDS-PAGE of the solubilized membranes of SRBC, followed by Western blotting with anti-Forssman antisera revealed that the gel front corresponding to the glycolipids exhibited a strongly positive reaction. To our knowledge, carbohydrate structures belonging to the globo-series of Forssman glycolipid are only expressed in glycolipids, *i.e.* not in the *N*-glycosidic and *O*-glycosidic structures of glycoproteins. However, as shown in Fig. 4, two bands corresponding to molecular weights of 80 and 40 kDa were stained with anti-Forssman antisera, and their staining with antibodies was not affected by extraction of lipids with an organic solvent, suggesting the presence of the Forssman structure in the glycoproteins of SRBC. In fact, the hemolytic activity toward SRBC of anti-SRBC antisera was completely abolished by liposomes prepared from the lipid extracts of SRBC. In addition, although several proteins participated in production of the antibodies on immunization with SRBC, only 24.5% of the total hemolytic activity was revealed to be due to the antibodies toward proteinous antigens. Thus, Forssman glycolipid was thought to be the major species-specific antigen in SRBC for the rabbit immune system.

The relative proportions of anti-glycolipid antibodies in anti-HRBC and anti-RRBC antisera were lower than those in anti-SRBC and anti-GRBC antisera. In particular, antibodies against Gb_4Cer and GM1 were not detected in anti-HRBC and anti-RRBC antisera, respectively, even though they were yielded on immunization with the purified glycolipids.

As to GM1 on RRBC, we previously found that its reactivity was confined to small ligands on flow cytometric analysis, that is, cholera toxin B-subunit (56 kDa in molecular weight) had the ability to bind with GM1 readily bound to RRBC, but anti-GM1 antibodies (150 to 970 kDa in molecular weight) could not bind to RRBC at all [16, 27]. Thus, one of the reasons why anti-GM1 antibodies were not produced on immunization with RRBC was supposed to be the masking of GM1 by coexisting molecules on RRBC, and the conformation of GM1 probably differs from those of Gg_4Cer, the unknown glycolipid ($IV^2Fuc\alpha$, $IV^3Gal\alpha$-Gg_4Cer), and FGM1, all of which effectively produced antibodies to themselves in anti-RRBC antisera. However, the titer of anti-Gg_4Cer antisera was close to that of anti-FGM1 antisera in anti-RRBC antisera, though the concentration of FGM1 was ten-times higher than that of Gg_4Cer in RRBC, suggesting that the ability to generate anti-glycolipid antibodies with high titers is independent of the concentrations of antigenic glycolipids on the cell surface.

In our previous studies [28, 29], rabbit anti-Gg_4Cer antisera with titers of more than 1:50,000 permitted attenuation of the activity of endogenous natural killer cells in nude mice and an increase in the growth of human cancer cells in nude mice after transplantation. The levels of antibody titers for different carbohydrate structures seemed

to be largely dependent on the inheritable background. Consequently, as another reason, one can suggest that immune reactions toward Gg_4Cer, the unknown glycolipid and FGM1 precede that toward GM1 among the antigenic glycolipids, and no production of anti-GM1 antibodies is observed on immunization with RRBC.

The overall profile of glycolipids on individual cells must be important for immune recognition. The preferential production of anti-Forssman and anti-Gg_3Cer antibodies on immunization with SRBC and GRBC was probably due to the significantly higher concentrations of Forssman glycolipid (71%) and Gg_3Cer (92%) in the total glycolipids of SRBC and GRBC, respectively. Because of the masking effect of terminal sialic acids on biological recognition [30, 31], $IV^3NeuAc\alpha$-nLc_4Cer and GD1a, comprising 30% and 15% of the total glycolipids in HRBC and RRBC, respectively, might interfere with the immune recognition of antigenic glycolipids coexisting on these erythrocytes. Analyses of the relationship between immune recognition and glycolipid composition are now in progress with liposomes and sialidase-modified erythrocytes in our laboratory to elucidate the pathological events in several antibody-mediated diseases.

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