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CHARACTERIZATION OF ANTI-FORSSMAN (ANTI-Fs) ANTIBODIES IN HUMAN SERA: THEIR SPECIFICITY AND POSSIBLE CHANGES IN PATIENTS WITH CANCER[1]

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The majority of normal human sera hemolyze sheep erythrocytes in the presence of complement ("hemolyzers"), whereas sera of the remaining population do not lyze sheep erythrocytes ("nonhemolyzers"). The antibodies responsible for this lytic activity are now identified as being specific to Forssman (Fs) glycolipid, predominantly of the IgM class, and only weakly cross-reactive with blood group A glycolipid. These conclusions are based on the following observations: 1) "Hemolyzer" sera produced complement-dependent lysis of liposomes containing Fs glycolipid, whereas "nonhemolyzer" sera reacted very weakly with the same Fs liposomes; the level of hemolytic activity generally paralleled the reactivity of the sera with Fs liposomes. 2) Human "anti-Fs" did not cross-react with liposomes containing either globoside, the precursor to Fs, or blood group A glycolipid. 3) The complement-dependent hemolysis of sheep erythrocytes by hemolyzer sera was specifically inhibited by Fs liposomes but not by either A- or globoside liposomes. 4) In five of six cases tested, hemolysin activity was present in the IgM fraction but not the IgG fraction, whereas in the remaining case the activity was present in the IgG fraction.

We have previously described the presence of Fs glycolipid in the normal gastrointestinal mucosa of a minor percentage of Taiwanese cancer patients, whereas in the majority of cases Fs was absent in the normal mucosa but present in the tumors derived therefrom. The sera of cancer patients displayed a decreased anti-Fs reactivity as compared with sera of a control group suggesting that Fs-positive tumor tissue may affect serum anti-Fs levels.

Several early reports described the presence in the sera of a majority of normal individuals of antibodies that lysed sheep erythrocytes, whereas the remaining sera did not lyse sheep erythrocytes (1, 2, reviewed by Buchbinder, Ref. 3). Although these findings were published in 1928–1929, they received little attention until recently when a possible relationship between the serum hemolysin and Forssman (Fs)[2] antigen status of tissue was suggested (4, 5). The Fs antigen is a glycosphingolipid whose structure is shown in Table I.[3] Recently we reported that in the majority of Taiwanese patients studied, Fs glycolipid was present in the gastrointestinal tumor tissue but not detectable in the adjacent normal mucosa (Fs^- individuals); in the remaining cases (Fs^+ individuals) Fs was present in the normal mucosa but undetectable in the tumor tissue (13). Thus, the alloantigenic distribution of Fs antigen in tissue suggests that the anti-sheep hemolysin present in the majority of human sera could be an alloantibody specific for Fs alloantigen. Furthermore, Levine (4) recently studied the anti-sheep hemolysin titer of over 1000 sera including those of cancer patients and observed that the incidence of hemolyzers in patients with cancer was significantly lower than that of normal sera. A gradual decrease in the incidence of hemolyzers with advancing age was also reported. This decrease of sheep hemolysin titer was ascribed to the increasing level of anti-Fs-Fs immune complexes (5).

The anti-sheep hemolysin of human sera was absorbed by guinea pig kidney tissue but not by bovine erythrocytes (4); therefore, the activity was assumed to be due to anti-Fs antibodies. However, it has never been clearly demonstrated that these antibodies are specific for the Fs antigen and not for other unknown structures in tissues and membranes or for structures related to Fs such as the blood group A antigen or globoside, the precursor of Fs hapten (see Table I). In several special cases the specificity of such human antibodies has been studied. A human monoclonal IgM produced in one case of Waldenström macroglobulinemia reacted with both Forssman glycolipid and globoside (14). Also, an autoantibody produced in a Donath-Landsteiner paroxysmal hemoglobinuria patient reacted with P erythrocytes but not with erythrocytes of the rare phenotypes P_1^k, P_2^k, and p (15). Such "anti-P" antibodies were reported recently to react not only with globoside but also with Fs glycolipid (16). Also, the antibodies of a blood group p individual reacted by complement (C) fixation not only with globoside, the P antigen, and ceramide trihexoside, the P^k antigen, but

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[2] Abbreviations used in this paper: PBS, phosphate-buffered saline 140 mM NaCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM $NaPO_4$, pH 7.4; 4 Me UmP, 4-methyl umbelliferone phosphate; DMPC, dimyristoyl phosphatidylcholine. Fs, Forssman antigen; anti-Fs, Forssman antibody.

