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The cytotoxicity and molecular mechanisms of the *Clostridium perfringens* NetB toxin

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ABSTRACT. The necrotic enteritis toxin B-like (NetB) toxin secreted by *Clostridium perfringens* is a key virulence agent in the pathogenesis of avian necrotic enteritis, a disease that causes significant economic loss to the poultry industry worldwide. NetB was purified from *Clostridium perfringens* type G (CNEOP004) that was isolated from chickens with necrotic enteritis in Japan. EC_{50} of this purified NetB toward chicken liver-derived LMH cells was 0.63 µg/ml. *In vivo* pathogenicity of NetB to chicks produced characteristic lesions of necrotic enteritis. Analysis of the localization of the NetB monomer and oligomer molecules on LMH cells showed that both molecules of the toxin were localized in non-lipid raft regions. Moreover, removal of cholesterol with the cholesterol depletion assay carried out in LMH cells detected both oligomers and monomers of the NetB molecule. These data suggest that the NetB toxin may recognize membrane molecules different from cholesterol in non-raft region. Furthermore, NetB-binding molecules on LMH cell membranes using the toxin overlay assay with immunoblotting showed that protein molecules of different molecular sizes were bound to NetB on non-lipid raft fractions. Further studies are necessary to characterize these protein molecules to examine their specific association with NetB binding and oligomerization.

KEY WORDS: *Clostridium perfringens*, cytotoxicity, necrotic enteritis, necrotic enteritis toxin B-like

Clostridium perfringens (*C. perfringens*) is the main causative pathogen of necrotic enteritis (NE) in humans and animals [12, 28]. *C. perfringens* is a prolific toxin producer and generates various toxins [17, 23]. *C. perfringens* is divided into various types, including A, B, C, D, E, F and G according to the production of major toxins: alpha (α), beta (β), epsilon (ε), iota (ι), enterotoxin (CPE) and the necrotic enteritis toxin B-like (NetB) toxin [17]. Poultry-specific conserved pathogenic virulence, which is attributed to the NetB toxin [4], gave rise to the new strain (type G) that was previously described under *C. perfringens* type A.

NE in chickens caused by *C. perfringens* re-emerged upon the ban on the use of antimicrobial growth in poultry production [4, 29]. This disease has led to huge economic losses in the poultry industry worldwide [30]. Alpha toxin had once been thought to be the virulent factor for NE [1], but alpha toxin was shown not to be an essential virulent factor in this disease process [10]. NetB, which was only identified in *C. perfringens* isolated from chicken NE, was shown to have the most important role in the pathogenesis of chicken NE [10, 11].

NetB is a member of the beta-pore-forming toxin (PFT) family, which is a member of the *Staphylococcus aureus* alpha-hemolysin-like β-PFT family [16]. PFTs are secreted as a water-soluble monomer and interact with the target cell membrane. The cellular action involves initial binding to a receptor followed by oligomerization and cytopathic pore formation. Oligomerization is a common feature of many PFTs involving lipid raft regions in target membranes. Lipid rafts are sphingolipid-cholesterol-rich detergent-resistant membranes that are utilized by a large number of pathogens as a target of entry in sensitive cells. After binding to the receptor, the PFT monomer molecules generally use the fluidity of the cell membrane to reduce the intermolecular distance between the monomers and oligomerizes to form pores on the host membranes, leading to cell death. Cholesterol plays a critical role in modulating the fluidity of the membranes. It can condense with sphingolipids and lipids with fully saturated acyl chains that are available in cell membranes and form detergent-resistant membranes or lipid rafts [24, 26]. Caveolae is one of the lipid rafts, which is rich in cholesterol, sphingolipids and various proteins including caveolins. Caveolin has three family members, whereas caveolin-I and-II are abundant in various cell types, including epithelial cells, fibroblasts, and endothelial cells and, therefore, is

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frequently used as a lipid raft marker in PFT binding studies [5]. Lipid rafts are reported to have varying impact on the cytotoxicity of PFT produced by many bacteria such as aerolysin (*Aeromonas* sp), α-toxin (*C. septicum*), ε- and ι-toxins (*C. perfringens*) [7, 19, 21, 22]. On the other hand, the amount of cholesterol on liposome membranes in NetB has been shown to affect oligomelization and pore formation [20]. However, the exact role of cholesterol and lipid rafts in the cytotoxicity induced by NetB is unknown. Therefore, we examined the interaction of NetB on LMH cells derived from chicken hepatocellular carcinoma. LMH cells are the only cell line sensitive to NetB. Strikingly, the present study found that the binding, oligomerization and pore formation of NetB occurred in non-lipid raft regions without the need for the presence of cholesterol in the host cell membrane. This study also detected protein molecules of different molecular sizes, which were bound to NetB within the non-lipid raft fractions.

