Microtiter Assay for Detecting Campylobacter spp. and Helicobacter pylori with Surface Gangliosides Which Bind Cholera Toxin

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Campylobacter jejuni with Gm1 ganglioside in the core of its lipopolysaccharide has been associated with Guillain-Barré syndrome. Since this epitope may be of considerable pathophysiologic importance and since this ganglioside binds cholera toxin, a rapid screening assay to detect bacteria that bind cholera toxin as an indication of Gm1 on their surfaces was developed. In the assay, bacterial lawns were grown on agar plates, harvested with phosphate-buffered saline, boiled, and incubated with a standard concentration of cholera B subunit. Preparations from strains with Gm1 were observed to inhibit the binding of cholera B subunit to Gm1 in a microtiter enzyme-linked immunosorbent assay. By using this assay with two groups of strains, 37 positive strains were detected among the 197 tested. Species with positive isolates included C. jejuni, Campylobacter coli, and Helicobacter pylori. The assay is capable of testing large numbers of isolates and should prove useful in future clinical and epidemiological studies of bacteria with this epitope.

Infections with Campylobacter jejuni are sometimes followed by the development of Guillain-Barré syndrome (GBS) (15). Although the exact mechanism of this development is not known, some have suggested that Gm1 ganglioside in the core of the lipopolysaccharide of certain strains of C. jejuni may stimulate an immune response to this epitope in patients with this infection (6, 7, 9, 10, 15, 19–23, 25). This immune response in susceptible patients is thought to then lead to an autoimmune peripheral neuropathy because the Gm1 in the bacteria is identical to that in nerve cells.

It is clear that certain strains of Campylobacter have Gm1 epitopes (1, 2, 22) as well as epitopes of other gangliosides (26); however, methods for detecting these ganglioside-bearing strains are tedious and have not allowed the screening of large numbers of strains. Gm1 ganglioside is the natural receptor for cholera toxin (CT) in mammalian cells (8), and microtiter enzyme-linked immunosorbent assay (ELISA) methods have been used for detecting and quantitating CT, as well as heat-labile toxin of Escherichia coli (3, 16). In the CT assay, Gm1 ganglioside is used to coat microtiter plates and test materials are applied to the plate in a standard ELISA format. Detection of the toxin is done with specific anti-CT antibodies. Based on an adaptation of this Gm1 ELISA for CT, an inhibitory ELISA was developed to detect strains of C. jejuni which bind CT to their surfaces and are thereby able to block the binding of CT to the Gm1 bound to the microtiter plate.

MATERIALS AND METHODS

Gm1 ELISA for CT. The ELISA for detecting CT has been described previously (16). Briefly, the test is performed by first coating microtiter plates with Gm1 ganglioside (1 μg/ml in phosphate-buffered saline [PBS]) (Sigma) and then blocking with 0.1% bovine serum albumin (BSA)–PBS. Cholera B subunit (Sigma) is then added (0.2 μg/ml, diluted in 0.1% BSA–PBS), followed by the additions of monoclonal anti-B-subunit antibody (donated by Ann-Mari Svenson) and horseradish peroxidase-conjugated anti-mouse antibody (Jackson Laboratory), and color development with o-phenylenediamine (Sigma) with appropriate washing between each reagent addition in the assay. After 15 min, the plate is read in an ELISA reader at an optical density (OD) of 450 nm.

Preparation of test bacteria. For the detection of Gm1 epitopes on bacterial cell walls, the test bacteria are first grown as a lawn on either chocolate agar or Mueller-Hinton agar for 48 h in a gas pack jar with Campy-BAP gas packs (BBL) at 37 or 42°C. The bacteria are harvested with 5 ml of PBS (pH 7.2, 0.01 M), transferred to a test tube, and placed in a boiling water bath for 15 min to kill the bacteria. After boiling, the suspension can be stored for weeks since lipopolysaccharide is extremely stable.

