Differential Activities of Plant Polyphenols on the Binding and Internalization of Cholera Toxin in Vero Cells*

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Plant polyphenols, RG-tannin, and applephenon had been reported to inhibit cholera toxin (CT) ADP-ribosyltransferase activity and CT-induced fluid accumulation in mouse ileal loops. A high molecular weight fraction of hop bract extract (HBT) also inhibited CT ADP-ribosyltransferase activity. We report here the effect of those polyphenols on the binding and entry of CT into Vero cells. Binding of CT to Vero cells or to ganglioside GM1, a CT receptor, was inhibited in a concentration-dependent manner by HBT and applephenon but not RG-tannin. These observations were confirmed by fluorescence microscopy using Cy3-labeled CT. Following toxin binding to cells, applephenon, HBT, and RG-tannin suppressed its internalization. HBT or applephenon precipitated CT, CTA, and CTB from solution, creating aggregates larger than 250 kDa. In contrast, RG-tannin precipitated CT poorly; it formed complexes with CT, CTA, or CTB, which were demonstrated with sucrose density gradient centrifugation and molecular weight exclusion filters. In agreement, CTA blocked the inhibition of CT internalization by RG-tannin. These data suggest that some plant polyphenols, similar to applephenon and HBT, bind CT, forming large aggregates in solution or, perhaps, on the cell surface and thereby suppress CT binding and internalization. In contrast, RG-tannin binding to CT did not interfere with its binding to Vero cells or GM1, but it did inhibit internalization.

Cholera toxin (CT), a heterohexameric AB₅ toxin produced by Vibrio cholerae, is responsible for the life-threatening diarrheal disease. The B subunits bind to cell surface ganglioside GM1 (1, 2), following which the holotoxin is endocytosed by epithelial cells and delivered to the Golgi and endoplasmic reticulum (3, 4), where reduction of a disulfide bond on CTA and proteolysis produce fragments A1 and A2. CTA1, which is enzymatically active, then enters the cytoplasm (5–7), where, in the presence of NAD, it catalyzes the ADP-ribosylation of Gαₛ (8–10), resulting in persistent activation of adenylyl cyclase (11). The subsequent increase in cyclic AMP (12) induces the secretion of fluid and electrolytes into the lumen of the small intestine (13).

Natural products, like herbal medications, have long been used to treat diarrheal disease and are useful therapeutic agents. Recently, inhibition of the action of bacterial toxins by plant polyphenols, composed of highly condensed tannins, has been reported (14–16). These include applephenon, HBT, and RG-tannin, derived from apple, hop bract, and Daio (Rhei rhizoma), respectively. RG-tannin and applephenon suppress CT-catalyzed ADP-ribosylation and CT-induced fluid accumulation in rabbit or mouse ileal loops (14, 15). RG-tannin is most likely a procyanidine polymer (mainly 8-mer) containing galloyl groups. Applephenon is composed of chlorogenic acid, catechins, and condensed catechins and procyanidins. Its anti-CT activity was attributed to the highly condensed tannin fraction (14), apple-condensed tannin (ACT), which contains procyanidine polymers (less than 15-mer and mostly 3–6-mer) (17). HBT was extracted from hop bract polyphenols, which contain procyanidine polymers (about 10–30-mers) with molecular weights around 6,000. HBT inhibited Shiga toxin 1 (Stx-1), which is produced by enterohemorrhagic Escherichia coli (16). Stx-1 is another AB₅ toxin with B subunits that bind to Gb₃ globotriaosyl ceramide on the cell surface (18, 19), leading to its endocytosis. The A subunit of Stx-1 is a 33-kDa protein with RNA N-glycosidase activity that blocks protein synthesis in eukaryotic cells. HBT interfered with Stx-1-induced inhibition of protein synthesis and lethal toxicity in a mouse inoculated with enterohemorrhagic Escherichia coli producing Stx-1. HBT also suppressed CT ADP-ribosyltransferase activity. It is not clear, however, whether inhibition of fluid accumulation by RG-tannin or applephenon is, in fact, the result of suppression of CT enzymatic activity.

