Microbiol. Immunol., 45(2), 111–117, 2001

Cytokine Responses to Recombinant Cholera Toxin B Subunit Produced by *Bacillus brevis* as a Mucosal Adjuvant

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Received June 16, 2000; in revised form, November 22, 2000. Accepted November 27, 2000

***Abstract*: We attempted to clarify the mechanism of the mucosal adjuvanticity of recombinant cholera toxin B subunit (rCTB), which is inherently uncontaminated with the holotoxin produced by *Bacillus brevis* and has a powerful mucosal adjuvant activity, on cytokine responses compared with that of cholera toxin (CT). rCTB had no ability to stimulate cyclic AMP formation in mouse peritoneal macrophages (Mϕ). Cytokine production by non-immunized Mϕ cultured with rCTB or CT and by the spleen cells of mice co-immunized intranasally with ovalbumin (OVA) and rCTB or CT was examined. rCTB alone did not induce interleukin (IL)-1α/β or IL-6 production by Mϕ, but combination of rCTB with lipopolysaccharide (LPS) enhanced both IL-1α/β production. Conversely, CT plus LPS suppressed IL-1α/β production more than LPS alone. Both rCTB and CT suppressed IL-12 secretion induced by interferon γ (IFN γ) plus LPS. IL-2, IL-4, IL-5, and IL-10 were secreted by mouse spleen cells restimulated with OVA after intranasal co-administration of OVA together with rCTB, and in response to CT, the same cytokines were secreted. The different effect of rCTB on Mϕ from that of CT may mean a difference between the mechanisms of rCTB and CT during the early stage of an immune response.**

***Key words*: Recombinant cholera toxin B subunit (rCTB), Mucosal adjuvant, Interleukin**

Oral and nasal mucosal immunizations have received a great deal of attention in recent years, because they stimulate both mucosal and systemic immunities, and antigen-specific mucosal immune responses can be expressed at all mucosal sites (26). In addition, the inoculation procedure is simple, reliable, and cheap, and injection needles are unnecessary. However, mucosal immune responses to protein antigen alone usually could not be demonstrated, or could only be weakly demonstrated, and therefore, mucosal adjuvants that can sufficiently enhance mucosal antigen-specific IgA antibody response levels are required for immunization. Cholera toxin (CT), *Escherichia coli*-derived heat-labile toxin, and their mutated modified proteins are generally well-known as mucosal adjuvants (4, 8, 22, 41, 42). CT is composed of two subunits, a toxigenic A subunit (CTA), which is involved in ADP-ribosylation and a pentameric B subunit (CTB) which binds with GM1 ganglioside present in most mammalian cells. Possible mechanisms of action of CT as a mucosal adjuvant include (a) enhancement of uptake of co-administered antigen across either the follicular or surface epithelial layer, (b) enhancement of antigen presentation by increasing the expression of costimulatory surface molecules and cytokines such as IL-1 and IL-6 of antigen presenting cells, (c) induction of both antigen- and CT-specific CD4+ T cells, (d) enhancement of the IgG1 and IgA isotype production of B cells by IL-4 produced by primed T cells, and (e) inhibition of CD8+ T cells in the

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Abbreviations: cAMP, cyclic AMP; CT, cholera toxin; D-PBS, Dulbecco's phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IL, interleukin; LPS, lipopolysaccharide; Mϕ, mouse peritoneal macrophages; OVA, ovalbumin; rCTB, recombinant cholera toxin B subunit.

epithelial layer that may produce inhibitory cytokines (9). Some investigators have suggested that the adjuvant mechanism of CT is linked to the ability to ADP-ribosylate and to stimulate cyclic AMP (cAMP) formation (23), and that the adjuvant activity of natural CTB may be due to the presence of the trace amounts of CTA that are frequently present in CTB preparations obtained from CT holotoxin (6, 20, 33, 38). We purified a large amount of recombinant cholera toxin B subunit (rCTB) secreted by *Bacillus brevis* carrying pNU212-CTB (14, 43), and it showed powerful adjuvant properties for biologicals without the holotoxin (15, 16).

In the present study, to identify the differences between the effects of rCTB and CT on mucosal adjuvanticity, we examined cytokine production by the spleen cells of mice previously immunized with ovalbumin (OVA) plus rCTB or CT and by non-immunized mouse peritoneal macrophages (Mϕ) stimulated with rCTB or CT.