Inhibitory ELISA. Fivefold dilutions of the bacterial suspension are placed in wells of a second microtiter plate (100 μl/well). Equal volumes of cholera B subunit (0.2 μg/ml, diluted with 0.1% BSA–PBS) are added to each well and incubated for 1 h. The OD at 490 nm of the bacterial B-subunit suspension is determined to confirm that the bacterial growth is adequate for a valid test. (If the OD is less than 0.1 in the undiluted well, the results are not accepted.) After incubation, the mixture is transferred to the Gm1 ganglioside plate and the assay for CT is completed as described above. Controls include a medium-only (negative) control which does not inhibit development of color in the ELISA and a positive control which contains a strain known to produce Gm1. Strain 93-13 was included in each assay as a positive control.

Negative strains do not inhibit the development of color relative to the negative control. Positive strains inhibit the development of color and are defined as strains which inhibit the OD by >50% relative to the negative control. To provide a semiquantitative estimate of the amount of CT-binding activity in the bacteria, a titer of the CT-binding activity was determined, and the titer is expressed as the OD of the undiluted bacterial preparation multiplied by the inverse of the highest dilution which still inhibits the ELISA color development by at least 50%.

Bacterial strains tested. The test was developed with C. jejuni 93-13, a GBS-associated strain which produces Gm1 ganglioside. The strain was isolated in northern China and was kindly donated by Irving Nachamkin. Test isolates included 24 strains of C. jejuni from the Naval Medical Research Institute and 175 strains (including several species) isolated in South Africa. Among the strains provided by the U.S. Navy were 21 stool isolates from American soldiers and marines who developed diarrhea during a 1-month joint military exercise in Thailand. The three others from the Navy included one previously used in a Campylobacter volunteer challenge study (4), the O:19 type strain (14), and a strain (serotype O:10) isolated from a patient with Miller-Fisher syndrome (a variant of GBS), which was found to produce GD3 (17). The South African strains included isolates of C. jejuni previously reported to be associated with GBS (5), isolates of C. jejuni, Campylobacter coli, Campylobacter hyointestinalis, Campylobacter jejuni subsp.
RESULTS

As previously reported, when cholera B subunit is tested in the Gm1 assay, color development occurs within 15 min after addition of the substrate (16). When the B subunit is first incubated with the positive control strain, however, color development is inhibited. The inhibition is dependent on the number of bacteria incubated with the B subunit as indicated by waning inhibition with serial dilutions of the bacteria. The titration results in Fig. 1 show that the control strain could be diluted to >1:625 before losing its inhibitory capabilities.

The assay clearly divided the strains into those which did not inhibit the assay (i.e., OD was >80% of the control) and those which inhibited it greatly (i.e., OD in the undiluted well was <10% of the control; 90% inhibition). Serial dilutions of these strains demonstrated that most could be diluted considerably; the median titer was 400. The highest titer was 50,000, which was found in a strain of C. coli. A description of the positive strains is shown in Table 1.

Two strains from the U.S. Navy from soldiers with diarrhea were positive, but neither was associated with GBS or Miller-Fisher syndrome. The strain with GD3 (17) was negative in our assay for CT binding.

Thirty-five of 173 strains from South Africa were positive. Among the positive strains from South Africa, several were noteworthy. Two isolates of C. jejuni were serotype O:41 and were previously isolated from patients identified from a cluster of GBS cases (5), and these were the only strains from GBS patients. PCR restriction fragment length polymorphism analysis of these two strains indicated that they represented a single clone (11). Other positive isolates included a strain of C. jejuni isolated from a fatal case of dysentery in an ostrich, a C. coli strain, and nine isolates of H. pylori, thus expanding the known species range of the CT-binding epitopes. Among the biotype 2 C. jejuni isolates, about half were positive, but no positive isolates were identified among the Campylobacter species other than C. jejuni and C. coli. None of the E. coli strains was positive. Quantitatively, strains of C. jejuni appeared to have more CT-binding ability than did H. pylori since the mean titers were 400, 425, and 29 for C. jejuni biotype 1, C. jejuni biotype 2, and H. pylori, respectively.

DISCUSSION

Because of the association between strains of Campylobacter and GBS, interest has developed regarding the finding that certain strains from this genus have ganglioside on their surfaces. It is theorized that an immune response to the ganglioside might lead to an autoimmune neuropathy because of the molecular identity between the epitope on the bacterial cells and the same epitope on nerve cells. The assay described here was thus developed to provide a rapid screening test to detect strains having such CT binding as an indication that they likely have ganglioside on their surfaces. The efficiency of the test is illustrated by the fact that the first lot of South African strains (n = 79) was tested during a 2-day period, suggesting that the assay can be adapted for large scale epidemiological studies in which a large number of isolates must be screened.