Because polyphenols are generally believed to precipitate proteins (20, 21), we thought that RG-tannin, applephenon, and/or HBT might act by preventing CT binding to or internalization by cells. We report here that RG-tannin did not inhibit CT binding to cells but formed complexes with CT and thereby suppressed internalization from the cell surface. On the other hand, HBT or applephenon formed large aggregates with CT that interfered with its binding to and internalization by cells, indicating that the mechanism of RG-tannin action was different from those used by HBT or applephenon.

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1 The abbreviations used are: CT, cholera toxin; CTA, A subunit of cholera toxin; CTB, B subunit of cholera toxin; Stx-1, Shiga toxin 1; HBT, hop bract tannin; ACT, apple condensed tannin; HBSS, Hank’s balanced salt solution; HRP, horseradish peroxidase; BSA, bovine serum albumin; PBS, phosphate-buffered saline; GM1, Gal-Ga1NAc[NeuAc]-Gal-Glc-Cer.
FIG. 1. Effect of polyphenols on CT binding to Vero cells. A, Vero cells were incubated at 4 °C for 30 min with HBT (○), applephenon (□), RG-tannin (○), or ACT (△) and 20 nM biotin-labeled CT before quantification of bound CT as described under “Materials and Methods.” Data are means ± S.D. of values from triplicate assays in one experiment representative of three. B, effect of polyphenols on viable Vero cell number. Cells were incubated at 4 °C for 30 min with HBT (○), applephenon (□), RG-tannin (○), or ACT (△) as indicated before determination of cell number as described under “Materials and Methods.” Data are means ± S.D. of values from triplicate assays. C, effect of treatment of Vero cells with polyphenols on CT binding. Cells were incubated at 4 °C for 30 min with HBT (○), applephenon (□), RG-tannin (○), or ACT (△) as indicated and washed before CT binding at 4 °C for 30 min. Data are means ± S.D. of values from triplicate assays. D, effect of polyphenols on the CT-stimulated cAMP content. Vero cells were incubated with the indicated concentrations of polyphenols at 37 °C for 30 min and then washed before the addition of labeled CT and incubation at 37 °C for 30 min. CAMP activity was determined as described under “Materials and Methods.” Data are means ± S.D. of values of three samples. E, effect of polyphenols on bound CT. Cells were incubated with labeled CT at 4 °C for 30 min, and after washing the cells, HBT (○), applephenon (□), RG-tannin (○), or ACT (△) was added. Cells were then incubated for 30 min, and CT binding was quantitated. Data are means ± S.D. of values of three samples.

MATERIALS AND METHODS

Reagents—CT and CTA were purchased from List Biological Laboratories, and CTB was from Calbiochem. Applephenon 50 and ACT were kindly provided by Dr. Yanagida (Nikka Whisky Distilling Co., Ltd.). HBT was prepared by Asahi Beer Co., and RG-tannin was prepared by Teumura & Co. Applephenon, RG-tannin, and HBT were dissolved in PBS, and HBT was passed through a 0.22-μm filter. 2-Mercaptoethanesulfonic acid, HRP-conjugated streptavidin, and BM blue substrate for peroxidase were purchased from Aldrich, Amersham Biosciences, and Roche Applied Science, respectively.

Cell Viability—The number of viable cells was determined using a cell counting kit (Dojindo Laboratories, Kumamoto, Japan), and viability of cells was evaluated by trypsin blue dye exclusion. The cell counting kit is based on the same principle as the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (22). Cells, grown on a 96-well plate, were incubated with 10 μl of cell counting reagent (company-supplied) at 37 °C for 1 h before color was quantified at 450 nm. Absorbance at 450 nm as a measure of cell number correlated well with the number counted directly using a hemacytometer. For Trypan blue dye exclusion assays, cells were detached from the plate by incubation with 0.25% of trypsin plus 0.02% EDTA. Equal volumes of cell suspension with 0.3% trypan blue in PBS were mixed before total and stained (dead) cells (at least 100 cells) were counted.