Materials and Methods

Animals. Female BALB/c mice aged 7 weeks were purchased from Japan SLC Co. (Shizuoka, Japan).

Test materials. rCTB was prepared by cultivating *Bacillus brevis* bearing pNU212-CTB at 30 C for 5 days (14) and purified from the culture supernatant by affinity chromatography using D-galactose immobilized agarose. The rCTB preparation contained predominantly stable pentameric form and its GM1 binding ability was similar to that of the native CTB (43). It contained little or no leucocytosis-promoting factor, as described previously (34), and it elicited no *in vitro* cytotoxicities, local histopathological or vascular permeability-increasing reactions in the experimental animals (10).

Other materials. The following materials were obtained from their manufacturers: chicken egg albumin type VII, lipopolysaccharide (LPS) from *Escherichia coli* type O111:B4 and fetal calf serum were purchased from Sigma Chemical Co. (SIG, St. Louis, Mo., U.S.A.), recombinant mouse IFN γ from Genzyme (Cambridge, Mass., U.S.A.), CTs from SIG and List Biological Laboratories Inc. (LBL, Campbell, Calif., U.S.A.) and CTBs from SIG, LBL, and Research Biochemicals Inc. (RBI, Natick, Mass., U.S.A.).

Analysis of LPS. All test materials were confirmed to be free of LPS contamination by the *Limulus* test (Endospecy; Seikagaku Corporation, Tokyo).

Immunization of mice. Mice were immunized intranasally under light ether anesthesia with 20 μl of solution containing 50 μg of OVA, with and without 10 μg of rCTB or CT, on days 0, 14, 21, 28, and 35, and sacrificed on day 42.

Assay for total IgG antibody. Mice were bled from the tail vein at weekly intervals after immunization. Antibody responses to OVA were detected by enzyme-linked immunosorbent assay (ELISA).

Preparation and culture of spleen cells. Spleen cells were collected from the immunized mice and suspended in RPMI-1640 (Nissui Pharmaceutical Co., Ltd., Tokyo) containing 10% heat-inactivated fetal calf serum (FCS-RPMI) at a cell density of 1×10^7 cells/ml. The cell suspension was plated at 500 μl/well onto a 24-well culture plate, and 500 μl of 1 mg/ml OVA solution in the same medium were added. After incubation at 37 C for 24 hr in a CO_2 incubator, the culture supernatants or lysates were collected for the cytokine assays.

Preparation and culture of macrophages. Mϕ that had not been previously immunized with rCTB, CT, or other antigens were collected in Dulbecco's phosphate-buffered saline (D-PBS) 4 days after intraperitoneal injection of 2 ml of sterilized 4.05% Brewer thioglycollate medium (Difco Laboratories, Detroit, Mich., U.S.A.). The ascitic fluid was centrifuged at $500 \times g$ for 10 min, and the cells were washed twice with D-PBS at 4 C and finally suspended in FCS-RPMI. The suspended cells were allowed to adhere to a 24-well plate at 37 C for 2 hr, and the non-adherent cells were washed off with D-PBS warmed to 37 C. The Mϕ monolayers were covered with 1 ml of FCS-RPMI and incubated overnight. The final count of the adherent cells was approximately 1×10^6 per well. rCTB was added to the well with 1 ml of FCS-RPMI, with or without stimulants for IL-1 or IL-12 secretion (1 μg/ml LPS, 100 U/ml IFN γ) (1, 3, 36). After incubation at 37 C for 24 hr, the 24-well plates were centrifuged at $500 \times g$ for 10 min to sediment the detached cells, and the supernatant was assayed for cytokine content.

Induction and assay for cAMP. Mϕ (1×10^6 cells/ml in a 24-well tissue-culture plate) in FCS-RPMI were incubated with test materials at 37 C for 2 hr, and the reaction was stopped by adding 50 μl of 1 N HCl. The cells were lysed by repeated freeze-thaw cycles and then centrifuged at $500 \times g$ for 5 min. The supernatants were used for cAMP assay. The concentration of cAMP was determined with a commercial cAMP-test kit (Cayman Chemical Co., Mich., U.S.A.).