MATERIALS AND METHODS

Bacterial strain and culture conditions

Clostridium perfringens type G (CNEOP004) was isolated from chickens with NE in Japan. To evaluate the ability of this strain to produce toxins, the following media were used: Brain heart infusion (BHI) medium (Difco; Becton, Dickinson and Co., Franklin Lakes, NJ, USA), trypticase peptone glucose (TPG) medium [5% trypticase (BBL™), 0.5% proteose-peptone (Bacto™; Becton, Dickinson and Co.), 0.4% D-glucose, 0.1% sodium thioglycolate, pH 7.2], cooked meat medium (CMM medium) [cooked meat medium (Difco; Becton, Dickinson and Co.) with 0.3% D-glucose, 0.2% soluble starch (Difco; Becton, Dickinson and Co.), corn peptone-thioglycolate (C-TGC) medium [1.7% casein peptone (Sigma Chemical Co., St. Louis, MO, Germany), 0.6% D-glucose, 0.3% corn meal (Sigma), 0.25% NaCl, 0.07% Bacto agar (Difco; Becton, Dickinson and Co.), 0.05% sodium thioglycolic acid, 0.025% L-cysteine, 0.01% sodium sulfite, pH 7.0], and soybean peptone-thioglycolate (S-TGC) medium [1.7% casein peptone (Sigma), 0.6% D-glucose, 0.3% tryptic soy broth (Bacto™; Becton, Dickinson and Co.), 0.25% NaCl, 0.07% bacto agar (Difco; Becton, Dickinson and Co.), 0.05% sodium thioglycolic acid, 0.025% L-cysteine, 0.01% sodium sulfite, pH 7.0] and heart infusion (HI) medium (Difco; Becton, Dickinson and Co.).

Assay of hemolytic activity

The hemolytic activity (HU_{50}) of NetB was assayed in round-bottomed microtiter plates (Becton, Dickinson and Co.). Test samples were serially diluted two fold with Dulbecco's phosphate buffer saline (PBS), and 100 µl of each diluted sample was added to 2 wells followed by addition of 100 µl of a 2% (v/v) suspension of chicken erythrocytes, which had been washed with Dulbecco's PBS twice by centrifugation at 1,500 × *g* for 10 min at 4°C. The plates were incubated for 1 hr at 37°C with shaking, then centrifuged at 200 × *g* for 5 min at 4°C. The absorbance of the supernatant was measured at 540 nm, which is the absorption wavelength of hemoglobin. The percent hemolysis was calculated for each sample, from which the amount of toxin inducing 50% hemolysis was determined. The hemolysis caused by 4% Triton X-100 was considered 100%.

NetB purification

NetB was purified from the supernatants of *C. perfringens* CNEOP004 cultured in 3 l of TPG (Becton, Dickinson and Co.) at 37°C for 36 hr under anaerobic conditions. The toxin was purified by fractionating the culture supernatant with 50% saturated ammonium sulphate [$(NH_4)_2SO_4$] at 4°C overnight and centrifugation at 11,000 × *g* for 20 min at 4°C. The precipitate was dissolved in 10 mM Tris-HCl buffer (pH 7.2) and dialyzed against the same buffer for 30 hr. After centrifugation at 11,000 × *g* for 10 min at 4°C, the supernatant was subjected to Q Sepharose ™ Fast Flow (GE Healthcare, Uppsala, Sweden). Fractions with hemolytic activity were collected from the flow-through fractions. The collected fractions were precipitated with 60% saturated $(NH_4)_2SO_4$ at 4°C overnight. After centrifugation, the precipitate was dissolved in 50 mM acetate buffer (pH 5.0) and was subjected to SP-650M cation exchange chromatography (Tosoh, Tokyo, Japan). The elution was performed in a stepwise procedure using 50 mM acetate buffer (pH 5.0) adjusted to a sodium chloride concentration of 50 mM, 75 mM, 100 mM, or 500 mM. Among the eluted fractions, the fractions with hemolytic activity were collected. After confirming the degree of purification by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [13] using a 12.5% gel, the purified toxin was dialyzed with PBS for 60 hr, concentrated by ultrafiltration using YM-10 membrane (Merk Millipore, Darmstadt, Germany) and stored at −80°C.

Cell line

LMH cells derived from chicken hepatocellular carcinoma are the only cells sensitive to NetB. LMH was maintained in Waymouth's medium (Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal calf serum at 37°C in 5% CO_2. The cells were grown to confluence in 25 cm^2 flasks before seeding for experiments.

