Isoantigenic expression of Forssman glycolipid in human gastric and colonic mucosa: Its possible identity with “A-like antigen” in human cancer

(Forssman-positive and Forssman-negative populations/tumor/globoside/blood group)

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ABSTRACT The heterogenous Forssman antigen is a glycolipid, a ceramide pentasaccharide with the structure GalNAc1→3GalNAcβ1→4Galα1→4Glc→ceramide. Forssman-positive animals are capable of synthesizing this compound in tissues or in erythrocytes, in contrast to the Forssman-negative species, including humans, which are incapable of adding the last carbohydrate in the sequence of the Forssman antigen, namely αGalNAc. The Forssman glycolipid and its precursor globoside were examined in twenty-one samples of surgically extirpated gastrointestinal mucosa and tumors derived therefrom. The results revealed that a few patients had chemically and immunologically detectable levels of the Forssman glycolipid as a normal component of their gastrointestinal mucosa (F⁺ population); in contrast, the majority of patients did not contain this glycolipid in their normal mucosa (F⁻ population). Whereas the F⁺ population included blood group A, B, and O, the F⁻ population did not correspond to a blood group A. The Forssman status in tumors taken from the F⁻ or F⁺ population showed the following striking features: (i) all tumors derived from F⁻ mucosa possessed Forssman glycolipid, whereas (ii) none of the tumors originating in F⁺ mucosa contained Forssman glycolipid. Globoside, the immediate precursor of Forssman antigen, was distributed equally among F⁺ and F⁻ mucosa and the tumors derived therefrom. Thus, the expression of Forssman antigen in gastrointestinal mucosa appears akin to that of an isoantigen. Furthermore, the Forssman antigen that appears in tumors of the F⁻ population could represent a human tumor-associated antigen. In view of the strong crossreactivity of Forssman antigen with blood group A determinants, the appearance of Forssman antigen in human tumors could be related to the “A-like antigen” (or “neo-A antigen”) of human tumors reported previously [Hakomori, S., Koscielak, J., Black, K. J., & Jeanloz, R. W. (1967) J. Immunol. 99, 31-38; Hakkinen, L. (1970) J. Natl. Cancer Inst. 44, 1183-1193].

The appearance of incompatible blood group antigens foreign to the host may occur in some human tumors. In 1951 Levine et al. (1, 2) reported a case which suggested the synthesis of P and P₁ antigen in gastric cancer tissue of a rare p individual. A possible conversion of blood group O or B to an A-like antigen in human gastrointestinal tumor was suggested by two independent studies: (a) rabbit antisemur directed against a “human tumor glycolipid” fraction agglutinated blood group A erythrocytes more strongly than O or B erythrocytes although the glycolipid was prepared from the tumor of a patient of blood group O (ref. 3; see also Discussion in the present paper); and (b) Hakkinen (4) described the presence of A-like antigen in stomach cancers from patients whose blood group status was group O or B. The active fraction was described as a “sulfoglycoprotein.”

In view of the extensive crossreaction between the Forssman

Abbreviations: TLC, thin-layer chromatography; CTT, ceramide tetrasaccharide; CF, ceramide pentasaccharide; F⁺ and F⁻, positive and negative for the presence of Forssman antigen.

and blood group A antigens (5, 6), the A-like activity observed in those studies could have been due to the presence of a Forssman antigen. In fact, Kawanami (7) described the presence of Forssman glycolipid in one case of gastric cancer although the glycolipid composition of normal mucosa was not examined. Consequently, a thorough study has been undertaken using the surgical samples collected by one of the authors (S.M.W.) in Taiwan, where there is a high incidence of gastric cancer.

The heterogenous Forssman antigen (8) is a glycolipid (9, 10) whose structure was identified as GalNac₁→3GalNAcβ₁→4Galα₁→4Glc→ceramide (11, 12). Forssman-positive animals, irrespective of species, are characterized by having a glycolipid of this structure (13-16), whereas Forssman-negative animals, including humans, are considered to be unable to complete the carbohydrate structure, although they can synthesize the immediate precursor of the Forssman antigen, which has been identified as globoside (11).