[3] The immunodeterminant of Forssman antigen, namely the terminal disaccharide GalNAcα1 → 3GalNAcβ1 → R, has been found in several other "carrier" structures, all of which are Forssman active: a ceramide tetrasaccharide of hamster fibroblasts, GalNAcα1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → Ceramide (9); a polysaccharide of Streptococcus type c (10); and a ceramide-heptasaccharide and -octasaccharide of dog gastric mucosa (11, 12).

TABLE I

Fs glycosphingolipid and closely related structures[a]

Globoside (6)	GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Ceramide
Forssman (7)	GalNAcα1 → 3GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1Ceramide
A^b glycolipid (8)	GalNAcα1 ↘ 3 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ1 → 1Ceramide 2 ↗ L-Fucα1

[a] Note that Fs and globoside share the same structure except for the nonreducing terminus; Fs and A^b glycolipid share the terminal GalNAcα1 → 3 structure. A^b glycolipid is one of the variants of A-active glycolipids present in human erythrocytes (8).

also with Fs (17, 18). In view of the apparent alloantigenic distribution of Fs glycolipid in human tissue and the possible change of antibody levels against this hapten, it was of considerable interest to determine if the anti-sheep erythrocyte hemolysin of human sera was really specific for Fs antigen.

MATERIALS AND METHODS

Glycolipids. The following glycolipids were isolated and purified as previously described (19): Forssman glycolipid from goat erythrocytes; globoside and A^b glycolipid (one of the A glycolipid variants) from human erythrocytes (structures shown in Table I).

Sera. Anti-Forssman and anti-globoside sera were prepared by injecting New Zealand White rabbits with mixtures of the purified glycolipid and bovine serum albumin emulsified in complete Freund's adjuvant as previously described (20). After ammonium sulfate precipitation of the globulin fraction, antibodies against the albumin were removed by passage through a bovine serum albumin-Sepharose column. Human anti-A blood grouping serum was obtained from Ortho Diagnostics, Raritan, N. J. Normal sera were obtained from healthy laboratory personnel. Sera from Taiwanese ulcer and cancer patients were obtained from Dr. S.-M. Wang, Department of Surgery, National Taiwan University Medical School, Taipei, Taiwan. All sera were heat inactivated at 56°C for 30 min.

Liposome lysis assay. Liposomes were tested for their sensitivity to antibody-C lysis by a slight modification of the method of Six *et al.* (21). Lipid mixtures were prepared consisting of 1 μmole dimyristoyl phosphatidylcholine (DMPC), 0.75 μmole cholesterol, 0.1 μmole dicetyl phosphate, and either 25 μg globoside, 20 μg Forssman, or 15 μg A^b glycolipid. DMPC was chosen as phospholipid because a previous study indicated that a human monoclonal IgM antibody displayed much greater reactivity with liposomes prepared with a saturated phosphatidylcholine than with sphingomyelin liposomes (22). Multicompartment liposomes were prepared from these lipid mixtures, and the final volume of the liposome suspension was adjusted to 500 μl. The lysis assay detects release of the fluorogenic substrate 4-methylumbelliferone phosphate from the liposomal aqueous compartments and its subsequent conversion by alkaline phosphatase to an intense fluorophore. Each assay tube contained 950 μl of buffer (20 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$), 5 μl of alkaline phosphatase (2.5 μg/ml), 10 μl of rabbit or human serum diluted as indicated in the *Results*, and 25 μl of guinea pig serum as the C source. The reaction was started by the addition of 10 μl of liposomes (2 mM DMPC).