Cytotoxicity assay

LMH cells were suspended in culture medium and inoculated into a 96 well plate at a density of 2 × 10^5 cells/ml. Cells were grown to 80 to 90% confluence for 2 days at 37°C under 5% CO_2. After removal of the medium and washing with Dulbecco's PBS, the toxin diluted with Waymouth's medium (Thermo Fisher Scientific Inc.) supplemented with 1% fetal calf serum was added to each well. The plates were incubated for 24 hr at 37°C in 5% CO_2. Cell Titer 96 Aqueous One Solution Reagent (Promega, Madison, WI, USA) was added and incubated for another 1 hr at 37°C in a 5% CO_2 atmosphere. The absorbance of each well was measured at 490 nm, and the absorbance of 4 wells was averaged for each toxin concentration. We considered LMH to have 0% cell lethality when cells were incubated in medium alone without NetB, and the absorbance of the medium alone was considered as

100% cell lethality. The data were represented as percent viability to determine the concentration of toxin causing 50% cell death (EC_{50}) as described previously [14].

In vivo toxicity of NetB

Zero point five ml of NetB (100 µg/ml in PBS) was intraperitoneally administered to 2-day-old chicks (Hy-Line system; Takeuchi Incubation Co., Ltd., Nara, Japan) after withdrawal of feed for 24 hr. After the death of the chicks, their digestive tracts were examined for gross necrotic lesions. For histopathological examination, intestinal tissues from infected and control chicks were processed. The tissues were collected and fixed in 4% phosphate-buffered paraformaldehyde solution, and 4-µm-thick sections were prepared from paraffin-embedded tissue blocks using standard methods. The sections were then stained with hematoxylin and eosin (HE).

Preparation of affinity purified rabbit anti-NetB polyclonal IgG

The NetB toxin was used for antigen immunization as described elsewhere [2]. Briefly, the toxin (200 µg/ml) was detoxified by treatment with formalin at a final concentration of 0.4% (v/v) and kept at 37°C for 7 days. After intraperitoneal administration of 20 µg of toxoid to mice (ddY strain, male 4 weeks old; SLC Co., Ltd., Hamamatsu, Japan), the animals were kept under observation for 4 days to examine their survival status. For the first immunization, rabbits (Japanese white, male, 14 weeks old, Oriental Yeast, Tokyo, Japan) were injected with 20 µg of toxoid intradermally, emulsified in an equal volume of Freund's complete adjuvant (Wako Pure Chemical Co., Osaka, Japan). Subsequently, the animals were injected with the same dose of toxoid emulsified in an equal volume of Freund's incomplete adjuvant (Wako Pure Chemical Co.) intradermally 3 times every 2 weeks. Two weeks after the fourth immunization, 20 µg of NetB toxoid alone was inoculated subcutaneously as a booster. Two weeks after the booster, whole blood was collected from the heart under anesthesia, and serum was collected. The IgG fraction was isolated from the rabbit serum as described by Harlow and Lane [18]. Thereafter, the IgG against the toxin was purified with a HiTrap NHS Sepharose column (GE Healthcare) according to the manufacturer's instructions.

Immunoblotting

Samples were run on SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) by using a semi-dry transfer cell (Trans blot SD, Bio-Rad laboratories Inc., Hercules, CA, USA). The membrane was then blocked with 5% skim milk in Tris buffer saline (TBS; 25 mM Tris-HCl, [pH 7.5],0.14 M NaCl) at room temperature for 30 min followed by treatment with affinity-purified rabbit anti-NetB IgG (5 µg/ml) or rabbit anti-caveolin-1 polyclonal IgG (ECM Biosciences, Versailles, KY, USA) (1:2,000) at room temperature for 1 hr. The membrane was washed with TBST (0.05% Tween 20 in TBS) and incubated with 3,000-times diluted peroxidase-labeled goat anti-rabbit IgG (GE Healthcare) at room temperature for 30 min. Detection was performed using a chemiluminescence kit (Super Signal® West Femto Maximum Sensitivity Substrate; Thermo Fisher Scientific Inc.). The method was based on the manual attached to the kit. For observation and photographing of chemiluminescence, a lumino image analyzer LAS-3000 (GE Healthcare) was used.