MATERIALS AND METHODS

Tissues. Normal mucosa and cancer tissues from gastric and colonic mucosa were obtained from surgically extirpated specimens obtained in the National Taiwan University Hospital and its associated hospitals. All cases were diagnosed by histological examination, and the ABO blood group of patients was determined by standard procedures. Immediately after the specimens were extirpated, the tissues were cleaned by rinsing with saline and frozen in dry ice and stored at −70°. The samples were shipped to this institute in dry ice. After thawing, the tumor tissues and normal mucosa were carefully separated and weighed.

Preparation of Glycolipid Fractions, Including Ceramide Tetra- and Pentasaccharide. The total glycolipid fraction was prepared from the weighed tissue by the acetylation procedure ("procedure A") (19), and was further separated into neutral glycolipids and gangliosides by DEAE-Sephadex A-25 according to the procedure of Yu and Ledeen (20). The total

* The immunodeterminant of Forssman antigen must reside on the terminal disaccharide GalNac₁→3GalNAcβ₁→R, because a ceramide tetrasaccharide with GalNac₁→3GalNAcβ₁→3Galα₁→4Galβ₁→ceramide of hamster fibroblast is equally active as the ceramide pentasaccharide (17), and a polysaccharide of Streptococcus type C containing the terminal disaccharide is Forssman-active (18).

† In the early literature, reviewed by Buchbinder in 1935 (6), most primates were Forssman-negative in erythrocytes and tissues. However, humans of blood groups A and AB were found by immunological means to be Forssman-reactive, but these results could have been due to crossreactions with blood group A structures.
neutral glycolipid fraction was further separated into various classes of glycolipids by a small column (15 x 0.8 cm) of porous silica gel (latribeads, Iatron Co., Tokyo) eluted with a chloroform/methanol/water system of increasing polarity (21). Ceramide tetrasaccharides were mainly eluted with chloroform/methanol/water 70:30:2.5 (vol/vol) (fraction 5) and the ceramide pentasaccharides, with chloroform/methanol/water 66:34:3.0 (fraction 6). Because these fractions slightly overlapped, aliquots of fraction 5 and 6 were combined for immunological assay, but chemical analysis was carried out on separate fractions.

Separation of Forssman Glycolipid and Its Identification. Because the Forssman glycolipid in the free state was not separable by thin-layer chromatography from the other ceramide pentasaccharides of fraction 6, the fraction was acetylated in pyridine/acetic anhydride. Excellent separation of the acetylated Forssman glycolipid was accomplished on TLC using solvent 1,2-dichloroethane/acetone (55:45, vol/vol). Under these conditions the other ceramide pentasaccharides ran much faster than Forssman glycolipid (see Fig. 1). Following this purification step, the Forssman glycolipid was deacetylated. An aliquot was degraded by \( \alpha\)-N-acetylglactosaminidase of pig liver (22) according to the procedure previously described (9). Another portion was methylated (23) and identified by electron impact mass fragmentography (16) using a Finnigan 3500 mass spectrometer.

Immunological Analysis. Preliminary analysis of the total glycolipid fraction failed to demonstrate any Forssman reactivity. For this reason the immunological assays described below were run on the purified ceramide tetra- and pentasaccharide fractions. Anti-Forssman and anti-globoside sera were prepared by injecting rabbits with mixtures of the purified glycolipid and bovine serum albumin emulsified in complete Freund’s adjuvant as previously described (24). Antibodies against the albumin were removed from the sera by passage through a bovine serum albumin-Sepharose column (25). The titer of anti-Forssman serum for hemolysis of sheep erythrocytes was about 1:2500, while the titer of antigloboside serum for hemagglutination of trypsinized human erythrocytes was 1:128 to 1:256.