Hemolysis of sheep erythrocytes. The hemolyzer status of human sera was determined by a tube assay as previously described (5). In addition, in a separate assay the hemolysin titer was quantitated by serial dilution of the test sera in microtiter plates, followed by addition of 25 μl each of guinea pig serum (diluted 1:30) and a 1% suspension of sheep erythrocytes.

For hemolysin inhibition studies, glycolipid liposome stock solutions were prepared by mixing glycolipid, egg lecithin, and cholesterol (mole ratio 1:50:50) in organic solvent, evaporating under N_2, dissolving in hot ethanol, and diluting in hot PBS to a final glycolipid concentration of 20 μg/ml. Twenty-five microliters of each liposome suspension were diluted in a 2-fold series in a microtiter plate and incubated with three hemolytic doses of the appropriate serum for 60 min at 37°C. Then the mixture was incubated with 25 μl each of C and 1% sheep erythrocytes. The extent of hemolysis was determined after 30 min at 37°C.

Separation of human IgM and IgG. One to two milliliter aliquots of human sera were chromatographed on a Sepharose CL 6B column (1.6 x 64 cm) in PBS, pH 7.4. Separation of IgM and IgG fractions was achieved as indicated by immunodiffusion tests by using rabbit anti-human IgM and goat anti-human IgG, which were gifts from Dr. K. Schwartz of this Center. The volumes of IgM and IgG pools were restored to that of the applied serum aliquot by using a collodion bag (Schleicher and Schuell, Keene, N. H.).

RESULTS

Specificity of C-dependent liposome lysis. Rabbit antiserum prepared against globoside reacted strongly with globoside liposomes, to a lesser extent with Fs liposomes, but not at all with liposomes containing blood group A glycolipid (A^b glycolipid; Table II). Such cross-reactivity of anti-globoside antibodies with Fs was previously observed by C fixation (20) and by liposome lysis (23).

In contrast, rabbit antibodies prepared against purified Fs glycolipid reacted strongly with Fs liposomes but did not react with liposomes containing globoside or with liposomes containing blood group A glycolipid. Although rabbit anti-Fs glycolipid antisera do not react with blood group A liposomes, human anti-A blood grouping serum showed significant cross-reaction with Fs liposomes but not with globoside liposomes (Table II).

Specificity of anti-sheep erythrocyte hemolysin in normal human sera. Several early studies reported that the majority of normal human sera possessed hemolytic activity against sheep erythrocytes, whereas a minority had weak or undetectable levels of such hemolysins (1–3). Table III indicates that the sera of such "nonhemolyzers" produced minimal C-dependent damage to Fs liposomes. In contrast, the strength of reaction

of hemolyzer sera with Fs liposomes paralleled the hemolysin titer. It should be noted that this correlation was found with DMPC liposomes but not with liposomes prepared with dipalmitoyl lecithin or sphingomyelin. Lysis of A^b liposomes by these sera was independent of both hemolysin titer and reactivity with Fs liposomes. Furthermore, neither rabbit nor human anti-Fs hemolyzed human erythrocytes of any ABO blood type (data not shown). Hence, this human "anti-Fs" appears like rabbit anti-Fs (Table II) to be specific for the Fs structure and does not cross-react significantly with A^b glycolipid. None of the human sera tested during this study reacted with globoside liposomes.

Immunoglobulin class of human anti-sheep hemolysin. IgM and IgG fractions of six hemolyzer sera were separated by Sepharose CL 6B chromatography. In five of these cases the hemolysin activity was localized in the IgM fraction (Table IV). In the remaining case, the IgG fraction contained the hemolysin activity. In a separate study, the hemolysin activity of mother and umbilical cord sera were compared. Among a group of 296 maternal sera, 87% had detectable hemolysin activity. However, of the 270 cord sera tested from this group only three contained detectable hemolytic activity. In two instances (case 25, mother and cord blood type A, and case 258, mother and cord blood type B) the hemolysin activity of the maternal sera was higher than that of the cord sera, whereas the cord serum of the remaining case (case 97, mother and cord both blood type O) was stronger than the maternal serum. The nature of the antibody class or subclass in these exceptional cases is being investigated. Thus, in almost all cases, human anti-Fs antibodies are IgM, which does not cross the placenta.