Assay for cytokines. The concentrations of cytokines in the supernatants were determined with a commercial ELISA kit: InterTest™ (Genzyme) or BIOTRAK™ (Amersham, Buckinghamshire, England).

Results

Effects of rCTB and CT on cAMP Production by Mϕ

To evaluate whether the adjuvant activity of rCTB

involves ADP-ribosyltransferase activity, we investigated whether it would increase the level of cAMP production in peritoneal Mϕ from non-immunized mice. As shown in Fig. 1, commercial CT induced a high level of cAMP production, and all commercial CTBs indicated almost the same level as 0.01 μg CT, whereas rCTB elicited no or little accumulation of intracellular cAMP even at a concentration of 10 μg.

Effects of rCTB and CT on Cytokine Production by Mϕ

Cytokine production (IL-1α/β, IL-6, and IL-12) by Mϕ was examined after incubation of non-immunized mouse peritoneal Mϕ with rCTB. rCTB or CT alone did not induce IL-1α/β, but rCTB markedly potentiated LPS-stimulated IL-1α/β production by Mϕ, whereas CT suppressed IL-1 production induced by LPS (Fig. 2). CT clearly stimulated IL-6 secretion, whereas rCTB alone induced IL-6 on a similar level to that elicited by non-treated Mϕ (Fig. 3). Under the present conditions, a great deal of IL-6 was produced in the presence of LPS. LPS produced 21.1±4.2 ng/ml of IL-6, and rCTB plus LPS produced 20.2±3.0 ng/ml of IL-6. There is no apparent difference between samples with and without rCTB. Both rCTB and CT suppressed secretion of IL-12 secretion induced by IFN γ plus LPS (Fig. 4). The effect of rCTB on induction of IL-1 and IL-6 by Mϕ is different from that of CT.

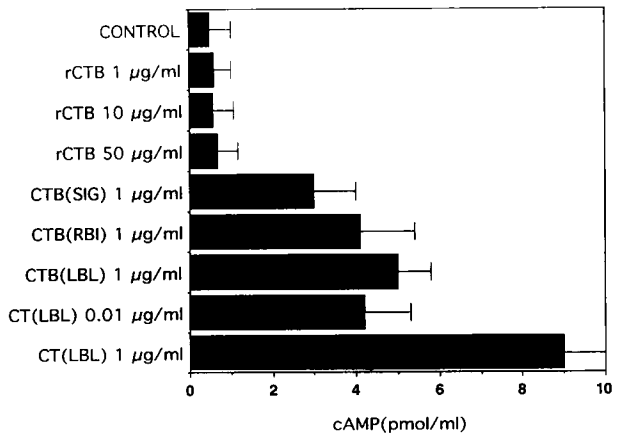

Fig. 1. cAMP production by Mϕ (1×10^6 cells) incubated *in vitro* with CT, CTB, or rCTB for 2 hr at 37 C. The bars indicate standard deviations. Control: non-treated cells.

Cytokine Production by Mouse Spleen Cells Restimulated with OVA after Intranasal Co-Administration of OVA Plus rCTB or CT

Cytokine secretion by mouse spleen cells after intranasal immunization with OVA plus rCTB was assayed to determine how rCTB influenced the immune system. Total IgG antibody titers detected by ELISA were transformed into logarithms to base 10. Serum antibody titers for mice which were immunized with OVA plus rCTB, OVA plus CT and OVA alone were 4.0±0.7, 4.2±0.5 and 1.2±0.4, respectively. Intranasal administration of OVA plus rCTB induced high levels of IgG antibody responses, almost the same level as OVA plus CT. The spleen cells of the groups immunized with OVA plus rCTB or CT secreted IL-2, IL-4, IL-5, and IL-10, but the spleen cells from the group immunized with OVA alone did not (Fig. 5). The amount of IFN γ produced by spleen cells stimulated *in vitro* with OVA made using OVA plus rCTB, OVA plus CT and OVA alone were 120±5.0, 111±11 and 107±8 pg/ml, respective-

