NOTE Immunology

Binding of Vibrio anguillarum to Neutral Glycosphingolipids from Intestinal Mucosa of Rainbow Trout (Oncorhynchus mykiss)

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(Received 11 June 2003/Accepted 17 September 2003)

ABSTRACT. To test whether glycosphingolipids (GSLs) on the intestinal mucosa of rainbow trout (Oncorhynchus mykiss) serve as a binding receptor for Vibrio anguillarum, we analyzed neutral GSLs from rainbow trout intestinal mucosa and investigated the binding of bacteria to neutral GSLs. Two kinds of neutral GSLs, designated N-1 and N-2, were identified on high-performance thin-layer chromatography (TLC) plates. In TLC immunostaining tests, V. anguillarum bound only to galactosylceramide (GalCer), lactosylceramide and N-1 having the same TLC mobility as GalCer, but neither to glucosylceramide nor to N-2. These results suggest that N-1 is GalCer (Galβ1–1Ceramide), and also that N-1 (GalCer) on rainbow trout intestinal mucosa act as a receptor for V. anguillarum.

KEY WORDS: glycosphingolipid, rainbow trout, Vibrio anguillarum.

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Vibrio species have been considered responsible for many disease outbreaks in cultured fish. In particular, Vibrio anguillarum, which causes vibriosis in marine and freshwater fish, is a highly pathogenic bacterium and has become a severe problem for fish farming industry [1, 16]. Adherence of bacteria to the mucosal surfaces is crucial to the initial phase of infection. In infectious diseases, many pathogens and bacterial toxins recognize the carbohydrate structure on the surface of cells as a receptor for binding [11]. Thus, an understanding of the membrane biology of the intestinal mucosa of fish may be useful for the prevention and cure of fish vibriosis.

Glycosphingolipids (GSLs) are ubiquitous components of the plasma membrane of cells. They are well known to be relevant to various membrane-related biological functions, such as the structural rigidity of membrane, cell surface receptor for various substances, cell surface markers, and antigens [6]. On the cells, in addition, they are important in receptor functions with respect to bacteria, bacterial toxins, and viruses [11]. Until now, Balebona et al. have suggested that adhesion of Vibrio strains to mucus and fish cells is not only mediated by hydrophobic interaction, but also by specific receptor interaction [2]. It has also been demonstrated that intestinal tract is a site of adhesion [7, 13], colonization, and proliferation [14] of V. anguillarum. In addition, oral or rectal administration of V. anguillarum to fish results in a systemic infection [5, 13] in which V. anguillarum is transported across the intestinal epithelium by endocytosis [5]. Thus, it is likely that the GSLs expressed on the mucosal epithelial cell surface of fish intestine acts as a binding receptor for V. anguillarum infection. However, few studies on the GSLs in the intestinal mucosa of fish have been reported [3, 9], but research on interaction between GSLs and adherence of V. anguillarum has not yet been presented. In the present investigation, we show that V. anguillarum can utilize glucosylceramide (GlcCer), as a binding receptor on the epithelial cell surface of the fish intestine.

Lactosylceramide (LacCer), galactosylceramide (GalCer) and GlcCer were commercially obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A.). Structures of these neutral GSLs are shown in Table 1.

Rainbow trout (Oncorhynchus mykiss) were commercially obtained form a rainbow trout nursery (Chihayaakasaka, Osaka, Japan). The fishes (200–300 g) were anesthetized by tricaine (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) and intestinal tract was collected. The intestine from the fish was washed with phosphate-buffered saline (PBS, pH 7.2). The mucosa was gently scraped off and lyophilized.

The neutral GSL preparation was performed according to the method of Watarai et al. [18] with slight modifications. Briefly, lipids were extracted sequentially from the lyophilized mucosal scrapings with 10 volumes each of chloroform–methanol (2:1, 1:1, and 1:2, v/v) and chloroform–methanol–water (30:60:8, v/v/v) at room temperature overnight. The lipid extracts were combined and evaporated to dryness in vacuo, and then the residue was dissolved in chloroform–methanol (1:1, v/v). The redissolved extracts were subjected to mild alkaline hydrolysis, followed by acetylation and purification on a Florisil column. The GSL fraction was dissolved in a minimum amount of a mixture of

Table 1. Structures of neutral glycosphingolipids

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical structure</th>
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<tbody>
<tr>
<td>Glucosylceramide</td>
<td>Glcβ1–1Ceramide</td>
</tr>
<tr>
<td>Galactosylceramide</td>
<td>Galβ1–1Ceramide</td>
</tr>
<tr>
<td>Lactosylceramide</td>
<td>Galβ1–4Glcβ1–1Ceramide</td>
</tr>
</tbody>
</table>

a) Gal, D-galactose; Glc, D-glucose.
chloroform–methanol-water (30:60:8, v/v/v) and applied to a DEAE-Sephadex A-25 column (acetate form) by the method of Ledeen et al. [12]. The neutral GSLs were eluted with chloroform-methanol-water (30:60:8, v/v/v).

V. anguillarum strain PT-24 was gifted from Kitasato Institute (Tokyo, Japan). The bacteria were cultivated in heart infusion broth (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 1% (w/v) NaCl for 24 hr at 20°C with continuous shaking. Formaldehyde solution was then added up to a concentration of 1%. The suspension was gently shaken overnight to deactivate the bacteria. The formalin was removed by centrifuging the cells three times with PBS. The bacterial pellet obtained after centrifugation was suspended in PBS and used for immunization and thinlayer chromatography (TLC) immunostaining test.

