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論　文　名	The cytotoxicity and mechanism of *Clostridium perfringens* NetB toxin (ウエルシュ菌が産生する NetB 毒素の病原性発現に関する研究)
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Introduction

Avian necrotic enteritis by *Clostridium perfringens* is such an emergent disease that causes significant economic loss to the poultry industry worldwide. Necrotic enteritis in chickens caused by *C. perfringens* is a contagious disease in which sudden and sporadic deaths occur due to coccidial infection, disturbance of the intestinal microflora by antibiotics, and high protein feeding. Clinical signs include loss of energy, feather reversal, and diarrhea, and small intestinal lesions are observed on gross examination. In the subclinical form of the disease, the intestinal mucosa is damaged, resulting in decreased digestion and absorption of nutrients, decreased weight gain, and increased feed requirements. This disease causes a loss of approximately 200 billion yen annually to the poultry industry worldwide, especially to the broiler industry, and there is an urgent need for measures to reduce the incidence of this disease.

C. perfringens is a Gram-positive, anaerobic, large bacillus that is classified into types A to G according to the production pattern of its major toxins (alpha, beta, epsilon, iota, enterotoxin and Necrotic Enteritis Toxin B-like (NetB toxin). It causes gas gangrene and necrotic enteritis in livestock and poultry. For more than 30 years, the pathogenic factor of chicken necrotic enteritis has been considered to be the alpha toxin (phospholipase C) produced by *C. perfringens*. However, there has been a remarkable breakthrough in both our theoretical understanding and practical application in 2006 when a mutant of *C. perfringens* in which the alpha-toxin gene was knocked out was found to cause necrotic enteritis, indicating that alpha-toxin is not an essential pathogenic factor for necrotic enteritis. In 2008, a novel protein toxin with 38% amino acid sequence homology to beta toxin produced by *C. perfringens* was identified in chicken isolates with necrotic enteritis and named NetB toxin. Its *netB* knockout mutant does not cause necrotic enteritis in chickens but reintroduction of *netB* into the knockout strain does, indicating that NetB toxin is an essential virulence factor for the development of necrotic enteritis

The author therefore isolated *netB*-carrying bacteria from the feces of necrotic enteritis-prone chickens and healthy chickens in Japan analyzed the characteristics of the bacteria in Chapter 1. The NetB toxin was then purified from the isolates and examined the species specificity of the toxin activity using various strains of cells and erythrocytes from various animal species and its pathogenicity to chickens in Chapter 2. In Chapter 3, the author analyzed the molecular dynamics of NetB toxin on the target cell membrane leading to pore formation. This thesis has provided several intriguing results, which will improve the understanding of the molecular mechanism of NetB toxin binding, oligomerization and cytotoxicity in avian cells.

Chapter 1. Molecular epidemiological analysis of *netB*-carrying *C. perfringens* isolated necrotic enteritis affected chickens

The author isolated *C. perfringens* from specimens obtained in Japan and examined the presence or absence of the major toxin gene and *netB* and compared the chicken erythrocyte hemolytic activity of each strain. Pulsed-field gel electrophoresis (PFGE) was also used to compare the genes and drug susceptibility of the isolates. In this study, *NetB toxin*-carrying *C. perfringens* was isolated from feces of chickens with necrotic enteritis and healthy chickens at several farms, indicating that *NetB toxin*-carrying *C. perfringens* is distributed in Japan and

infects healthy chickens. However, *NetB toxin*-negative bacteria were also isolated from chickens with necrotic enteritis, suggesting that there may be other factors associated with the development of necrotic enteritis. In DNA sequencing of isolated *C. perfringens, NetB toxin* is a very well conserved gene and that the *NetB toxin*-carrying strains in Japan are almost identical to those distributed worldwide. In PFGE analysis, three strains which were isolated from chickens with necrotic enteritis on different farms and harbored *NetB toxin*, were all classified as different types. These results suggest that conjugative plasmids containing *NetB toxin* are not easily transmitted, and that there may be a single or small number of *NetB toxin*-carrying strains spreading in the same area. In addition, each type of *NetB toxin*-carrying strains isolated in this study indicates that there are different *NetB toxin*-carrying strains in different regions.

Chapter 2. Purification and toxicity of *Clostridium perfringens* NetB toxin

The NetB toxin was purified from the supernatants of *C. perfringens* type G (CNEOP004) isolated from chickens with necrotic enteritis in Japan. To select a suitable medium for producing large amount of toxin a few media along with the culture time were examined. Haemolytic activity showed the highest rate in culture supernatants in tryptose peptone glucose (TPG) medium for 36 hours. The toxin was purified by fractionating the culture supernatant with ammonium sulphate precipitation, anion chromatography and cation exchange chromatography. Finally, a band of NetB toxin (33kDa) was observed by these purification procedures. EC_{50} of this purified NetB toxin toxin toward LMH cells was 0.63 μg/m*l*.