Sucrose density gradient ultracentrifugation

LMH cells cultured in a 25 cm^2 flask were washed with PBS at 37°C and suspended in 2 ml of NetB solution prepared at 10 µg/ml with Waymouth 's medium (Thermo Fisher Scientific Inc.). After incubating at 37°C for 1 hr, the mixture was washed with PBS at 4°C and added to 0.35 ml of morpholinoethanesulfonic acid (MES)-buffered saline (MBS; 25 mM MES, 150 mM NaCl, 2 mM EDTA, pH 6.5) containing 1% Triton X-114 and 0.1% protease inhibitor cocktail (Sigma). After keeping the mixture on ice for 1 hr, the sample was treated with 0.35 ml of 80% sucrose-containing MBS. Upon mixing properly, the sample was transferred to an ultracentrifuge tube (Ultra-Clear ™ Centrifuge Tubes 11 × 60 mm; Beckman Coulter, Brea, CA, USA), and 2 ml of 30% sucrose-containing MBS was added followed by layered with 1.3 ml of MBS containing 5% sucrose. After ultracentrifugation using with a SW60Ti (Beckman) at 250,000 × *g* for 18 hr at 4°C, 0.4 ml of fractions were collected in order from the bottom. To concentrate the sample, 100 µl of 30% trichloroacetic acid was added to each fraction, mixed, and allowed to stand on ice for 30 min. The mixture was centrifuged at 15,000 × *g* for 10 min at 4°C, and 500 µl of acetone was added to the precipitate and mixed. After centrifugation at 15,000 × *g* for 10 min at 4°C, the supernatant was removed, and the pellet was suspended in PBS after complete evaporation of acetone.

Cholesterol removal from cell membranes

To examine the inhibitory effect of cholesterol-interacting agents, LMH cells were incubated at 37°C for 30 min in the presence or absence of 5 mM methyl-β-cyclodextrin (MCD; Sigma) in 2.5 ml of Waymouth's medium (Thermo Fisher Scientific Inc.) and were washed twice with PBS. The removal of cholesterol was confirmed using a cholesterol measurement kit (Cholesterol-E-Test; Wako) according to the manufacturer's instructions. Each cell was treated with NetB (10 µg/ml) for an additional 1 hr at 37°C. After washing, cells were suspended in 0.1 ml of MBS containing 1% Triton X-114 and 0.1% protease inhibitor cocktail. After incubating for 1 hr on ice, the detergent-insoluble fractions were separated from the supernatant by centrifugation at 15,000 × *g* for 15 min and were subsequently resuspended in 1 ml of PBS. Samples were subject to SDS-PAGE and immunoblotting using affinity-purified rabbit anti-NetB (10 µg/ml) or anti-actin (Sigma) (1:100) IgG. The reactive bands were developed by chemiluminescence. The LAS-4000 system was used to visualize signals and quantified using densitometry software (MultiGauge; FUJIFILM Corp., Tokyo, Japan). The densities of monomers and oligomers in each lane were shown as relative values when we normalized the density of actin in the same lane to100.

Toxin overlay assay

LMH cells cultured in a 25 cm^2 flask were washed with PBS at 37°C and suspended in 0.35 ml of MBS containing 1% Triton X-114 and 0.1% protease inhibitor cocktail (Sigma) and left on ice for 1 hr. After centrifuging the suspension at 15,000 × *g* for 15 min at 4°C, the supernatant was transferred to a new tube as a soluble fraction, and the precipitate was suspended in 200 µl of cell lysate containing 0.1% protease inhibitor cocktail and collected as an insoluble fraction sample. Sucrose density gradient ultracentrifugation was performed as described above. The fraction collected was subjected to SDS-PAGE using 13 well SuperSep ™ Ace, 5–20% and transferred to a PVDF membrane. The membrane was blocked with TBS containing 5% skim milk for 30 min at room temperature. The membrane was washed 3 times with TBST and incubated with the NetB solution prepared at 10 µg/ml with TBST containing 0.5% skim milk at room temperature for 1 hr. Immunoblotting was performed using the primary and secondary antibodies according to the method as described above.

Other methods

The Bradford method [3] and the BCA method [25] were used for protein quantification. The amount of protein was calculated as the amount corresponding to bovine γ-globulin. A series of animal experiments were conducted in accordance with the Osaka Prefecture University Graduate School of Life and Environmental Sciences Animal Experiment Guidelines, "Act on the Protection and Management of Animals" and "Standards for Breeding and Storage of Experimental Animals and Pain Reduction".

RESULTS

NetB purification and its cytotoxic activities

To select a medium producing a large amount of toxin, CNEOP004 was cultivated in BHI medium, TPG medium, CMM medium, C-TGC medium, S-TGC medium, HI medium, and the hemolytic rates in each medium were examined. The hemolysis rate was 50% (BHI medium), 65% (TPG medium), 6% (CMM medium), 0% (C-TGC medium, S-TGC medium) and 1% (HI medium) (Supplementary Fig. 1). Among the media used, TPG medium showed the highest hemolysis rate and, therefore, was adopted as the medium for NetB purification. In addition, to examine the optimal culture time for NetB purification, the strain was inoculated into 10 ml of TPG medium and incubated at 37°C for 12 hr intervals until 72 hr. Subsequently, the hemolysis rate of the culture supernatant was 29% at 12 hr, 52% at 24 hr, 72% at 36 hr, 71% at 48 hr, 67% at 60 hr and 70% at 72 hr. The absorbance at 600 nm was 1.500 at 12 hr, 1.531 at 24 hr, 1.539 at 36 hr, 1.458 at 48 hr, 1.478 at 60 hr, and 1.478 at 72 hr. Taken together, the maximum growth of this strain was determined to be after 36 hr of incubation. Therefore, 36 hr was adopted as the culture time for NetB purification (Supplementary Fig. 2). Finally, a band of NetB (33 kDa) was observed by purification procedures using $(NH_4)_2SO_4$ precipitation, anion chromatography and cation chromatography (Fig. 1). Furthermore, the volume, protein content, hemolytic activity, and the recovery rate in each purification stages are shown in Supplementary Table 1. EC_{50} of this purified NetB toxin toward LMH cells was 0.63 µg/ml (data not shown).

