財團法人明日醫學基金會專題研究計畫申請書

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研究型別	■個別型計畫 ☐整合型計畫					
計畫歸屬	☐自然處 ☐工程處 ■生物處 ☐人文處 ☐科教處 ☐永續會					
申請機構/系所(單位)	中國醫藥大學 醫學系					
本計畫主持人姓名	賴志河	職稱	副教授	身分證號碼		
本計畫名稱	中文	細胞膜膽固醇於細胞致死腫脹毒素誘導細胞致病所扮演的角色				
	英文	Role of cholesterol-enriched microdomains in cytolethal distending toxin-induced pathogenesis of cells				
整合型總計畫名稱						
整合型總計畫主持人				身分證號碼		
全程執行期限	自民國 101 年 1 月 1 日起至民國 101 年 12 月 31 日					
研究學門(請參考本申請書所附之學門專長分類表填寫)	學門代碼	名稱(如為其他類，請自行填寫學門)				
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項 目 名 稱	說明	單位	數量	單價	金額	備註
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消耗性器材	細胞培養耗材：無菌培養皿、無菌吸管、培養基、胎牛血清、液態氮、CO_2 氣體及離心管等	年	1	60,000	60,000	
消耗性器材	Reporter assay kit，ELISA assay kit 分析 IL-8，NF-κB 及 AP-1 promoter activity，每組十瓶(盤)，共五組	組	5	10,000	50,000	
消耗性器材	分子生物學實驗藥品與耗材：限制酶、聚合酶、電泳膠、緩衝液及 DNA marker 等	年	1	20,000	20,000	
消耗性器材	蛋白質電泳試劑與耗材：電泳配製試劑、protein marker、PVDF membrane 及濾紙等	批	1	30,000	30,000	
消耗性器材	吸管、玻璃瓶、塑膠瓶及血清瓶等耗材	年	1	28,000	28,000	

消耗性器材	清潔劑、酒精、有機溶劑及化學試劑等	批	1	12,000	12,000	
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Abstract

Campylobacter spp. is the most common zoonotic disease and causes of diarrhea worldwide. During slaughter and in subsequent processing steps, poultry carcasses may become contaminated with *Campylobacters*. Human disease likely results from improper handling or incomplete cooking of the contaminated poultry. *Campylobacter*-associated enterocolitis is typically associated with a local acute inflammatory response and involves intestinal tissue damage in both animal and human. Cytolethal distending toxin (CDT), produced by *Campylobacter*, is a putative virulence factor that induces cell-cycle arrest and apoptosis in eukaryotic cells. However, none of report explored the molecular mechanisms of *Campylobacter* CDT and its biological applications. Our preliminary study has been demonstrated that cell intoxication by CdtB delivery into the nucleus through the association of CdtA/CdtC and membrane cholesterol-rich microdomains. Translocation of CdtB into nucleus induced cell cycle arrested at G2/M phase. Therefore, in this proposal, we will extend our research to investigate the specific cholesterol-recognition sites on CdtA and/or CdtC in detail. Studies from this proposal, we anticipate inspire the future inauguration for application of agricultural microbe in biomedical industries.

Keywords: *Campylobacter jejuni*, cytolethal distending toxin, cholesterol

A. BACKGROUND AND SIGNIFICANCE

1. Infection of *Campylobacter jejuni*

Campylobacter jejuni (*C. jejuni*) is one of the most common causative agents of food-borne infectious illnesses in humans (1). Watery diarrhea is commonly seen in children infected with *Campylobacters* (2). Infection by this pathogen usually occurs in humans through consumption of contaminated poultry products (3). However, the virulence factors responsible for induction of host diarrhea remain to elucidate. Several reports have been revealed that *C. jejuni* adheres to and invades into host cells (4,5). Infection of cells with *C. jejuni* stimulates nuclear factor (NF)-κB, which mediates the production of several proinflammatory cytokines (6). In addition, *C. jejuni* induces the release of interleukin (IL)-8 from infected epithelial cells (7). Moreover, a bacterial membrane-associated protein–cytolethal distending toxin (CDT)–has subsequently been identified as one of the virulence factors required for the induction of IL-8 (8). Both bacterial invasion and secretion of CDT are thought to be correlated with *C. jejuni*-induced local acute inflammatory responses and involved in host enterocolitis.