Anti-V. anguillarum antibody was induced in female New Zealand White rabbits by subcutaneous immunization. Rabbits were injected with 100 µg of V. anguillarum preparation with Freund’s complete adjuvant. At 2 weeks, each rabbit was injected subcutaneously with 100 µg of V. anguillarum preparation with Freund’s incomplete adjuvant. At 1 week after the final immunization, each rabbit was bled out and serum was collected. Immunoglobulin G (IgG) of immune serum was fractionated from antiserum by precipitation with 50% ammonium sulfate, followed by affinity chromatography on Affi-Gel Protein-A (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

The immunostaining of neutral GSLs on a TLC plate was performed according to the method of Watarai et al. [17] with slight modifications. Briefly, neutral GSLs were spotted on a high-performance TLC (HPTLC) plate (Silica Gel 60 F-254, Merck, Darmstadt, Germany), and developed with chloroform-methanol-water (65:35:8, v/v/v). The dried plate was soaked for 1 min in a 0.02% solution of polyisobutylmethacrylate dissolved in hexane, allowed to air-dry, and then blocked by incubation in PBS containing 1% bovine serum albumin and 0.02% NaN3 at 37°C for 30 min. It was then rinsed 5 times with PBS containing 0.1% Tween 20 (washing buffer) and incubated with V. anguillarum solution (1 x 10^12 cells/ml) at 4°C overnight. After that, the plate was washed 5 times with washing buffer. After washing, the plate was incubated with the 1:100 dilution of rabbit anti-V. anguillarum antibody at 37°C for 90 min. The plate was washed 5 times with washing buffer and reincubated with the 1:1,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) at 37°C for 90 min. As the final step, it was washed 5 times with washing buffer and incubated with peroxidase substrate solution, which consisted of 2 ml of 4-chloro-1-naphthol (Sigma) (0.3% in methanol), 5 volumes of 100 mM Tris-HCl buffer (pH 7.4) containing 200 mM NaCl and 0.01% of H2O2 for 10 min at room temperature. The reaction was stopped by washing with distilled water.

Figure 1 shows an HPTLC profile of the neutral GSLs obtained from rainbow trout intestinal mucosa. Two kinds of neutral GSLs were found in the sample analyzed. They are designated as N-1 and N-2 from top to bottom on HPTLC. N-1 GSL, which was the predominant component in rainbow trout intestinal mucosa, and migrated similar to GalCer on HPTLC. N-2 GSL migrated slower than LacCer.

In order to define whether V. anguillarum recognizes rainbow trout intestinal neutral GSLs, we developed the TLC immunostaining test using neutral GSLs from rainbow trout intestinal mucosa. As shown in Fig. 2, V. anguillarum bound to LacCer, GalCer and N-1, but not to GlcCer and N-2. This result suggests that N-1 GSL on the epithelial surface of rainbow trout intestinal tract would act as a receptor for the binding of V. anguillarum to intestine.

In the present study, two kinds of neutral GSLs, N-1 and N-2, were detected in rainbow trout intestinal mucosa (Fig. 1). Binding of bacteria to GSLs separated by TLC appears to be a convenient way of detection and characterization of GSL receptor. In this study, only N-1 with an Rf value similar to that of GalCer standard was reactive with V. anguillarum on the HPTLC plate. N-2 was not recognized by V. anguillarum (Fig. 2). These findings suggest that N-1 is GalCer (Galβ1–1Cer), and also that N-1 (GalCer) on rainbow trout intestinal mucosa possesses receptor activity for V. anguillarum. In fact, GalCer standard showed reactivity...
to *V. anguillarum* on HPTLC plate (Fig. 2). In TLC immunostaining test, in addition, LacCer (Galβ1–4Glcβ1–1Cer) and GalCer (Galβ1–1Cer) standards, which contain Galβ1-terminal structure, are recognized by *V. anguillarum* (Fig. 2). However, *V. anguillarum* did not bind to GlcCer (Glcβ1–1Cer). These findings suggest that the epitope recognized by *V. anguillarum* may be the terminal Galβ1-structure in GSLs.

It has been reported that adhesion of *Vibrio* strains to specific receptors on fish mucosal cells is an important initial step in infection [2]. In this study, it was shown that GalCer (Galβ1–1Cer) (N-1) from rainbow trout intestinal mucosa would be a binding receptor for *V. anguillarum*. It is well known that adhesion of bacteria to the mucosal surfaces is mediated by fimbriae [4, 8, 10, 15]. Thus, it is likely that fimbriae play a role in the binding of *V. anguillarum* to N-1 (GalCer) on the rainbow trout intestinal mucosa. However, further studies are necessary to clarify whether fimbriae contribute to adherence of *V. anguillarum* to N-1 GSL.

In conclusion, this is a first description of the presence of binding site for *V. anguillarum*, such as N-1 (GalCer), in the rainbow trout intestine. Further studies are in progress to examine the *in vivo* role of the N-1 (GalCer) as binding site for *V. anguillarum*, and to verify whether the affinity and localization of *V. anguillarum* to the rainbow trout intestine are related to the expression of the N-1 (GalCer). Furthermore, the results of the present study would be useful for understanding of the mechanisms of infection of *V. anguillarum* and prevention and treatment of vibriosis.

This work is partly supported by a Grant-in Aid from the Japanese Society for the Promotion of Science (NO. 13660325), and by a Research Fund from Scientific Feed Laboratory Co., Ltd.

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