Pathogenicity of NetB in chicks

Chicks died within 3 hr of intraperitoneal administration of NetB (50 µg). Necropsy showed extensive bleeding from the duodenum to the jejunum and thinning of the caecum, but no lesions were found in other organs. The intestinal mucosae in the duodenum, jejunum, and caecum were found to be desquamative with bleeding, but no abnormal findings were observed in the colon. Characteristic microscopic lesions in moderate cases included focal, multifocal to coalescing necrosis of enterocytes, whereas in severe cases, coagulative necrosis of the entire superficial mucosa separating underlying viable lamina propria with infiltration of mild to moderate inflammatory cells was observed (Fig. 2).

Localization of the NetB monomer and oligomer molecules on the cell membrane

To analyze the localization of the NetB monomer and oligomer molecules on the cell membrane, LMH cells were incubated with NetB, dissolved using a surfactant, fractionated by sucrose density gradient ultracentrifugation, and immunoblotting was performed using anti-NetB IgG. In addition, immunoblotting with an antibody of caveolin, which is a lipid raft marker, was simultaneously performed as an index of localization. NetB monomer and oligomer molecules were confirmed to be localized in fractions with higher specific gravity than raft fractions where caveolin was present (Fig. 3). Moreover, the monomer and oligomer molecules existed in the same fraction.

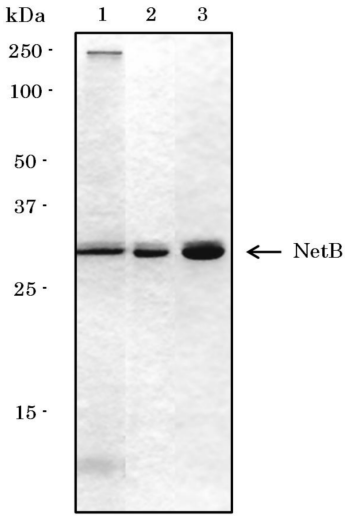

Fig. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified native necrotic enteritis toxin B-like (NetB) (approximately 33 kDa) stained with Coomassie Brilliant Blue in each step of purification of the toxin. Lane 1: ammonium sulphate precipitation of culture supernatant, 2: after anion chromatography, 3: after cation chromatography. CNEOP004 strain was inoculated into trypticase peptone glucose medium and cultured at 37°C for 36 hr, after which NetB was purified.