Cell Enzyme-linked Immunosorbent Assay—Vero cells (2 × 10⁴ cells/well) were cultured in Eagle’s minimal essential medium with 10% fetal calf serum in 96-well plates overnight. Biotin-labeled CT was prepared according to the supplier’s instructions using EZ-Link Sulfo-N-hydroxysulfosuccinimide-SS-Biotin (Pierce). Approximately 84% of the label was incorporated into CTA. Cells were washed with cold Hanks’ balanced salt solution with 0.1% of BSA (HBSS-BSA) three times before the addition of 100 μl of 20 nM CT in HBSS-BSA, incubated at 4 °C for 30 min, washed three times with cold HBSS, and fixed for 20 min with 0.25% glutaraldehyde in PBS. After blocking with 3% BSA in PBS for 1 h, cells were incubated with HRP-conjugated streptavidin for 1 h, and HRP was detected with BM blue substrate. After a 20-min incubation, the reaction was stopped with 1 M H₂SO₄, and color was measured at 450 nm. Background absorbance was subtracted. Between each step, cells were washed with PBS three times.

Internalization of toxin was quantified as follows. After binding of biotin-labeled CT and washing to remove unbound toxin, HBSS-BSA, warmed to 37 °C, was added, and the cells were incubated at 37 °C for the indicated time before washing immediately with cold HBSS, fixation, and addition of 0.5 μl of 2-mercaptoethanesulfonic acid, which does not enter cells, to reduce disulfide bonds and release biotin from CT bound to the cell surface. After additional incubation at room temperature for 30 min, cells were permeabilized with 1% Triton X-100 for 15 min and treated with HRP-conjugated streptavidin as described above to quantify internalized toxin.

GM1 Enzyme-linked Immunosorbent Assay—25 μl of GM1 ganglioside (10 μg/ml in ethanol) were added to each well of a 96-well plate, which was incubated at 37 °C until the ethanol had evaporated, and thereafter, the assay was performed at room temperature. The plate was washed three times with 0.1% Tween 20 in PBS (TPBS) and blocked for 1 h with 1% BSA in TPBS. Then 100 μl of biotin-labeled CT (5 nM) without or with polyphenols as indicated were added, and after incubation for 1 h, the plate was washed to remove unbound toxin before the addition of HRP-streptavidin and incubation for 1 h. HRP was detected using BM blue. Between each step, the plate was washed three times.

Fluorescein-labeled CT Binding and Internalization—CT was labeled with Cy3 using a Fluorolink-Ab Cy3 labeling kit (Amersham Biosciences). Cells (2 × 10⁴/well) were seeded in 6-well plates containing coverslips and incubated at 37 °C overnight. After washing, cells were incubated at 4 °C for 30 min in HBSS-BSA containing Cy3-labeled CT with or without polyphenols (200 μg/ml). Then cells were washed to remove unbound toxin, before fixation or incubation at 37 °C for 10 min with HBSS-BSA containing polyphenols. Cells
were fixed with 3% paraformaldehyde and inspected by fluorescence microscopy (Olympus GX-FLA).

Precipitation of CT—Polyphenols as indicated were incubated with 20 nM biotin-labeled CT in PBS at room temperature for 30 min before centrifugation (14,700 × g, 15 min). Supernatant was discarded, and pellet was dissolved in 80 μl of SDS-sample buffer (0.25 M tris, pH 6.8, 4% SDS, 40% glycerol, and 0.005% bromophen blue). After boiling for 5 min, proteins were separated by SDS-PAGE in 14% gel and transferred to polyvinylidene difluoride membranes. At the same time, standard samples of biotin-labeled CT (0.1, 0.2, 0.3, and 0.4 μg) were treated in parallel and used to quantify recovery. Biotin was detected using HRP-conjugated streptavidin followed by ECL Western blotting detection reagent (Amersham Bioscience). Chemiluminescence was detected by LAS-1000, and the density of each sample was quantified using Image Gauge software (Fujifilm). The amount of CT precipitated was calculated as CTA, because most of the label was found in CTA. Data were expressed as a percentage of the total CT applied. Precipitation of CTA or CTB was determined similarly using biotin-labeled CTA or CTB.