2. Cytolethal distending toxin

Cytolethal distending toxin (CDT) is a tripartite protein toxin composed of three subunits—CdtA, CdtB, and CdtC (9)—encoded by an operon comprising *cdtA*, *cdtB*, and *cdtC* (10). Several bacterial species have been identified to contain CDT toxin including *C. jejuni* (11), *Shigella dysenteriae* (12), *Escherichia coli* (13), *Haemophilus ducreyi* (14), *Aggregatibacter actinomycetemcomitans* (15), and *Helicobacter hepaticus* (16). CDT holotoxin functions as an AB_2 toxin in which CdtA and CdtC serve as the binding (B) unit and CdtB is the active (A) unit (17). Previous study revealed that both of CdtA and CdtC can interact with cell membrane and enable translocation of the holotoxin across the cell membrane (18). The nuclear-translocated CdtB subunit exhibits type I deoxyribonuclease (DNase) activity, which causes DNA damage and in turn leads to cell-cycle arrest at the G2/M phase (19).

Studies of CdtA and CdtC functions are relatively limited compared with those of CdtB. Both CdtA and CdtC adopt lectin-type structures homologous to ricin, a plant toxin (18,20). The crystal structure of the *H. ducreyi* CDT revealed that there are two important binding elements of the toxin; one is an aromatic patch in CdtA, and the other is a deep groove at the interface of CdtA and CdtC (18). A structure-based mutagenesis study further demonstrated that mutations of the aromatic patch or groove impair toxin binding to the cell surface and reduce cell intoxication (21). A study of CDT from *A. actinomycetemcomitans* also revealed that both CdtA and CdtC not only bind to the cell surface but are associated with membrane lipid rafts (22). Lipid rafts are microdomains that contain large fractions of cholesterol, phospholipids, and glycosylphosphatidylinositol-anchored proteins (23). Studies *in vitro* showed that the structure of lipid rafts is stabilized in the cold non-ionic detergents such as Triton X-100 , but it can be disrupted by the cholesterol depletion agent methyl-β-cyclodextrin (MβCD) (24). A recent study of *A. actinomycetemcomitans* CDT revealed that the CdtC subunit contains a cholesterol recognition/interaction amino acid consensus (CRAC) region, which is required for CdtC binding to lipid membrane microdomains (25). This finding indicates that cholesterol provides an essential ligand for CDT binding to the cell membrane and

also serves as a portal for CdtB delivery into host cells for the induction of cell intoxication.

3. Rational and significance

Very few studies reported the association of *C. jejuni* or its toxin with cell receptors. Our recent study revealed that the association of the *C. jejuni* CDT subunits with the host membrane is mediated through a cholesterol-dependent manner (26). Binding of CDT subunits (CdtA and CdtC) to the cell membrane, delivery of CdtB into the nucleus, and G2/M arrest were all reduced when cellular cholesterol was depleted. Our preliminary results provide evidence that membrane cholesterol plays an essential role in the binding of *C. jejuni* CDT subunits to membrane rafts, which enhances cell cycle arrest and cell death in host cells. Further investigation regarding the specific cholesterol-recognition sites on CdtA and/or CdtC in detail will be executed in this proposal.

B. PRELIMINARY RERULTS

To develop and characterize the biological functions of CDT harboring anti-cancer activities, we first establish recombinant protein expression system to express and purify CDT derived from *C. jejuni*. We also have performed several preliminary studies to explore molecular mechanisms that explanation the toxin entry, delivery, and cytotoxicity of host cells.