Several points about the assay should be highlighted. First, the organisms are grown on standard agar and are harvested with PBS. They are then boiled to eliminate the biohazard of these potentially virulent strains. Gm1 ganglioside remains stable through boiling and storage at room temperature; thus, it is possible to keep boiled specimens for long periods or to ship them through the mail, since after boiling, they are not a biohazard and their activity will not be lost. Second, the assay was highly reproducible, giving consistent results even with bacterial preparations prepared several months earlier. Third, while the assay was developed for Campylobacter, it was also used with other species, and strains of H. pylori giving positive results were also found. Thus, it should be possible to search for CT binding in other species of bacteria or families of microorganisms. The assay used cholera B subunit for convenience since the B subunit and the holotoxin utilize the same Gm1-binding site. A comparison of B subunit and holotoxin in the assay was not carried out, but there is no reason to expect that the results would differ.

CT binding was found in many but not most strains of Campylobacter, and there was a clear separation between those which did and did not inhibit the binding. Positive strains continued to inhibit the binding even when highly diluted. While the strains tested do not represent a random sample of these species, the fact that this epitope is relatively common among Campylobacter strains while GBS occurs rarely suggests that other factors (e.g., genetic predisposition) are also important in the development of GBS. Alternatively, the occurrence of

![Graph showing optical density (OD) vs. dilution of bacteria](http://jcm.asm.org/)
CT binding may, like Penner serotype, be a marker for strains with GBS potential.

Some limitations of the assay should also be noted. The assay described here detects CT binding but does not detect Gm1. Gm1 is the natural receptor for CT and binds it avidly, but it may not be the only ganglioside which binds CT. Thus, if strains have Gm1 on their surfaces, they would be expected to be positive in this assay; however, other gangliosides from Campylobacter have been reported (26) but the CT-binding properties of these other gangliosides are not established. One strain tested in this study has been found to have GD3 (17), and this strain was negative in the CT-binding assay. For practical purposes, however, it seems that Gm1 is the epitope most likely to lead to CT binding in these strains.

Although other bacterial and viral antigens have been associated with GBS, C. jejuni is the only bacterium previously reported to have Gm1. Specifically, Gm1 had not been associated with GBS, the epitope most likely to lead to CT binding in these strains. This strain was negative in the CT-binding assay. For practical purposes, however, it seems that Gm1 is the epitope most likely to lead to CT binding in these strains.

The relatively common occurrence of CT-binding epitopes on the surfaces of these bacteria suggests that the epitope might confer survival advantages. Two such advantages can be hypothesized. First, strains with Gm1 epitopes may be protected somewhat from immune responses of the host, since during most infections, this epitope might be perceived as a “self” antigen and would be less likely to stimulate a vigorous immune response. A second possibility relates to a possible physiological symbiosis between strains bearing the CT-binding epitope and other organisms (e.g., enterotoxigenic E. coli) producing the toxin. Since Campylobacter spp. are normally residents of animals, including birds and mammals, the environments of these host species may provide a potential survival advantage for Gm1-binding strains.

Clinical reports from China and Japan have suggested that infections with C. jejuni with serotype O:19 (Penner) are more frequently reported among GBS patients than are other serotypes (24). However, cases of GBS associated with other serotypes have also occurred, and a cluster of GBS cases associated with Penner O:41 was described in South Africa (5). The association between serotype and GBS suggests that serotype may be a marker for GBS-related strains, but the O-side chain responsible for the serotype-specific antigen per se is unlikely to be the true virulence mechanism if the virulent property is more directly linked to the presence of gangliosides which are found in the core. The core oligosaccharide structures are not constant within the species nor are they conserved even within the same serotype, suggesting that a direct association of this structure, rather than of the serotype, may be more appropriate for identifying high-risk strains.

In summary, this inhibitory CT ELISA should facilitate studies requiring testing of large numbers of bacterial strains. Through such studies, the species specificity of the CT-binding epitopes and their association with GBS can be studied more readily.

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