Sucrose Density Gradient Centrifugation—Polyphenols (100 μg) and biotin-labeled CT (10 μg) in PBS (500 μl) were incubated at room temperature for 30 min before transfer to the top of a linear gradient of 8–30% sucrose in PBS solution (4.3 ml) and ultracentrifugation (Hitachi model P55ST2; 200,000 × g, 4 °C, 16 h). Eight 600-μl fractions were then collected (from the top). Samples of each were subjected to SDS-PAGE in a 14% gel (nonreducing conditions), and proteins were transferred to polyvinylidene difluoride membranes. Biotin-labeled CT was detected with HRP-conjugated streptavidin and ECL reagent. CT in each fraction was quantified as CTA using Image Gauge software (Fujifilm) and calculated as follows: percentage of total = intensity of fraction/sum of intensities of eight fractions × 100. Similar experiments were performed using biotin-labeled CTA and CTB.

Assay of Complexes Using a Molecular Weight Exclusion Membrane Filter (Centricon)—Amicon Centricon YM-50 and YM-100 are molecular weight exclusion membranes. According to a company-supplied user guide, 90% of a ~100-kDa protein is recovered in retentate of YM-100, and 85% of a ~50-kDa protein is recovered in retentate using a YM-50 membrane. Biotin-labeled CT, CTA, or CTB (10 μg) was incubated with polyphenols (100 μg) in 500 μl of PBS at room temperature for 30 min. The mixture was then applied to YM-50 and YM-100, which were centrifuged at 2,000 × g for 5–10 min until the retentate volume was ~20 μl. Retentate was diluted to 500 μl with PBS before samples were taken for separation of proteins by SDS-PAGE in 14% gels (nonreducing conditions) and transfer to polyvinylidene difluoride membranes. Biotin-labeled tox was detected and quantified as described above.

cAMP Assay—cAMP was quantified using a cAMP enzyme immunoassay kit (Kayan Chemical Co.).

Polyphenols (200 μg/ml) and 3-isobutyl-1-methylxanthine (500 μM) were added to Vero cells (7 × 10⁵ cells/well) grown in 6-well plates and incubated at 4 °C for 30 min. Cells were then washed three times, and 20 nM CT was added in the presence of 3-isobutyl-1-methylxanthine (500 μM), followed by incubation at 37 °C for 30 min. Medium was then aspirated and 0.3 ml of 0.1 M HCl added in enzyme immunoassay buffer (company-supplied). After incubation for 20 min at room temperature, cells were scraped and centrifuged, and the supernatant was used for an assay following the manufacturer’s instructions.

RESULTS

Applephenon, ACT, and HBT, but Not RG-tannin, Suppressed CT Binding to Vero Cells—Binding and internalization of biotin-labeled CT by Vero cells was dependent on CT concentration, reaching a maximum at ~20 nM (data not shown). When 20 nM CT and the indicated concentrations of applephenon, ACT (highly condensed tannin fraction of applephenon), or HBT, were incubated with cells for 30 min, toxin binding to cells was inhibited (Fig. 1A). HBT and ACT were more potent than applephenon; HBT at 50 μg/ml, a concentration at which applephenon was ineffective, suppressed binding of CT by 80%. In contrast, RG-tannins, at concentrations up to 200 μg/ml, did not affect CT binding. Treatment of Vero cells with polyphenols did not alter cell number (Fig. 1B) or microscopic appearance (data not shown), suggesting a lack of cytotoxicity in these experiments. Further, incubation of cells with polyphenols did not decrease subsequent binding of CT (Fig. 1C) or decrease the cAMP response to CT (Fig. 1D). The amount of CT, bound to cells, did not decrease following incubation with polyphenols, compared with the control without polyphenols (Fig. 1E). These data suggested that the inhibitory effects of polyphenols did not result from nonspecific binding to the cells with masking of CT binding sites or competition by polyphenols with bound CT, leading to its displacement from the cells. Inhibitory effects of polyphenol were more robust when CT was incubated with it before addition to the cells (data not shown).