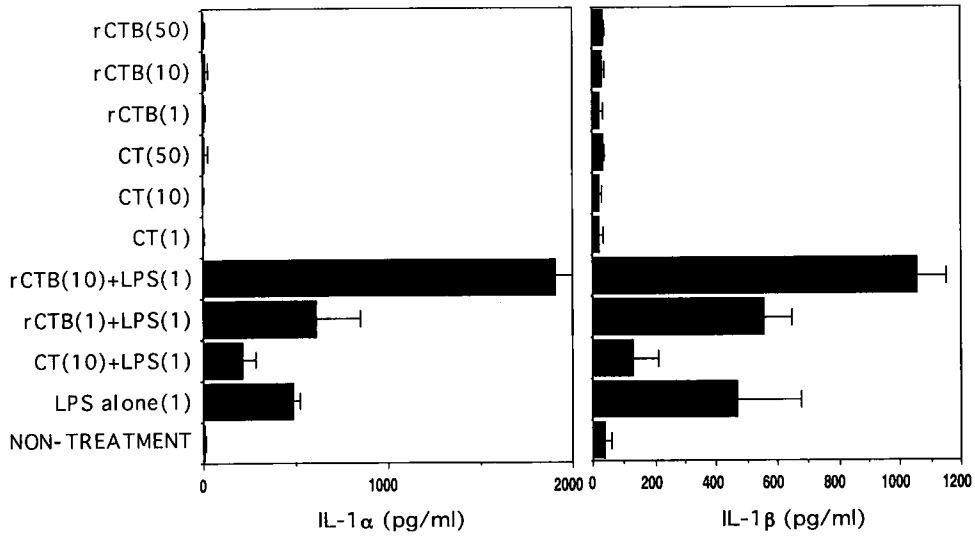

Fig. 2. Effect of rCTB or CT (LBL) on IL-1α/β production by non-immunized Mϕ (1×10^6 cells) incubated *in vitro* with and without LPS (1 μg/ml) for 24 hr at 37 C. Figure in parenthesis: μg/ml. The bars indicate standard deviations.

ly. There was no significant difference between rCTB and CT. IFN γ was not produced by spleen cells in the absence of OVA stimulation.

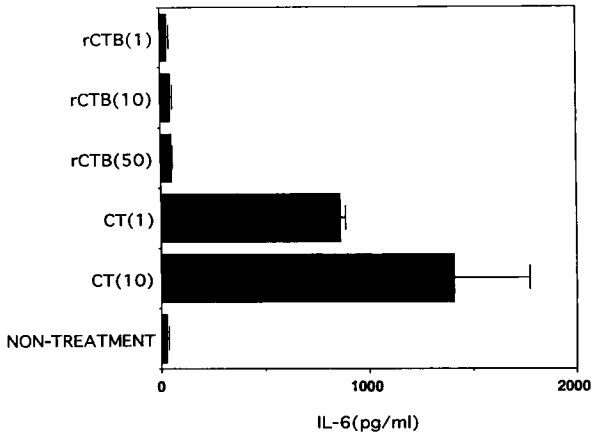

Fig. 3. Induction of IL-6 secretion by non-immunized Mϕ (1×10^6 cells) by incubation with rCTB or CT (LBL) *in vitro* for 24 hr at 37 C. Figure in parenthesis: μg/ml. The bars indicate standard deviations.

Discussion

Early studies on the mucosal adjuvanticity of CT and

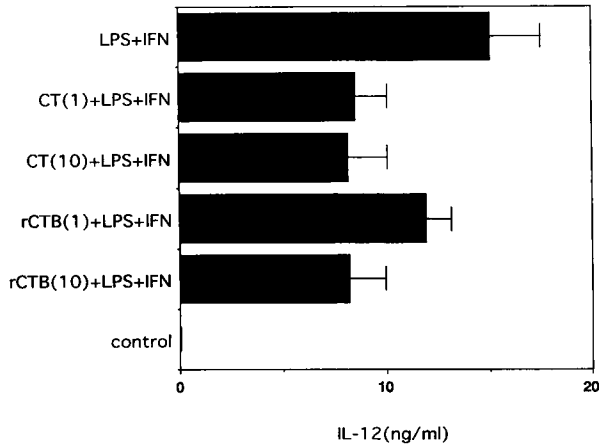

Fig. 4. Effect of rCTB or CT (LBL) on IL-12 production by non-immunized Mϕ (1×10^6 cells), incubated *in vitro* with and without LPS (1 μg/ml) and IFN γ (100 U/ml) for 24 hr at 37 C. Figure in parenthesis: μg/ml. The bars indicate standard deviations.