Haemolytic assay was done in erythrocytes of various animals and birds by measuring the amount of toxin inducing 50% hemolysis (HU_{50}). The results of erythrocyte sensitivity tests using the purified NetB toxin showed that the species specificity of NetB toxin is very narrow, limited to the pheasant order to which chickens belong and some birds, and it is thought to have little toxicity to mammals. Cytotoxicity tests were conducted using various cell lines, and the 50% cell lethal dose (EC_{50}) was calculated. Chick liver-derived LMH cells showed remarkably high sensitivity (EC_{50}: 0.63 μg/ml). chick embryo fibroblast (CEF) cells were about 1/14 of the sensitivity of LMH cells (EC_{50}: 8.7 μg / ml). In mammalian cell lines, IEC-6 cells (EC_{50}: 18 μg/ml), 3Y1 cells (EC_{50}: 33 μg/ml), and MDBK cells (EC_{50}: 31 μg/ml) were even less sensitive, and CRFK, MDCK, and Vero cells (EC_{50}: 8.7 μg/ml) were less sensitive. CRFK cells, MDCK cells, Vero cells, P3U1 cells, C2C12 cells, L929 cells, and Caco-2 cells were not lethal even at a NetB toxin concentration of 50 μg/ml. Chicks died within 3 hr of intraperitoneal administration of NetB toxin (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. Therefor it could be concluded that the NetB toxin was biologically active and the toxicity was more specific to birds. From these results, NetB toxin has shown the possibility of specifically binding to cell surface molecules expressed only on birds.

Chapter 3. Binding and oligomerization of NetB toxin on avian cells

As the author has seen in Chapter 2 that significant hemolytic activity and cytotoxicity happen only in an avian cell line so we attempted to investigate the oligomerization and pore formation capabilities of NetB toxin only on chicken liver derived LMH cells. LMH cells were reacted with freshly purified NetB toxin solution (10 μg/ml) followed by lysis with morpholinoethanesulfonic acid-buffered saline containing 1% Triton X-114. Subsequently, the cell lysates were fractionated by sucrose density gradient ultracentrifugation and run on SDS–PAGE followed by immunoblotting. Results showed that both monomer and oligomer molecule of NetB toxin were localized in non-lipid raft region. Furthermore, to analyze the involvement of cholesterol in the cell membrane in NetB toxin binding and oligomer formation, methyl-β-cyclodex trin (MCD) was applied to LMH cells to remove cholesterol. After cholesterol removal, NetB toxin was reacted, dissolved using a surfactant. Then the cell lysate was centrifuged into soluble fraction and an insoluble pellet and then these both were immunoblotted using an anti-NetB toxin antibody. It was also immunoblotted using anti-actin antibody to differentiate the band intensity between oligomers found in MCD treated cells and MCD untreated cells. The result showed that oligomer of NetB toxin can be detected in the cells even after removal of cholesterol. These data suggested that this NetB toxin may recognize different membrane receptors or use a different mechanism for membrane-protein interactions than usual pore forming toxins do. In order to analyze the localization of the NetB toxin binding molecules on LMH cells, a toxin overlay assay was done followed by immunoblotting. In this assay, the LMH cells were separated into detergent-soluble and detergent-insoluble fractions and the soluble parts were fractionated by sucrose density gradient ultracentrifugation. Results showed that molecules of approximately 60, 53, 47, 40 and 25 kDa that were bound to NetB toxin were detected in the non-lipid raft supernatant fractions.

Conclusions

The author found that *netB*-carrying *C. perfringens* is widespread in Japan and infects healthy chickens. *NetB toxin* is on a zygotic plasmid, but it is not easily transmitted, and only a single or a small number of *netB*-carrying strains are thought to spread in the same area. NetB toxin was also a pore-forming toxin with highly specific toxin activity restricted to the pheasant order, to which chickens belong, and some birds.

It was found that NetB toxin binds to the target cell in the non-raft region without the involvement of cholesterol in the cell membrane, and that the nearby monomers polymerize to form oligomers without being affected by the fluidity of the membrane. Thus, the molecular dynamics of NetB toxin, from binding to the cell to pore formation, was partially elucidated.

Since *netB*-carrying bacteria are widely detected even in healthy chickens, prevention of the onset of necrotizing enterocolitis, such as vaccination, is considered to be important. In the future, the identification of binding molecules to NetB toxin and the determination of the region on the NetB toxin molecule involved in the dynamics from binding on cell membranes to pore formation will elucidate the pathogenic mechanism of necrotic enteritis in chickens and contribute to the development of vaccines.