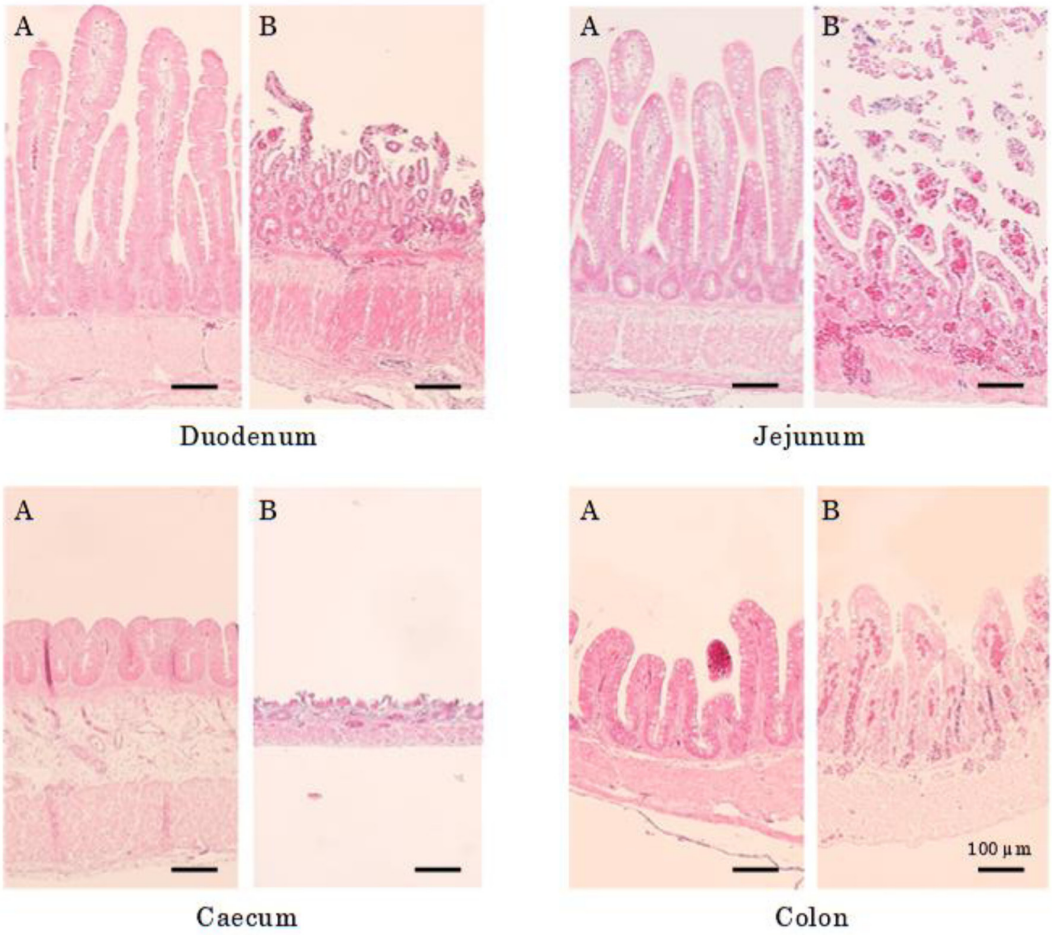

Fig. 2. Histological lesions in duodenum, jejunum, caecum and colon of chicks intraperitoneally inoculated with phosphate buffered saline (PBS) without (A) and with necrotic enteritis toxin B-like (NetB) (B).

The binding and oligomerization ability of NetB to cholesterol-depleted LMH cells

To analyze the involvement of cholesterol in the cell membrane in NetB binding and oligomer formation, MCD was added to LMH cells to remove cholesterol. After cholesterol was removed, NetB was added, dissolved using a surfactant, fractionated into soluble and insoluble fractions by centrifugation and subsequently immunoblotted using an anti-NetB antibody. MCD-untreated cells had a cholesterol concentration of 130 µg/ml, whereas that of MCD-treated cells was 30 µg/ml, confirming that cholesterol was removed by MCD treatment (Fig. 4). As a result of immunoblotting, monomer molecules and oligomer molecules of NetB were detected even in cells from which cholesterol was removed. Moreover, to quantify each band, immunoblotting using an anti-actin antibody was also performed at the same time. The relative value of the band intensity of the monomer and oligomers molecule as compared to the band intensity of actin in each lane, which was set to 100, was calculated. As a result, we determined that the levels of monomer and oligomer molecules in the cells from which cholesterol was removed were not decreased in both the soluble and insoluble fractions compared to control. In addition, there was significantly no difference in the ratio of oligomer and monomer with or without cholesterol removal (Table 1).

Localization of binding molecules to NetB

To analyze the localization of binding molecules to NetB in LMH cells, the cells were lysed with a surfactant, fractionated by sucrose density gradient ultracentrifugation, and a toxin overlay assay was performed. In addition, immunoblotting with an anti-caveolin antibody was also performed as an index of localization. As a result, molecules of approximately 60 kDa, 53 kDa, 47 kDa, 40, and 25 kDa that bind to NetB were detected only in the non- lipid raft fraction (Fig. 5).

DISCUSSION

NE is a re-emerging infectious disease in poultry causing huge economic losses to the livestock industry worldwide. NetB appears to play a key role in NE in poultry. Here, we characterized the cytotoxicity of the purified native NetB collected from the CNEOP004 strain both *in vitro* and *in vivo* and its oligomerization and pore formation on LMH cells. Our results showed very high cytotoxicity