The suppressive effects of HBT, applephenon, and ACT on CT binding to cells were confirmed by fluorescence microscopy using Cy3-labeled CT (Fig. 2). CT bound to Vero cells in the absence of polyphenols (control) and in the presence of RG-tannin; in the presence of applephenon or ACT, however, CT binding was much lower. CT bound very poorly to HBT-treated cells. After incubation of these cells at 37 °C for 15 min, toxin was internalized and accumulated in the perinuclear region in control and RG-tannin-treated cells but not in applephenon-
ACT-, or HBT-treated cells. To determine whether these cells were viable, cells treated with 200 μg/ml polyphenols were detached from the plate with trypsin, and total cell number and trypan blue exclusion were investigated. Treatment with polyphenols did not decrease the cell number (Fig. 2B) or cell viability by trypan blue dye exclusion (Fig. 2C) compared with control cells not exposed to polyphenol.

**HBT and Applephenon Suppress CT Internalization into Vero Cells**—To examine whether HBT and applephenon suppress internalization of CT, cells were treated with biotin-labeled CT at 4 °C for 30 min and then washed with HBSS to remove unbound CT before incubation at 37 °C with each polyphenol. CT internalization was quantified after removing biotin from the cell surface with 2-mercaptoethanesulfonic acid. CT internalization was suppressed by applephenon (Fig. 3A) or HBT (Fig. 3B) in a dose-dependent manner. Weak suppression was observed with RG-tannin (Fig. 3C). No morphological changes were observed under the microscope (data not shown), and there were no changes in viable cell number following incubation with polyphenol (Fig. 3D).

**RG-tannin Suppresses Internalization of CT**—RG-tannin did not suppress binding of CT to Vero cells but weakly suppressed CT internalization. We determined whether preincubation of CT and RG-tannin would further suppress internalization. CT and RG-tannin were incubated at 4 °C for 30 min before the addition to cells and incubation at 4 °C for 30 min. Binding of CT was not prevented by preincubation with RG-tannin (Fig. 4B). Cells were washed and incubated at 37 °C for the indicated times. As shown in Fig. 4A, RG-tannin suppressed internalization of CT in a dose-dependent manner.

**HBT and Applephenon Suppress CT Binding to Immobilized GM1**—CT binds to surface ganglioside GM1. We examined whether polyphenols suppress CT binding to immobilized GM1. Preincubation of polyphenols with immobilized GM1 did not decrease CT binding (Fig. 5A). CT binding, however, was suppressed in a dose-dependent manner by applephenon and HBT; some, but not significant suppression, was observed with RG-tannin (200 μg/ml) (Fig. 5B). Polyphenols Precipitate CT—To clarify how the polyphenols suppress CT binding or internalization, we examined whether polyphenols aggregate CT. Biotin-labeled CT was incubated with the indicated concentrations of polyphenols for 30 min at room temperature, after which the samples were centrifuged, and the resulting precipitate was resolved on gels (Fig. 6A). As evident from these data, polyphenols precipitated CT in a concentration-dependent manner. At 50 μg/ml, HBT, ACT, and applephenon reached a plateau and precipitated the maximum amount of CT. RG-tannin was less effective. Similar results were obtained with biotin-labeled CTA (Fig. 6B). Again, RG-tannin was less effective and ACT was more effective than applephenon. When CTB was used instead of CTA (Fig. 6C), again, RG-tannin precipitated less CTB than other polyphenols.
RG-tannin Complexes with CT—Interaction with RG-tannin and CT was further examined using sucrose density gradients (Fig. 7A). As biotin was mostly incorporated in CTA as described under “Materials and Methods,” only the CTA blot is shown. The primary peak was found in fraction 4 with both untreated CT and RG-tannin-treated CT. When the intensities of fraction 5 of untreated CT and RG-tannin-treated CT were compared, a statistically significant difference was observed (Fig. 7A, bottom), suggesting that RG-tannin-treated CT was recovered in a denser fraction than CT. All of the HBT-treated CT was recovered in the bottom fraction (Fig. 7A, top). Applephenon- and ACT-treated CT were also found in the bottom fraction (data not shown). These data suggested that HBT, ACT, and applephenon made large aggregates of more than 250 kDa with CT from the calibration using standard molecular weight markers, and RG-tannin complexed with CT. Since RG-tannin did not inhibit CT binding to Vero cells, we hypothesized that RG-tannin formed a complex with CTA but not CTB. As expected, CTA incubated with RG-tannin was recovered in a more dense fraction than CTA alone (Fig. 7B). However, shift of RG-tannin-treated CTB to a denser fraction was also observed (Fig. 7C), suggesting that RG-tannin might interact with both CTA and CTB complexes with CT. CTA or CTB incubated with HBT was recovered in the bottom fraction, consistent with formation of a large aggregate of more than 250 kDa (Fig. 7, B and C). ACT- or applephenon-treated CTA or CTB was also recovered in the bottom fraction (data not shown).
Effect of Polyphenols on Cholera Toxin