The quantity of ceramide tetra- and pentasaccharide fractions to be tested was based on the dry weight of the tissue residue remaining after extraction of glycolipids with chloroform/methanol. The aliquots of fraction 5 and fraction 6 glycolipids corresponding to 50 mg of the dry tissue residue were mixed with 100 \( \mu\)l of 5 mM sphingomyelin, 10 \( \mu\)l of 50 mM cholesterol, and 15 \( \mu\)l of 3 mM dietylphosphate solutions in chloroform/methanol (2.1, vol/vol). Multi-compartment liposomes were prepared from these lipid mixtures and the final volume of the liposome suspension was adjusted to 250 \( \mu\)l. These liposomes were tested both for their sensitivity to antibody–complement lysis by a slight modification of the method of Six et al. (26), and for hemolysis inhibition. The liposome assay detects release of a fluorescent substrate (4-methylumbellif erone phosphate) from the liposomal aqueous compartments and its subsequent conversion by alkaline phosphatase to an intense fluorophore. Each assay tube contained 950 \( \mu\)l of buffer (20 mM Tris-HCl, pH 8.0/150 mM NaCl), 5 \( \mu\)l of alkaline phosphatase (2.5 mg/ml), 10 \( \mu\)l of a 1:10 dilution of either anti-Forssman or antigloboside serum, and 25 \( \mu\)l of guinea pig serum as the complement source. The reaction was started by the addition of 10 \( \mu\)l of liposomes (2 mM sphingomyelin). In control experiments the anti-Forssman serum was found to be specific for Forssman liposomes, causing the release of 53% of the trapped marker from Forssman liposomes but only 0.4% from globoside liposomes (both preparations containing 25 \( \mu\)g of glycolipid per \( \mu\)mol of sphingomyelin). In contrast, rabbit anti-sheep hemolysin reacts more extensively with globoside liposomes (27).

The results of hemolysis inhibition and liposome lysis assays of the ceramide tetra- and pentasaccharide fractions of 21 samples of normal mucosae indicated a clear-cut distinction of two human populations: one group showed positive Forssman activity while the other lacked Forssman activity (designated F*+ and F*−, respectively; Tables 1 and 2). Sixteen of the twenty-one patients were F*−; this group encompassed all ABO blood groups, while four of the five F*+ patients were from blood group O. Because samples of stomach as well as colonic mucosa were represented in both F*+ and F*− populations, no correlation could be made between the F*− activity and the location of the mucosa. All normal mucosa showed strong reactivity with antigloboside in the liposome lysis assay.

In agreement with the results of immunological reactivity, the consistent presence of Forssman glycolipid was chemically demonstrated in F*+ mucosa when the ceramide pentasaccharide fractions were acetylated, and separated on thin-layer chromatography (TLC) (Fig. 1). In striking contrast, none of the F*− population yielded a detectable Forssman glycolipid spot. It should be noted that in the free state Forssman glycolipid overlapped with the TLC spots of other ceramide pentasaccharides, thus preventing assessment of its presence. The remaining ceramide tetra- and pentasaccharide fractions of fractions 5 and 6 have not been characterized chemically, but preliminary results suggest the presence of globoside, para- globoside, galactosylparagloboside, and several other complex glycolipids containing fucose.

Forssman glycolipid antigen in tumors derived from F*− and F*+ mucosa

Determination of Forssman activity by both hemolysis inhibition and liposome lysis assays revealed that all tumors derived from F*− mucosa contained significant levels of Forssman reactivity (Table 1). In striking contrast, the ceramide tetra- to pentasaccharide fractions of tumors derived from F*+ mucosa did not express Forssman activity (Table 2). All tumor fractions from both F*+ and F*− cases possessed strong globoside reactivity.

The chemical analysis of acetylated glycolipid fractions on TLC agreed with the immunological data; all the tumors derived from F*− mucosa contained chemically detectable Forssman glycolipid, whereas the tumors derived from F*+ mucosa did not contain Forssman glycolipid. Some examples are shown in Fig. 1. Several other acetylated glycolipid bands were present in the ceramide tetra- and pentasaccharide fractions. When these materials were purified on TLC as...
null
expression in human tissue, but it could be possible that expression of Forssman is essentially of an "oncofetal" nature (30) in F- individuals; namely, its synthesis may be repressed during development and de-repressed in tumors. The repression of Forssman synthesis may not take place with development in some tissues, thus yielding F+ individuals. The "incomplete synthesis" model (31) can be applied to explain the deletion of Forssman glycolipids in tumors derived from F+ tissue. Forssman expression may well depend on the cell type even in F- individuals, because differences of ganglioside expression in lymphocyte subpopulations are now known (32).

Whatever the mechanism of Forssman expression might be, the presence of Forssman antigen in tumors of F- individuals is of great significance because it could be recognized as a tumor-associated antigen. The apparent conversion of O or B antigens to an A-like antigen in tumors of O or B individuals

<table>
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<tr>
<th>Patient identification no., sex, age</th>
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All tumors were histologically identified as adenocarcinoma; numbers for hemolysis inhibition and liposome lysis have the same meaning as in Table 1.

