A Revised Structure for the Forssman Glycolipid Hapten*

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SUMMARY
A glycolipid hapten of the Forssman antigen of horse spleen was found to be a ceramide pentasaccharide, which contained 2 moles of N-acetylgalactosamine, 2 moles of galactose, and 1 mole of glucose per ceramide. An α-N-acetylgalactosaminidase of hog liver (Weissman, B., and Hinrichsen, D. F., Biochemistry, 8, 2034 (1969)) hydrolyzed the terminal α-N-acetylgalactosaminosyl residue with a simultaneous loss of the Forssman hapten activity. The resulting compound was identical with globoside (Yamakawa, T., Nishimura, S., and Kamimura, H., Jap. J. Exp. Med., 35, 201 (1965); Hakomori, S., Siddiqui, B. L., Y. T., Li, S. C., and Hellerqvist, C. G., J. Biol. Chem. 246, 2271 (1971)) and gave a specific precipitin reaction with anti-globoside antiserum. The oligosaccharide released from the Forssman hapten gave an intense Morgan-Elson reaction and 1 of the 2 moles of galactosamine was periodate resistant. With these results and those of methylation studies, the structure of the Forssman hapten was proposed as N-acetylgalactosaminosyl-α-(1 → 3)N-acetylgalactosaminosyl-β-(1 → 3)galactopyranosyl-α-(1 → 4)galactopyranosyl-β-(1 → 4)glucopyranosyl-(1 → 1)ceramide.

A hapten of the genetically defined heterophilic antigen, first described by Forssman (2), has been established to be glycosphingolipid (3–6). The chemical structure of the purified glycolipid hapten has been described as an anomer isomer of globoside with regard to the terminal galactosaminosyl residue, i.e. α-N-acetylgalactosaminosyl(1 → 3)galactosyl(1 → 4)galactosyl-(1 → 4)glucosylceramide (6). Recently, a significant change of the concentration and reactivity of Forssman antigen has been described with the changes of cell surface function, i.e. the appearance (7–8) or disappearance (9) of this antigen on the surface of transformed fibroblastic cells. In this communication, a revised chemical composition and structure of Forssman antigen prepared from horse spleen is described.

EXPERIMENTAL PROCEDURE
A glycolipid having both anti-sheep hemolysin activity and giving a precipitin with anti-Forssman glycolipid rabbit antiserum was isolated from horse spleen by the method described by Yamakawa, Irie, and Iwanaga (5), and further purified on thin layer chromatography. The “heterophile Forssman antiserum” showing hemolytic as well as precipitin reaction was purchased from Difco Laboratories, Detroit, Michigan. The anti-Forssman glycolipid rabbit antiserum used in the early stages of the experiment was donated by Dr. Maurice M. Rapport, New York Psychiatric Institute, New York. The antiserum was also prepared in our laboratory by injection of 1 mg of purified Forssman glycolipid, 5 mg of crystalline bovine serum albumin with the Freund’s adjuvant into rabbit according to the method described by Koscikaiak, Hakomori, and Jeanloz (9). The anti-globoside antiserum was prepared by the same method as that mentioned above (9). The reactivities of glycolipids with anti-Forssman antiserum and with antigloboside antiserum were tested by the double diffusion method using thin films of agarose on a microscope slide provided with plastic templates according to the method described by Sharpless and Lo Grippo (10). The glycolipid concentration tested was 100 μg/0.1 ml and 10 μl was placed in each well on the template. Globoside, ceramide trihexoside, and ceramide lactoside were prepared from human erythrocytes (11). α-N-Acetylgalactosaminidase (EC 3.2.1.40; α-2-acetamido-2-deoxy-o-galactose : acetamido-deoxygalactohydrolase) was prepared from hog liver according to the method described by Weissman and Hinrichsen (12). The enzyme preparation was freed of β-N-acetylhexosaminidase activity as tested with p-nitrophenyl β-N-acetylglucosaminidase, and globoside was not hydrolyzed. Further characterization of the globoside-like material produced by hydrolysis with α-N-acetylgalactosaminidase was carried out as described previously (13, 14). The composition of the carbohydrates was determined by gas chromatography after acetylation followed by hydrolysis (15). The method permitted complete hydrolysis of hexosaminidase linkage without destruction of neutral sugars, thus giving a correct ratio of individual neutral and amino sugars. The linkages of positions between neutral sugars were determined by

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† S. Kijimoto and S. Hakomori, unpublished results.
TABLE 1

Molar ratio of component sugars

The values were determined by gas chromatography as hexitol or galactosaminitol acetate after acetolysis followed by hydrolysis according to the method described by Yang and Hakomori (14). For the condition of periodate oxidation, see the text. The value for GalNAc was corrected by multiplying the factor, 1.2, on an empirical basis.