審査結果の要旨

Clostridium perfringens は嫌気性、芽胞形成のグラム陽性大桿菌であり、産生する毒素（α, β, ε, ι, CPE, NetB）の産生性の違いから A～G 型に分類される。本菌は土壌や下水など環境中および動物の腸内細菌叢から分離されており、動物に様々な疾患を引き起こす。その一つが鶏壊死性腸炎であり、A および G 型菌まれに C 型菌によって引き起こされる。鶏壊死性腸炎は、世界の養鶏業に大きな経済的損失をもたらす、致死性の高い急性の伝染性疾病であり、発生軽減への対策が急務となっている。鶏壊死性腸炎の病原因子は、本菌が産生する α 毒素（ホスホリパーゼ C）であると考えられてきた。近年、本菌が産生する β 毒素とアミノ酸配列の相同性が 38%の新規蛋白毒素（NetB 毒素）が、壊死性腸炎の発症鶏から発見され、現在では、NetB 毒素が鶏壊死性腸炎の発症に関わる必須の病原性因子であると考えられている。NetB 毒素は細胞膜に結合後、7 量体となり孔を形成することで細胞の溶解を引き起こす孔形成毒素であり、G 型菌のみが産生する。本研究では NetB 毒素の病原性発現機構を解明する目的で、日本国内の壊死性腸炎発症鶏より分離した株から精製した NetB 毒素を用いて、種特異性および標的細胞膜上における分子動態の解析を行い、以下の成果を得た。

第 1 章では、わが国の壊死性腸炎発症鶏および健常鶏から *C. perfringens* を分離し，*netB* の有無を調べ, 株間の鶏赤血球溶血活性を比較した。また、パルスフィールドゲル電気泳動（PFGE）を用いて、遺伝子の系統樹解析を行いさらに薬剤感受性を比較した。本研究により，複数の農場において壊死性腸炎発症鶏と健常鶏の糞便から *netB* を保有する *C. perfringens* が分離されたことから、*netB* を保有する *C. perfringens* が日本国内に分布し，健常鶏にも感染していることを証明した。しかし，壊死性腸炎を発症した鶏からは *netB* 陰性菌も分離されており，壊死性腸炎の発症には他の病原因子も関与している可能性が示唆された．分離された *C. perfringens* において、*netB* の塩基配列は非常によく保存されていた。PFGE 解析では，異なる農場の壊死性腸炎発症鶏から分離された *netB* 保有株は, いずれも異なるタイプに分類された。これらの結果から、日本において、地域ごとに異なる *netB* 保有株が存在することが明らかとなった。

第 2 章では、壊死性腸炎発症鶏から分離された *C. perfringens* 培養上清より 2 種類のイオン交換クロマトグラフィーを用いて NetB 毒素（33kDa）を精製した。様々な哺乳類・鳥類の赤血球および株化細胞に対する NetB 毒素の溶血・細胞致死活性を測定した。NetB 毒素の種特異性は非常に高く、溶血および細胞致死活性はニワトリが属するキジ目や一部の鳥類に限定されており、哺乳類の赤血球や株化細胞に対してはほとんど毒性を示さなかった。ニワトリ肝癌由来株化細胞である LMH 細胞に対する 50%細胞致死量（EC_{50}）は 0.63 μg/ml と非常に高値を示した。NetB 毒素（50 μg）を 2 日齢ニワトリの腹腔内に投与したとき、 3 時間以内にすべての個体が死亡した。剖検では十二指腸から空腸への大量出血と盲腸の菲薄化が認められた。組織学的には腸管粘

膜の剥離・脱落および出血が確認され、壊死性腸炎発症鶏と同様の病態を示した。以上より，NetB 毒素は生物学的に活性を有しており，その毒性は鳥類に特異的であると結論づけた。

第 3 章 では NetB 毒素の活性は鳥類に対してのみ有していることから LMH 細胞を用いて、細胞膜上での NetB 毒素のオリゴマー化と孔形成能を調べた。LMH 細胞と NetB 毒素を反応させた後、細胞溶解物をショ糖密度勾配超遠心分離法で分画し、SDS-PAGE で分析した後、免疫ブロッティングを行った。その結果、NetB 毒素のモノマー分子とオリゴマー分子の両方が細胞膜上の非ラフト画分に存在していた。さらに、コレステロールを除去した LMH 細胞に対しても NetB 毒素のオリゴマーが検出されたことから、NetB 毒素の細胞膜への結合やオリゴマー化にはコレステロールが関与しないことが明らかとなった。また、LMH 細胞上の NetB 毒素と結合する分子が toxin overlay assay により非ラフト画分に検出された。

以上、本研究により *netB* を保有する *C. perfringens* は日本に広く蔓延しており、健常鶏にも感染していることが明らかとなった。NetB 毒素は、ニワトリが属するキジ目や一部の鳥類に限定された種特異性の非常に高い活性を有する孔形成性毒素であった。NetB 毒素は、細胞膜上の非ラフト領域で標的細胞に結合し、近傍のモノマー同士がコレステロールの関与なく重合してオリゴマーを形成することを明らかにし、NetB 毒素の細胞への結合から孔形成までの分子動態の一部を解明した。これらの成果は、今後の細胞膜上の NetB 毒素結合分子の同定や、細胞膜への結合から孔形成に至る動態に関与する NetB 毒素分子上の領域決定の一助となり、鶏壊死性腸炎の発症メカニズムの解明さらには、ワクチン開発に繋がり、獣医学および畜産学の発展に貢献するものである。本論文の審査ならびに最終試験の結果と併せて博士（獣医学）の学位を授与することを適当と認める。