DISCUSSION

RG-tannin and condensed tannin fraction of apple polyphenol (applephenon) named ACT were reported to suppress CT-induced ADP-riboosylation of Gα1 or agmatine and fluid accumulation in mouse or rabbit ileal loops (14, 15); however, their precise mechanism of action was not determined. In this report, we focused on the effect of RG-tannin, applephenon, and ACT, on the binding and internalization of CT. We also used HBT, which is fractioned from hop bract and contains components similar to those in ACT. It suppressed ADP-riboosylation by CT and was recently found to be a potent inhibitor of Stx1 produced by Escherichia coli O157:H7 (16).

HBT, ACT, and applephenon, but not RG-tannin, inhibited the binding of CT to Vero cells; all of the polyphenols inhibited CT internalization. The polyphenols used in this report were not cytotoxic at the concentrations studied; incubation of cells with polyphenols did not inhibit subsequent binding of toxin, and cell function was apparently not impaired. Further, inhibition of internalization of bound CT was not due to the release of bound toxin by the polyphenols. These data suggested that the inhibitory effects of the polyphenols were not due to their nonspecific binding to cells or to cell damage but resulted from their interaction with CT in solution and on the cell surface, thereby preventing CT binding and internalization. HBT, ACT, and applephenon bound CT, resulting in large aggregates, which were sedimented by high speed centrifugation. These CT aggregates did not bind to GM1 or to Vero cells. RG-tannin, on the other hand, did not form large aggregates with CT similar to applephenon or HBT, but it did associate with CT as demonstrated by sucrose density gradient centrifugation and filtration through molecular weight exclusion membranes. RG-tannin did not inhibit CT binding to cells or to GM1 immobilized on plates. It did, however, complex with CTB as well as CT. It appears that the GM1-binding site of CTB was not masked by interaction with RG-tannin, although complex formation did affect CT internalization. ACT was more potent than applephenon for both inhibition of CT binding and precipitation of CT, which may explain why ACT was more effective than applephenon in inhibiting of CT ADP-riboosyl transferase activity (14). The different interactions of RG-tannin and other polyphenols with CT may reflect the presence in RG-tannin, but not in applephenon and HBT, of galloyl moieties.