FIG. 1. Thin-layer chromatograms of the acetylated ceramide tetrasaccharide (CTT) and ceramide pentasaccharide (CP) fraction of normal mucosa and cancer tissues derived therefrom. Chromatograms were developed with 1,2-dichloroethane/acetone (55:45) on silica gel G and stained with orcinol. (Left) Examples of blood group A cases. Lanes 1, 2, 7, and 12 are reference acetylated glycolipids. 1, globoside; 2, Forssman glycolipid; 7 and 12, mixture of globoside and Forssman glycolipid. 3 to 6: case 045 (F- colon mucosa and tumor; see Table 1). 3, CTT of normal colon mucosa; 4, CP of normal colon mucosa; 5, CTT of colon cancer; 6, CP of colon cancer. 8 to 11: case 050 (F+ cardia mucosa and cardia tumor, Table 1). 8, CTT of normal cardia mucosa; 9, CP of normal cardia mucosa; 10, CTT of cardia cancer; 11, CP of cardia cancer. 13 to 16: case 061 (F+ gastric mucosa and cancer therefrom; see Table 2). 13, CTT of normal stomach mucosa; 14, CP of normal stomach mucosa; 15, CTT of gastric cancer; 16, CP of gastric cancer.

(Right) Examples of blood group O cases. 1 and 2: case 019 (F- stomach mucosa and cancer therefrom, see Table 1). 1, CP of normal colon mucosa; 2, CP of colon tumor. 8 and 9: case 075 (F+ colon mucosa and cancer therefrom, see Table 2). 8, CP of normal colon mucosa; 9, CP of colon tumor. Lanes 3 to 7 are reference acetylated glycolipid. 3, galactosylparagloboside; 4, Forssman glycolipid; 5, globoside; 6, paragloboside; 7, a mixture of Le-glycolipid and Le-glycolipid. Y indicates a yellow spot which is an unknown, nonglycolipid compound probably derived from the acetylation procedure. (This film is sensitive to yellow and records it markedly.)
as mentioned in the introduction could well be ascribable to the presence of Forssman antigen in tumors from O, F− or B, F− individuals. The crossreactivity of blood group A and Forssman antigen is well known (5, 6); Forssman glycolipid is capable of inhibiting A-hemagglutination, and A-antigen inhibits hemolysis of sheep erythrocytes by anti-Forssman antibody (our own experience). However, further extensive study is necessary to identify the A-like (or "neo-A") antigen, occurring in clinical cases as was described by Hakkinen (4). A possible relation of "A-like immunogenicity" displayed by "human tumor glycolipid" fraction to the Forssman antigen was explored before, and the tumor glycolipid fraction did not inhibit hemolysis of sheep erythrocytes by anti-sheep hemolysin (3). However, this previous finding is compatible with our present results, namely Forssman glycolipid is a relatively minor component in tumors of F− mucosa as compared to coexisting glycolipids and the Forssman activity must be masked by other glycolipids. Because the immunogenicity of Forssman for rabbit is stronger as compared to other glycolipids, "A-like immunogenicity" was demonstrated with the tumor glycolipid fraction. Forssman glycolipid might be immunogenic in blood group O or B individuals. Previously, Levine suggested that immunotherapy of human tumors having illegitimate ABO and P (Tα) blood group antigens could be possible by immunization of patients with such antigens (1, 2). Immunologic suppression of tumors in O or B individuals by immunization with Forssman glycolipid suitably arranged on a carrier macromolecule might be possible.

Note Added in Proof. In contrast to the findings of Levine et al. (1, 2), the studies described above on antigens (either the precursor, glycolipid (F−), or the final product, Forssman antigen (F+)) do not directly reflect the presence of corresponding human erythrocyte antigens. In an attempt to identify these two classes without resorting to biochemical procedures, P. Levine, M. Celano, W. W. Young, and S. Hakomori screened more than 100 normal sera (in a 1/2 dilution) for their capacity to hemolyze sheep erythrocytes in the presence of complement. Two classes were identified in both tests, i.e., lysers (about 70%) and nonlysers. Double-blind studies are being carried out to determine if the lysers and nonlysers correspond with the F− and F+ populations, respectively. If so, the way will be open to carry out familial and racial studies on the two antigens by the immunological procedures outlined. Buchbinder (6) refers to two studies in 1928 and 1929 in which the same two classes were found upon screening normal human sera for their various capacities to hemolyze sheep erythrocytes.

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