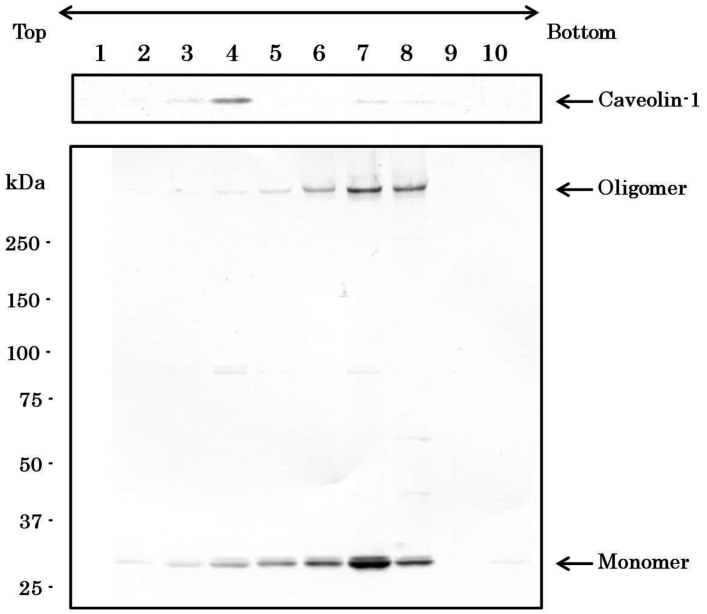

Fig. 3. Association of oligomerized necrotic enteritis toxin B-like (NetB) with cholesterol-rich microdomains (rafts). LMH cells were treated with NetB (10 µg/ml) at 37°C for 1 hr. After the mixture was washed with PBS at 4°C, cells were solubilized in cold morpholinoethanesulfonic acid (MES)-buffered saline (MBS) buffer containing 1% Triton X-114 on ice. The cell lysates were mixed with an equal volume of 80% sucrose–MBS buffer solution and loaded in a centrifuge tube for discontinuous sucrose density gradient centrifugation. After centrifugation, 0.4 ml gradient fractions were collected in order from the bottom of the tube. Aliquots of gradient fractions were applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes for oligomer and caveolin-1 detection. Following transfer, the membranes were treated with an affinity-purified rabbit anti-NetB IgG and anti-caveolin-1 antibody. Lanes 1–10, fractions from the top to bottom of the gradient.

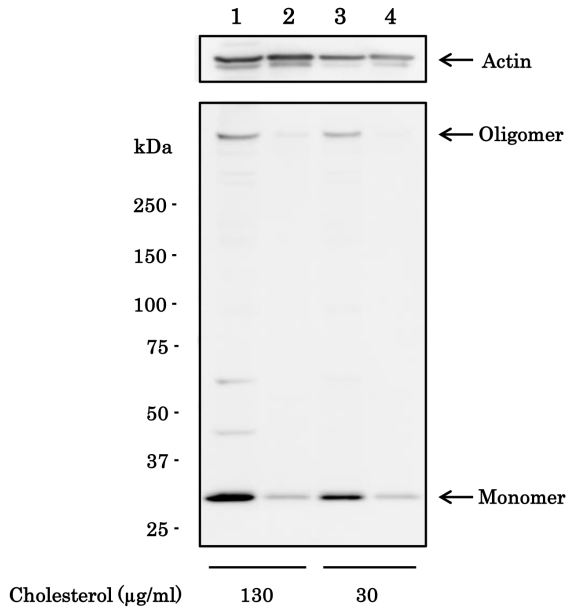

Fig. 4. Detection of oligomerized toxin on LMH cells pretreated with agents associating with membrane cholesterol. LMH cells were incubated in the presence or absence of 5 mM methyl-β-cyclodextrin (MCD) at 37°C for 30 min, washed and treated with necrotic enteritis toxin B-like (NetB) at 37°C for 1 hr. NetB treated cells were solubilized in cold lysis buffer containing 1% Triton X-114 and phase partitioned by centrifugation. The supernatants (even numbered) and pellets (odd numbered) were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting as described in Fig. 3. Lanes: 1 and 2, untreated; 3 and 4, MCD-treated.

Table 1. Relative values of the NetB oligomer and monomer with and without cholesterol removal.

Lane number [a)]	1	2	3	4
Oligomer	42.9	3.48	46.1	5.71
Monomer	155	30.5	169	55.6
Ratio of Oligomer (%)	27.6	11.4	27.3	10.3

a) Lanes: 1 and 2, untreated; 3 and 4, MCD-treated.

against the LMH chicken cell line (0.63 EC_{50}/ml) but no toxicity to mammalian cells such as Vero cells (>50 EC_{50}/ml) (data not shown). This observation is related to the fact that NE caused by the NetB-producing *C. perfringens* is restricted to chickens, suggesting that the receptor for NetB may bind to molecules that are specifically expressed only on the cell surface of chickens. Our *in vivo* study showed that the NetB toxin produced typical lesions of natural cases of NE and were consistent with lesions in chickens inoculated with *C. perfringens* Type G [15, 27]. Significant intestinal bleeding suggests that chicks may have died of hemorrhagic shock resulting from hemolysis of the erythrocytes and tissue necrosis in the duodenum, jejunum, and cecum (Fig. 2), which confirms that NetB is a major causative agent of NE in chickens. These results are likely due to bacterial growth and NetB production in the jejunum and ileum.