1. Expression and functional analysis of recombinant *C. jejuni* CDT subunits

We first investigated the activity of recombinant *C. jejuni* CDT using Chinese hamster ovary (CHO-K1) cells. Each *C. jejuni* CDT subunit was cloned and expressed with a His-tag in *E. coli* BL21-DE3. Recombinant CDT subunits were then purified and analyzed by SDS-PAGE (Fig. 1A). The purified recombinant CDT subunits were readily detected by western blot using monoclonal anti-His (Fig. 1B). Western blot was carried out to determine whether polyclonal antibodies generated against each subunit could recognize the individual CDT subunits when assembled and associated with cells. As shown in Fig. 1C, the individual recombinant CDT proteins were recognized by the respective polyclonal CDT antiserum (anti-CdtA, anti-CdtB, or anti-CdtC). The polyclonal antisera were further used to investigate the association of the CDT subunits and cell membrane lipid rafts.

To characterize the biological function of *C. jejuni* CDT holotoxin, we examined its ability to induce cell distention in CHO-K1 cells. Our results revealed that each of the purified recombinant CDT proteins alone had no effect on the cell cycle and morphology of CHO-K1 cells after co-culture for 48 h (Fig. 1D). However, upon cell exposure to CDT holotoxin (200 μM each subunit) for 48 h, cell-cycle analysis showed G2/M arrest and microscopy indicated cell distention (Fig. 2). These results demonstrated that the three recombinant *C. jejuni* CDT subunits interact with each other and deliver CdtB into the target cells, which resulted in cell-cycle arrest at G2/M.

2. Cholesterol is required for *C. jejuni* CdtA and CdtC association with the cell membrane

We then assessed whether CDT subunits can associate with the membrane cholesterol–rich microdomains (i.e., detergent-resistant membrane, DRM). CHO-K1 cells were first incubated with

200 μM of individual CDT subunits for 2 h at 4°C. Subsequently, the cells were analyzed by flow cytometry for the presence of CDT proteins on the cell membrane. As shown in Fig. 2 (upper panel), CdtA and CdtC were associated with the cell membrane. The mean channel fluorescence (MCF) for anti-CdtA and anti-CdtC was 40.2 and 108.3, respectively. However, the MCF for anti-CdtB was only 11.4. When CHO-K1 cells were pretreated with 10 mM MβCD for 1 h, the MCF was reduced for both anti-CdtA (22.8) and anti-CdtC (55.8) (Fig. 3, lower panel), but the MCF of anti-CdtB did not change when compared with the cells not treated with MβCD. These data suggested that both CdtA and CdtC but not CdtB are capable of interacting with DRM.