Os et al. (15) reported that RG-tannin (15 μg/ml) prevented completely the induction by CT (1.3 μg) of fluid accumulation. In our studies, however, RG-tannin at 10 or 50 μg/ml suppressed at 20 min CT internalization by Vero cells only 12 and 20%, respectively (Fig. 4). Mechanisms in addition to reduced internalization of the RG-tannin-CT complex, including inhibition of ADP-riboosylation, might be responsible for the suppression by RG-tannin of CT-induced fluid accumulation. It is also not clear whether the toxin-polyphenol complex is taken up by cells and delivered intact from Golgi to endoplasmic reticulum and then cytosol or whether polyphenols like RG-tannin, apple-

**TABLE I**

Analysis of CT-, CTA-, or CTB-polyphenol complex using molecular weight exclusion membranes

| Additions    | Percentage of toxin recovered in retentate of YM-100 |
|--------------|-------------------------------------------------------|
| CT           | CT A                                            |
| None         | 9.9 ± 5.0                                          |
| ACT (200 μg/ml) | 0.8 ± 0.1*                                         |
| HBT (200 μg/ml) | 99.9 ± 0.1*                                         |
| RG-tannin (200 μg/ml) | 75.6 ± 9.8*, 32.2 ± 13.7*                     |

* p < 0.01 to each control without polyphenol.

To confirm that RG-tannin complexes with CT, molecular weight exclusion membranes (Centricon) were used (Fig. 8 and Table I). Since 97% of CT (84 kDa) was recovered in retentate with a YM-50 molecular weight exclusion membrane (data not shown), we used a YM-100 membrane in which 10% of CT was recovered in retentate (YM-100 membrane excludes ~100,000 molecular weight protein). With YM-100, 76% of CT incubated with RG-tannin was recovered in retentate, and all of the HBT-CT and ACT-CT complexes were recovered in retentate. Similar experiments were performed using biotin-labeled CTA or CTB. Most of the CTA or CTB was recovered in the filtrate using a YM-100 membrane; however, RG-tannin-treated CTA or CTB was recovered 32 and 58%, respectively, in retentate. Almost all CTA or CTB was recovered in the retentate of a YM-100 membrane when treated with HBT or ACT. These data confirmed that RG-tannin interacts with both CTA and CTB and complexed with CT.

**FIG. 8.** Analysis of CT-, CTA-, or CTB-polyphenol complex using molecular size exclusion membranes. Biotin-CT, -CTA, or -CTB and polyphenols were incubated at room temperature for 30 min, before analysis on Centricon (Amicon) YM-100 membranes as described under “Materials and Methods.” Representative chemiluminescence images are shown. In the CT and polyphenol experiment, only an image of CTA is shown. Lanes 1–4, filtrate; lanes 5–8, retentate; lanes 1 and 5, toxin incubated with ACT; lanes 2 and 6, toxin incubated with HBT; lanes 3 and 7, toxin incubated with RG-tannin; lanes 4 and 8, toxin alone.

**FIG. 9.** CTA inhibits RG-tannin-induced suppression of CT internalization. Biotin-CT (20 nm) was incubated at 4 °C for 30 min with RG-tannin as indicated in the presence (○) or absence (□) of CTA (200 nm) before the addition to cells. Cells were then incubated at 4 °C for 30 min, and then cells were washed and incubated at 37 °C for 20 min. Internalized toxin was quantified as described under “Materials and Methods.” Data are means ± S.D. of values of four samples.
phenon, or HBT are internalized and interfere intracellularly with toxin activity.

Polyphenols are known to bind to proteins; however, the interaction depends on structure of the polyphenol and the protein surface structure (20, 21). RG-tannin was reported to interfere with CT activity but not with that of heat-stable enterotoxin of enterotoxigenic *Escherichia coli* (15). We found that RG-tannin differed from HBT and applephenon in its interaction with CT. Thus, polyphenols show specificity in their interactions with bacterial toxins. Specificity is thus observed at the level of the polyphenol and the toxin.

CT is thought to be endocytosed after binding to GM1 and localization to detergent-insoluble microdomains, lipid rafts, or caveolae (23–25). These polyphenols may interfere with an early step in endocytosis. How RG-tannin suppresses internalization of CT from the cell surface by complex formation or how internalization of CT already bound to the cell surface was suppressed by HBT and applephenon needs to be better understood. HBT may be another potential therapeutic agent along with applephenon and RG-tannin to protect against CT-mediated disease. Further, based on the present report, it may also be effective on CT that had already bound to cells.