Inhibition by glycolipid-liposomes of sheep erythrocyte hemolysin. The sheep erythrocyte hemolysin activity of rabbit anti-Fs and human (Pat) sera, both of which showed strict specificity for Fs by liposome lysis (Tables II and III), was strongly inhibited by Fs liposomes but not by A^b- or globoside-liposomes (Table V). In contrast, inhibition of human anti-A typing serum was nearly as effective with Fs liposomes as with A^b liposomes. Thus, this anti-A serum had a much broader range of specificity than anti-Fs sera.

TABLE II

Extent of cross-reactivity of glycolipid liposomes[a]

Antiserum Added	% Trapped 4-Methylumbelliferone Phosphate Released after 30-min. Liposome lysis[b]		
	Antigen incorporated:		
	Globoside	Forssman	A^b-glycolipid
Rabbit anti-globoside	62.3	4.9	0.3
Rabbit anti-Fs	0.5	59.6	2.0
Human anti-A typing serum	0.1	10.4	61.8

[a] Liposomes were prepared as described in *Materials and Methods* and contained either 25 μg globoside, 20 μg Forssman, or 15 μg A^b-glycolipid per μmole phospholipid. 4-Methylumbelliferone phosphate release was determined in the presence of 10 μl of either a 1:10 dilution of anti-globoside, a 1:10 dilution of anti-Fs, or a 1:4 dilution of human anti-A typing serum.

[b] Numbers for liposome lysis indicate the percent of trapped 4-methylumbelliferone phosphate released in 30 min. In the presence of heated (56°, 30 min) instead of native guinea pig serum the percent of trapped marker released was less than 2% in all cases.

TABLE IV

Anti-sheep erythrocyte hemolysin in fractionated human sera[a]

Serum	Hemolysin Titer		
	Whole serum	IgM fraction	IgG fraction
Pat.	1:64	1:2	1:32
Mic.	1:128	1:64	NIL[b]
Sto.	1:256	1:128	1:4
Sny.	1:32	1:32	NIL
61	1:8	1:8	NIL
78	1:64	1:16	NIL
8	1:64	1:16	NIL

[a] Human sera were fractionated on Sepharose CL 6B as described in *Materials and Methods.* Numbers for hemolysin titer have the same meaning as in Table III.

[b] NIL, no hemolysis even with undiluted serum fraction.

TABLE III

Anti-sheep erythrocyte hemolysin, anti-Forssman, and anti-A activities of normal human sera

"Non-hemolyzers"			Liposome Lysis[a] Antigen Incorporated:		"Hemolyzers"				Liposome Lysis[a] Antigen Incorporated:	
Serum	ABO type	Forssman	A^b-glycolipid	Globoside	Serum	ABO type	Hemolysin titer[b]	Forssman	A^b-glycolipid	Globoside
Rec.	A	0.8	0.3	0.5	Suj.	A	1:8	13.3	1.0	0.4
Mon.	O	0.3	9.7	0.1	11	B	1:16	18.1	37.2	NT[d]
Har.	AB	1.5	0.3	0.3	Bot.	A	1:16	22.4	0.8	0.7
Loj.	AB	0.7	0.3	0.7	Con.	A	1:16	31.4	0.8	0.9
Pug.	O	0	1.3[c]	0.3	1	O	1:16	38.7	68.9	NT
Hak.	O	2.8	1.9	NT	Pat.	A	1:16	40.5	0	NT
You.	O	3.0	23.5	NT	Lil.	B	1:32	32.5	1.9	0.8
					19	O	1:32	37.4	54.9	NT
					8	O	1:64	42.4	43.6	NT
					Ole.	A	1:64	52.0	0.8	0.9
					9	O	1:64	57.5	4.2	NT
					Gec.	A	1:64	68.6	1.1	1.0
					Mic.	A	1:128	66.3	0.8	1.5
					Sto.	B	1:256	58.1	2.8	0.7
					Rob.	A	1:256	66.3	1.1	1.0

[a] Numbers for liposome lysis have the same meaning as in Table II; 10 μl undiluted human serum were tested in each assay.