<table>
<thead>
<tr>
<th></th>
<th>Gal:Glu:GalNAc</th>
<th>Forssman hapten glycolipid</th>
<th>2.0:0.94:1.80</th>
<th>Globoside</th>
<th>2.0:0.93:0.80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forssman hapten glycolipid periodate oxidized</td>
<td>1.0:0.90:0.85</td>
<td>1.0:0.90:0.82</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Globoside periodate oxidized</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

* Periodate resistant under the given condition; a higher concentration of periodate was required for the oxidation of glycosyl residue adjacent to the lipid moiety.

methylation in dimethylformamide (16), followed by formolysis, hydrolysis, reduction, and acetylation (17). In a separate experiment methylation was carried out in dimethylsulfoxide (18). The partially methylated alditol acetates were determined by gas chromatography-mass spectrometry according to the method described (19). The oligosaccharide was liberated from the ceramide by the modified Wiegand's degradation (20). The linkage between two N-acetylgalactosamines was determined by combined results of two reactions: the indirect Ehrlich reaction of the liberated oligosaccharide, and the amino sugar analysis after periodate-oxidation. The method has been known to be useful for diagnosis of linkage of amino sugars (21) and has been used in this study rather than characterization of methylated amino sugars.

The indirect Ehrlich reaction was carried out by the Morgan-Elson (22) procedure adapted to the microscale (see Table II). Gal-β-(1 → 3)GlcNAc-β-(1 → 4)Gal-β-(1 → 4)Glc(lacto-N-tetraose) (23), Gal-β-(1 → 4)GlcNAc-β-(1 → 4)Gal-β-(1 → 4)Glc(lacto-N-neotetraose) (24), GalNAc-β-(1 → 3)Gal-α-(1 → 4)Glc-β-(1 → 4)Glu(globo-N-tetraose) (14, 20, 25), GlcNAc-β-(1 → 4)GluNAc(di-N-acetylchitobiose) (26) were run as references. Lacto-N-tetraose and lacto-N-neotetraose were donated by Drs. A. Kobata and V. Ginsburg of the National Institute of Arthritis and Metabolic Disease, and di-N-acetyl-

The cleavage of the fully or partially methylated galactosaminyl-galactosaminosyl linkage without destruction of methylated amino sugar was unexpectedly very difficult. This is possibly due to the presence of highly positive charge of di-N-methylamino group. Conventional methods, such as methanolysis, formolysis, or acetolysis, which have given a reasonable result for the partially methylated neutral sugars, were unsatisfactory for the methylated dihexosaminosyl group. Further basic study to find out a method applicable for studying methylation analysis of dihexosaminosyl group is being planned.

**FIG. 1.** Conversion of Forssman glycolipid hapten to globoside by hydrolysis with α-N-acetylgalactosaminidase of hog liver. Fifty micrograms (approximately 0.03 μmole) of Forssman glycolipid was dissolved in 100 μl of 0.05 M sodium citrate buffer pH 4.7 containing 100 μg of sodium taurocholate. Complete digestion was effected by slight heating and agitation in a Vortex mixer, followed by brief sonication in Balsonic cleaning bath (14). Diazyox enzyme solution (200 μl) containing 0.2 unit of the activity when determined with phenyl-α-N acetylgalactosaminidase was added. After 12 hours of incubation, shaken with 3 ml of chloroform-methanol (2:1, v/v), and centrifuged. The lower phase was placed on thin layer plate (Silica Gel H) and developed with chloroform-methanol-water 65:25:4; spots were indicated by 0.2% (w/v) orcinol in 2 M sulfuric acid. Lane 2: a purified Forssman hapten glycolipid; Lane 3: a Forssman hapten treated with α-N-acetylgalactosaminidase; Lane 1, 4: Reference a, globoside; Reference b, ceramide trihexoside; Reference c, lactosylceramide. The bands in Lane 3 below globoside (a) or above lactosylceramide (c) were nonorcinol-positive yellow bands due to contaminants present in this sample.
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FIG. 2. Disappearance of the reactivity with anti-Forssman antiserum and creation of the reactivity with anti-globoside antiserum by hydrolysis of Forssman glycolipid with an α-N-acetylgalactosaminidase. a (center), anti-Forssman antiserum (αF); b (center), anti-globoside antiserum (αG). F, Forssman glycolipid; FG, Forssman glycolipid hydrolyzed with α-N-acetylgalactosaminidase; G, globoside.