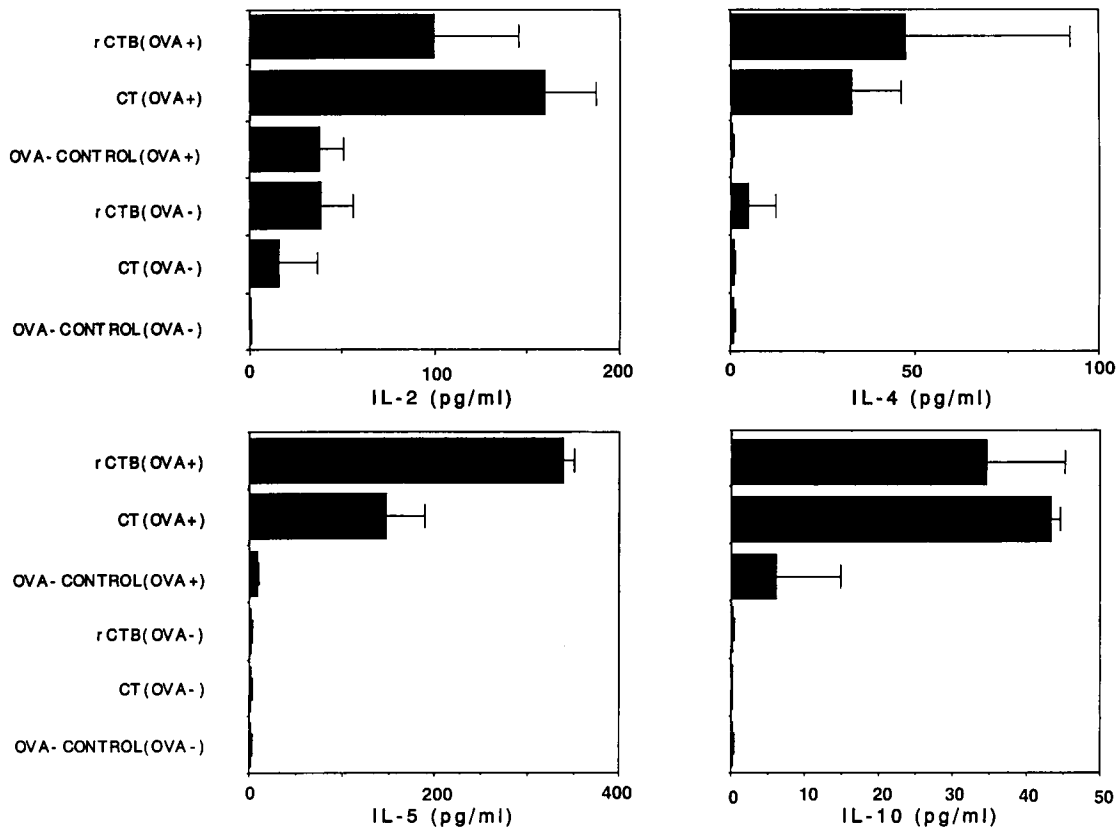

Fig. 5. Cytokine production by mouse spleen cells (5×10^6 cells/ml) incubated with and without OVA (500 μg/ml) after intranasal co-administration of OVA (50 μg/mouse) and rCTB (10 μg/mouse) or CT (1 μg/mouse) for 24 hr at 37 C. (OVA+) and (OVA−) indicate the secondary stimulation with and without OVA. OVA-control: cytokine production by the spleen cells of mice immunized with OVA alone. The bars indicate standard deviations.

CTB have reported that ADP-ribosyltransferase activity is indispensable for the adjuvant effect of CT (23), CTB possesses no mucosal adjuvanticity (20, 22, 23) and trace amounts of contaminated holotoxin are essential for the mucosal adjuvanticity of CTB purified from natural CT (6, 33). rCTB is devoid of A subunit and has no ADP-ribosyltransferase activity, which is closely related to the enterotoxicity of CT. Actually, it was shown in this study that rCTB did not elicit accumulation of intracellular cAMP, unlike natural or commercial CTB containing trace amounts of holotoxin (Fig. 1). Nevertheless, rCTB displays strong mucosal adjuvant properties (15, 16, 34, 43); it has been reported that mutant CTs which have no or little enzyme activity of A subunit also retain mucosal adjuvanticity (41, 42). Furthermore, rCTB induces no *in vitro* cytotoxicity and no local histopathological or vascular permeability-increasing reactions in the experimental animals (10).