[b] Numbers for hemolysin titer indicate the highest dilution of the respective human serum that caused complete lysis of sheep erythrocytes as described in *Materials and Methods.*

[c] The failure of many sera of type O or B individuals to lyze A^b-liposomes is consistent with previous data which indicated that less than half of the sera from normal individuals of type O or B hemolyzed type A erythrocytes (page 20 of Reference 24).

[d] NT, not tested.

Anti-Fs levels in the sera of Taiwanese patients with gastric ulcers or gastrointestinal cancer. The anti-Fs levels of sera from Taiwanese patients with gastrointestinal cancer were compared with sera from Taiwanese gastric ulcer patients as controls (Fig. 1). Significantly, a decreased anti-Fs reactivity was apparent in the cancer patients' sera as compared to the ulcer cases. Forty-two percent (8/19) of ulcer patients' sera were "high lyzers" (greater than 25% release of liposomal trapped marker) as compared with only 10% (2/19) of cancer patients' sera. Conversely, 47% (9/19) of the cancer patients' sera were defined as "nonlyzers" (less than 10% trapped marker release) whereas 26% of ulcer patients' sera were "nonlyzers". Since the aging process will effect the anti-Fs titer of sera (5) and a difference in the average age existed between the ulcer and cancer patient groups (ulcer 43 years *vs* cancer 53 yrs), an age-matched comparison was made. The results still showed a remarkable difference. The ratio of the incidence of the high, the medium, and the nonlyzer between 45 and 60 years in ulcer patients was 4/3/2, whereas in cancer patients it was 1/4/6, respectively. These results suggest that the presence of Fs-positive tumor tissue may decrease serum anti-Fs levels (see *Discussion*).

DISCUSSION

The results of this study clearly indicate that the antibodies in normal human sera causing C-dependent lysis of sheep erythrocytes are specific for Fs glycolipid structure, that is GalNAcα1 → 3GalNAcβ1 → 3Galα1 → R, based upon: 1) the specific lysis of Fs liposomes and 2) specific inhibition of sheep erythrocyte hemolysis by Fs glycolipid liposomes. A suspected cross-reaction of the anti-Fs antibodies in normal human sera with globoside and blood group A glycolipid was not observed, whereas a weak but definite cross-reaction was observed between rabbit anti-globoside and Fs glycolipid hapten. In both human and rabbit antisera, anti-globoside antibodies appear to cross-react well with the Fs structure (16), whereas anti-Fs antibodies do not cross-react with globoside. The occurrence of anti-Fs antibodies in sera is independent of blood group A, B, O; similarly the presence of Fs antigen in mucosal tissue was independent of the blood group ABH system (13).

TABLE V

Inhibition by glycolipid-liposomes of sheep erythrocyte hemolysis

Antiserum	Glycolipid Concentration[a]		
	Globoside	Forssman	A^b glycolipid
Rabbit anti-Fs	>20	0.31	>20
Human (Pat.) serum	>20	0.075	>20
Human anti-A typing serum	>20	0.31	2.5

[a] Numbers for hemolysis inhibition indicate the minimum concentration of glycolipid in μg/ml which produced complete inhibition of hemolysis.