PFT cytotoxicity, as mentioned previously [16], results in oligomerization and pore formation on target cell membranes. Several pore-forming toxins such as alpha-toxin of *C. septicum* and α-, δ-, ε-, ι-toxins of *C. perfringens* have been reported to oligomerize in cholesterol-rich lipid rafts [6, 8, 21, 31]. In the present study, analysis of the localization of the NetB monomer and oligomer molecules in LMH cells revealed that both molecules were present in the same fraction in the non-lipid raft region as determined by immunoblotting. Therefore, we speculated that, following binding to cells, NetB oligomerizes in the non-lipid raft rather than being assembled into lipid rafts using the fluidity of the membrane.

Reduction of approximately 77% in cholesterol by MCD on LMH cell membranes did not influence toxin binding to cells and oligomer formation (Fig. 4 and Table 1). In our previous study, we have reported that similar levels of cholesterol removal reduced oligomerization of *C. Septicum* alpha-toxin [8]. Therefore, if cholesterol is involved in the binding to cells and oligomerization of NetB, there should be some change in the amount of monomer and oligomer molecules in the MCD-treated group. A similar finding was noted in a report where toxicity of a viral toxin to swine enterocytes was not reduced after extraction of cholesterol with MCD from the cell membranes [9]. However, a study conducted by Savva *et al*. has reported that oligomerization of NetB was reduced by

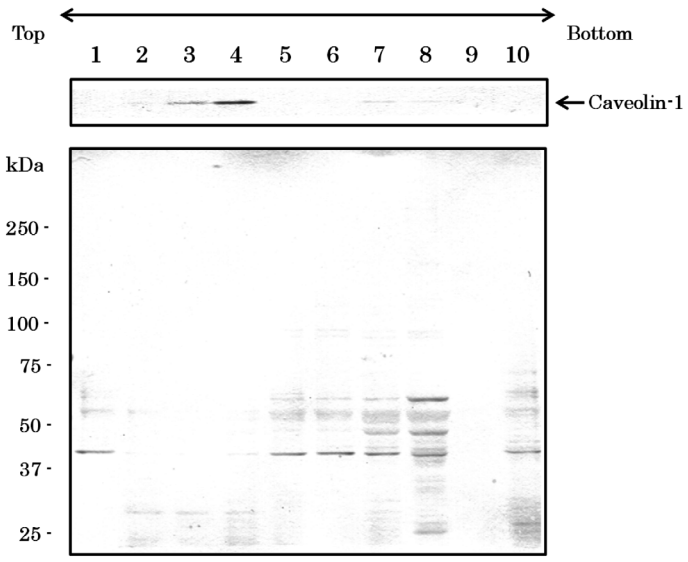

Fig. 5. Binding of necrotic enteritis toxin B-like (NetB) to LMH cells detected by the toxin overlay assay. LMH cells were solubilized in cold MBS buffer containing 1% Triton X-114 on ice. The cell lysates were mixed with an equal volume of 80% sucrose–MBS buffer solution and loaded in a centrifuge tube for discontinuous sucrose density gradient centrifugation. After centrifugation, 0.4 ml gradient fractions were collected in order from the bottom of the tube. Aliquots of gradient fractions were applied to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. After blotting, the membrane was treated with the NetB solution (10 µg/ml) at room temperature for 1 hr. After washing, Immunoblotting was performed using anti-NetB IgG and the secondary antibody. Lanes 1 to 10, fractions from the top to bottom of the gradient.

cholesterol depletion with 2-methyl-2,4-pentanediol (MPD) on liposome membranes [20]. In this report, NetB and MPD were simultaneously inoculated on cholesterol-containing liposomes. On the other hand, in our study, we previously treated LMH cells with MCD to reduce cholesterol before toxin treatment and then examined cell binding and oligomerization of NetB. This difference in experimental methods may affect the results. As we observed that NetB localized in the non-lipid raft region and its oligomerization was independent of cholesterol, there may be components other than cholesterol and raft proteins critically involved in the binding of this NetB toxin. We analyzed the localization of the NetB binding molecules on LMH cells, and our results showed that the monomers and oligomers of NetB were recovered from fractions in the non-lipid raft regions marked with the absence of lipid raft marker caveolin-1. Since the toxicity of NetB is highly limited to birds, it has very high species specificity. On the other hand, cholesterol and sphingolipid on cell membranes are not different among animal species. Therefore, the molecules on cell membranes related to the pathogenicity of NetB may possess avian specific structures. Further studies on the structural and functional analysis of NetB-bound molecules are needed to unambiguously establish their roles in the pore-forming mechanism of NetB.

In conclusion, the cytotoxicity of NetB occurs on non-raft and non-cholesterol regions rather than in raft areas on cell membranes. Our data offer a novel insight into the role of NetB toxin in host-pathogen interactions, and this knowledge could be used to devise effective control measures against NE. Further experiments are in progress to study the characteristics of the binding molecules involved in the pore formation mechanism of NetB.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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