In this study, the profiles of cytokines secreted by the spleen cells of mice immunized with OVA plus rCTB and *in vitro* by peritoneal Mϕ of non-immunized mice were examined as a clue to the mechanism of the adjuvant effect of rCTB as compared with CT. First, we tested their effects on secretion of IL-1, IL-6 and IL-12 which are secreted by Mϕ and may play an important role in the mechanism of the antibody production. Neither rCTB alone nor CT alone induced IL-1α/β when incubated with Mϕ (Fig. 2) although some reports have stated that IL-1 production is stimulated by incubating Mϕ with CT alone (2, 21). It is a well-known fact that LPS stimulates IL-1 production by Mϕ (1, 7). We found the difference between rCTB and CT in the presence of LPS. That is, rCTB potentiated LPS-stimulated IL-1α/β production by Mϕ whereas CT suppressed it. As for CT, it has been reported that IL-1 protein synthesis is blocked posttranscriptionally by the cAMP-dependent pathway or cAMP accumulation in Mϕ (17).

There are some reports on IL-6 production that link it to an intracellular cAMP dependent pathway (25, 44). Our results (Figs. 1 and 3) were highly consistent with theirs. As for the adjuvanticity of CT, IL-6 may play an important role (24, 25). Because rCTB alone did not induce IL-6 production, the mechanism of adjuvanticity of rCTB seemed to be different from that of CT.

IL-12 is involved in the differentiation of Th1 cells (36). Both rCTB and CT suppressed IL-12 secretion induced by LPS and IFN γ (Fig. 4). Therefore, rCTB might activate the immune system of the Th2-type not only by increasing IL-4 secretion by lymphocytes (Fig. 5) (27) but also by decreasing Th1 cells by suppressing IL-12 secretion by Mϕ. CT is generally known to activate the immune system of Th2 cells (12, 39, 40) but some investigators have suggested that CT activates both Th1 and Th2 cells (13). rCTB might affect the Th1/Th2 balance by the same mechanism as CT has shown in the latter. Whether rCTB interacts with Th1 cells will have to be examined in the future.

IL-2, IL-4, IL-5 and IL-10 were secreted by spleen cells restimulated *in vitro* with OVA after intranasal immunization with rCTB and OVA (Fig. 5). IL-4, IL-5 and IL-10 are the important cytokines for IgA secretion in mucosal immune systems (28, 29, 35, 37). The question has been raised as to the effect of IL-2 as a Th1-type cytokine on mucosal immune systems, but it might not be necessary to consider mucosal immunity from the standpoint of Th1/Th2 balance alone because some reports claim that IL-2 is also an essential cofactor for IgA switching by TGF-β (5, 18, 19). Since whole-spleen cell cultures composed of various types of cells were used in this experiment, as the first experiment, IL-2 may be secreted by several types of cells, as well as by precursor Th cells (30, 31) or Th0 cells (32). When rCTB was compared with other adjuvants on cytokine production by spleen cells, IL-2 and IFN γ were produced with OVA stimulation in case of Freund's complete adjuvant (data not shown), which is well-known to stimulate Th1 type immune response (11). rCTB may induce Th2 immune response by production of IL-4, IL-5 and IL-10 in spite of the absence of IL-2 and IFN γ.

Differences in the effects of CT and rCTB on Mϕ found in the presence of LPS (Fig. 2) may reflect the action of rCTB in the early stage of the immune response. There is little difference between cytokine production by spleen cells induced by rCTB and CT. Further studies on cytokine production following intranasal administration of rCTB using nasal associated lymphoid tissue separated from mucosal tissue and on the mechanisms involved in the increase of IL-1 production are in progress in our laboratory.

We are grateful to Dr. Masayoshi Kohase, Chief of Lab. of Cytokines, National Institute of Infectious Diseases, for his helpful discussion and suggestions.

This work was supported by a grant for Research on Health Sciences Focusing on Drug Innovation, No. 51138 from the Japan Human Health Sciences Foundation.

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