TABLE II

<table>
<thead>
<tr>
<th>Color yield (% to lacto-N-tetraose)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lacto-N-tetraose</td>
<td>100</td>
</tr>
<tr>
<td>Lacto-N-neotetraose</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Globo-N-tetraose</td>
<td>25</td>
</tr>
<tr>
<td>Reaction product of globoside, directly</td>
<td>21</td>
</tr>
<tr>
<td>Reaction product of Forssman glycolipid, directly</td>
<td>38</td>
</tr>
<tr>
<td>Di-N-acetyl-chitobiose</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION

The carbohydrate composition of the Forssman glycolipid was found to contain 2 moles of N-acetylgalactosamine, 2 moles of galactose, and 1 mole of glucose, in contrast to that of globoside which contained 1 less mole of galactosamine (Table I). The hydrolysis of the terminal N-acetylgalactosamine of Forssman hapten was catalyzed specifically by α-N-acetylgalactosaminidase of pig liver described by Weissmann and Hinrichsen (12), and the hapten was quantitatively converted, with a simultaneous loss of the Forssman activity, to a glycolipid which was indistinguishable from globoside on thin layer chromatography (Fig. 1) and showed an identical carbohydrate sequence and anomeric structure of globoside as described earlier (13, 14). A new immunological reactivity with anti-globoside antiserum was created, concomitant with the loss of Forssman activity (Fig. 2).

The permethylated Forssman hapten gave each approximately 1 mole of 2,4,6-tri-O-methylgalactitol, 2,3,6-tri-O-methylgalactitol, and 2,3,6-tri-O-methylglucitol which were identical with the partially methylated alditols yielded from permethylated globoside. Presence of di-O-methylhexitol was not demonstrated, thus excluding a possibility of any branched structure.

The results of carbohydrate analysis and methylation have indicated that Forssman glycolipid is a ceramide pentasaccharide containing an identical internal carbohydrate structure to globoside, but have indicated further that it should have an additional mole of galactosamine. Loss of Forssman activity by α-N-acetylgalactosaminidase and a simultaneous creation of a new reactivity with anti-globoside antiserum have indicated that α-N-acetylgalactosaminosyl residue is linked to the terminal β-N-acetylgalactosaminsyl residue of globoside. These results, together with the results of recent study on anomeric structure of globoside (14), Forssman glycolipid should have di-(N-acetyl)
galactosaminosyl residue attached to α-galactosyl-(1 → 4)-β-galactosyl-(1 → 4)glucosyleramide. The oligosaccharide separated from the Forssman hapten gave an intense Morgan-Elson reaction (80% higher color yield than that of globo-N-tetraose) (Table II) and one galactosamine was periodate resistant (Table I). Those results have suggested that the terminal and the penultimate galactosamine could be linked through the 1 → 3 rather than the 1 → 4 or the 1 → 6 linkage (see Footnote 2). Thus, the structure of the Forssman hapten is proposed as α-N-acetylgalactosaminosyl-(1 → 3)N-acetylgalactosaminosyl-β-(1 → 3)galactopyranosyl-α-(1 → 4)galactopyranosyl-β-(1 → 4)glucopyranosyl-(1 → 1)ceramide (Fig. 3).

The presence in dog intestine of two ceramide pentasaccharides, each containing 2 moles of galactosamine was reported by Vance, Shook, and McKibbin (27). The sequence of the carbohydrate composition in one glycolipid when studied by degradation with acid hydrolysis was described as GalNAc→GalNAc→Gal→Gal→Glu-ceramide. Those results have suggested that the terminal and the penultimate galactosamine residue attached to ar-galactosyl-(1 + 4)-β-glucopyranosylL(1 + 1)ceramide (Fig. 3).

The immunological specificity of Forssman activity could be determined by the terminal α-N-acetylgalactosaminosyl-(1 → 3) N-acetylgalactosaminosyl residue, rather than α-N-acetylgalactosaminosyl residue. Free rotation or steric flexibility of the α-N-acetylgalactosaminosyl-(1 → 3) linkage could be greatly hampered by the presence of a bulky 2-acetamido group at the carbon 2 position of the penultimate galactosamine, thus the steric stability of the terminal α-N-acetylgalactosaminosyl determinant could be greater than any other possible structure. The structural rigidity of this determinant group is similar to that of blood group A or B determinant, in which the flexibility of α-N-acetylgalactosaminosyl residue is considered to be prevented by the presence of fucoseyl residue at the carbon 2 position of the penultimate galactose residue (28). It is interesting to note that, of a number of genetically defined carbohydrate groups, only those which have a nonreducing terminal with greater steric rigidity appear to be strongly antigenic and thus have greater immunogenetical significance.

The structure proposed for Forssman hapten in this paper encourages the idea that globoside may be a direct biosynthetic precursor of Forssman antigen.
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