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REFERENCES

1. Cuatrecasas, P. (1973) *Biochemistry* **12**, 3558–3566
2. Holmgren, J., Lonroth, I., Mansson, J., and Svennerholm, L. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72**, 2520–2524
3. Lencer, W. I., Hirst, T. R., and Holmes, R. K. (1999) *Biochim. Biophys. Acta.* **1450**, 177–190
4. Fujinaga, Y., Wolf, A. A., Rodighiero, C., Wheeler, H., Tse, B., Allen, L., Jobling, M. G., Rapoport, T., Holmes, R. K., and Lencer, W. I. (2003) *Mol. Biol. Cell.* **14**, 4783–4793
5. Tse, B., and Rapoport, T. A. (2002) *J. Cell Biol.* **159**, 207–215
6. Schumitz, A., Herrgen, H., Winkeler, A., and Herzog, V. (2000) *J. Cell Biol.* **148**, 1203–1212
7. Teter, K., Allyn, R. L., Jobling, M. G., and Holmes, R. K. (2002) *Infect. Immun.* **69**, 6166–6171
8. Moss, J., and Vaughan, M. (1977) *J. Biol. Chem.* **252**, 2455–2457
9. Casse, D., and Seizinger, Z. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 3307–3311
10. Gill, D. M., and Meren, R. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3050–3054
11. Casey, P. J., and Gilman, A. G. (1988) *J. Biol. Chem.* **263**, 2577–2580
12. Tausiss, R., and Gilman, A. G. (1995) *J. Biol. Chem.* **270**, 1–4
13. Field, M., Fromm, D., al-Awqati, Q., and Greenough, W. B., III (1972) *J. Clin. Invest.* **51**, 796–804
14. Saito, T., Miyake, M., Toba, M., Okamatsu, H., Shimizu, S., and Noda, M. (2002) *Microbiol. Immunol.* **46**, 249–255
15. Ohi, H., Matsoura, D., Miyake, M., Ueno, M., Takai, I., Yamamoto, T., Kudo, M., Moss, J., and Noda, M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3042–3046
16. Tagashira, M., Yahiro, K., Morinaga, N., Moss, J., and Noda, M. (2003) *US-Japan Cholera and Other Bacterial Enteric Infections, 38th Joint Panel Meeting*, pp. 45–48, U. S. Department of Health and Human Disease, Public Health Service, National Institutes of Health, Bethesda, MD
17. Yanagida, A., Kanda, T., Takahashi, T., Kamimura, A., Hamazono, T., and Honda, S. (2009) *J. Chromatogr. A* **1200**, 251–259
18. Lindberg, A. A., Brown, J. E., Stromberg, N., Westling-Ryd, M., Schultz, J. E., and Karlsson, K. A. (1987) *J. Biol. Chem.* **262**, 1779–1785
19. Lingwood, C. A., Law, H., Richardson, S., Petrie, M., Brunton, J. L., De Grandis, S., and Karmali, M. (1987) *J. Biol. Chem.* **262**, 8834–8839
20. Haslam, E. (1996) *J. Nat. Prod.* **59**, 205–215
21. Charlton, A. J., Baxter, N. J., Khan, M. L., Moir, A. J., Haslam, E., Davies, A. P., and Williamson, M. P. (2002) *J. Agric. Food Chem.* **50**, 1583–1601
22. Mosmann, T. (1983) *J. Immunol. Methods* **16**, 55–83
23. Wolf, A. A., Jobling, M. G., Wimer-Mackin, S., Ferguson-Maltzman, M., Madara, J. L., Holmes, R. K., and Lencer, W. I. (1999) *J. Cell Biol.* **141**, 917–927
24. Torgersen, M. L., Skretting, G., van Deurs, B., and Sandvig, K. (2003) *J. Cell Sci.* **114**, 3737–3747
25. Le, P. U., and Nabi, I. R. (2003) *J. Cell Sci.* **116**, 1059–1071