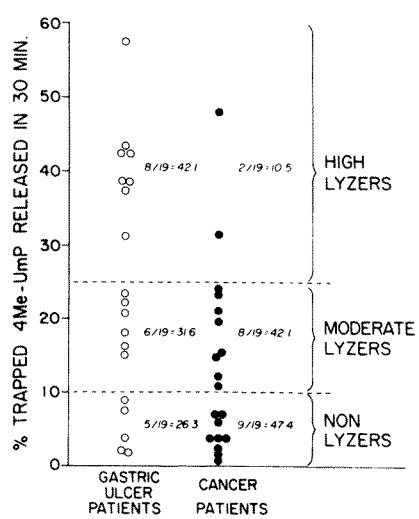

Figure 1. Anti-Fs levels in sera of Taiwanese patients with gastrointestinal cancer or gastric ulcer. Ten microliters of each human serum were tested for reactivity with Fs-liposomes as described in Table II and *Materials and Methods.* ○, ulcer patient serum; ●, cancer patient serum.

In five of six cases studied, the anti-sheep erythrocyte hemolysin activity was found primarily in the IgM fraction (Table IV). Thus, "naturally occurring" human anti-Fs antibodies belong to the IgM class as do antibodies against blood group antigens and other carbohydrate-containing antigens (24). If Fs antigen is indeed a human tumor-associated antigen (13), the outlook for specific immunotherapy may not be favorable due to the IgM nature of human anti-Fs. Although experimental models of passive immunization have produced conflicting results as to the most effective class or subclass of immunoglobulin for controlling tumor growth (25), it is clear that IgG can function not only in C-mediated lysis of cells but also in K cell, antibody-dependent cellular cytotoxicity. Furthermore, in the 1951 case reported by Levine *et al.* (26) a patient of genotype pp possessed IgG as well as IgM antibodies against the P_1 blood group antigens in her tumor tissue. It has been proposed that the high titer IgG of this case may have been responsible for the patient's 22-year survival after surgery without evidence of metastasis (4). It is possible in the Fs system, as in the case of ABO blood group antibodies, that intentional immunization with Fs antigen may produce "immune" anti-Fs of the IgG class that could have a greater effect on tumor growth than the "naturally occurring" IgM antibodies.

Early reports classified human sera as either possessing or lacking lysins for sheep erythrocytes (1-3). Similarly, we grouped normal sera as either hemolyzers or nonhemolyzers (Table III) and found significant anti-Fs activity only in the former category based on the liposome lysis assay, through which the specificity of the reaction has been unequivocally demonstrated. The origin of the anti-Fs antibodies is no doubt in response to exposure to the widely distributed Fs antigen (10). Inability to respond to this immunogen could be the result of at least two possibilities. First, the immune response against Fs antigen could be under genetic control, such that certain persons could not synthesize anti-Fs. This possibility is presently being investigated. Second, Fs antibodies might be produced in all persons but would be absorbed onto any tissues containing the Forssman antigen. Humans generally have been considered to lack Fs, primarily because it has never been detected in their erythrocytes. However, we have recently reported that Fs glycolipid was present in the gastrointestinal mucosa of a minority of Taiwanese cancer patients tested but was undetectable in the majority of cases (13).

Our results indicate that the levels of anti-Fs in Taiwanese cancer sera are generally lower than that of a control group, namely in sera of Taiwanese gastric ulcer patients (Fig. 1). The actual level of serum anti-Fs in cancer patients may be the result of a number of factors, including nonspecific effects such as debilitation and immunosuppression. In addition Fs-positive tumor tissue may absorb anti-Fs from the serum. Alternatively, Fs antigen may be shed from the tumor resulting in the formation of immune complexes (27); this possibility is the basis for the "self nonself" concept for cancer recently proposed by

Levine (5). In this regard it will be important to follow anti-Fs levels of cancer patients throughout the disease process to determine what effects anti-Fs has on tumor recurrence or metastasis after surgery.

It is also of prime importance to determine if a correlation exists between tissue Fs antigen status and the serum level of anti-Fs. However, due to the effects that Fs-positive tumor tissue may have on anti-Fs levels (described above), such a correlation may be more obvious if tissues and sera of nonmalignant patients are first investigated. Such studies are now in progress.

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