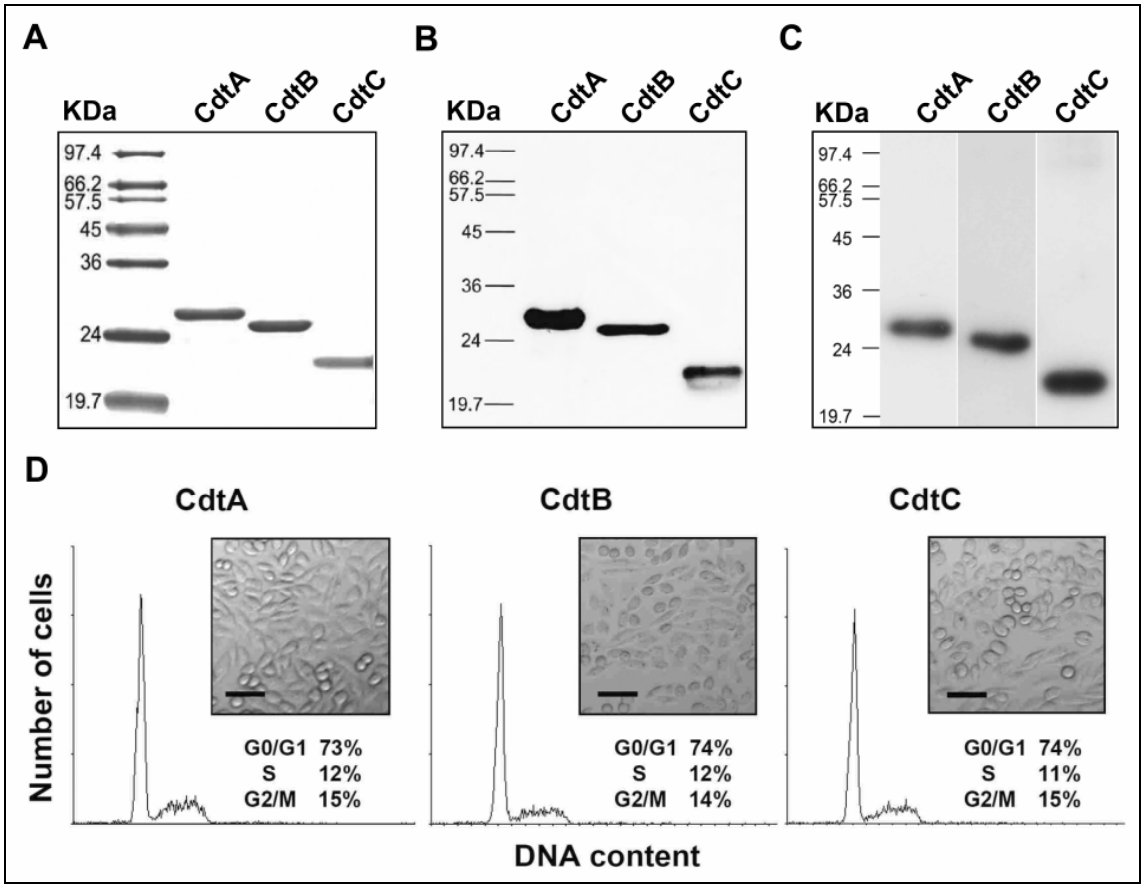

Figure 1. Purification and characterization of each recombinant *C. jejuni* CDT subunit. (A) Recombinant CDT proteins were expressed and purified. Each CDT subunit was subjected to SDS-PAGE and stained with Coomassie brilliant blue. (B) Each cloned CDT subunit was isolated and purified from bacterial extracts and verified by western blot analysis with a monoclonal antibody specific to the His-tag. (C) Western blot was conducted on extracts of CHO-K1 cells exposed to the CDT holotoxin and assessed by antisera against CdtA, CdtB, and CdtC, respectively. Molecular weight markers (kDa) are shown at left. (D) CHO-K1 cells were exposed to each purified recombinant CdtA, CdtB, or CdtC at 37°C for 48 h.

3. Delivery of CdtB into cells requires the association of CdtA and CdtC with lipid microdomains.

To further test whether CdtB transport to the host cells is dependent on the association of CdtA and CdtC with membrane lipid rafts, cells were exposed to CDT holotoxin (200 μM each subunit) at 37°C for 1 h to 6 h. As expected, pronounced cytoplasmic distribution of CdtB (green) was

observed at 2 h, and significant nuclear localization after 6 h in the absence of MβCD (Fig. 4A, upper panel). However, intracellular distribution of CdtB was dramatically reduced upon pretreatment with 10 mM MβCD (Fig. 4A, lower panel). We then further examined whether localization of CdtB in the cell nucleus was dependent on the presence of cholesterol. Cells were left untreated or treated with 10 mM MβCD for 1 h and exposed to CDT holotoxin for 0 to 6 h at 37°C. As shown in Fig. 3B, the localization of CdtB in the nucleus increased after incubation in the absence of MβCD, but CdtB localized in the nucleus was almost completely blocked upon MβCD treatment. Taken together, these observations suggest that the association of *C. jejuni* CdtA and CdtC with lipid rafts is important for CdtB binding and transport to target cells.

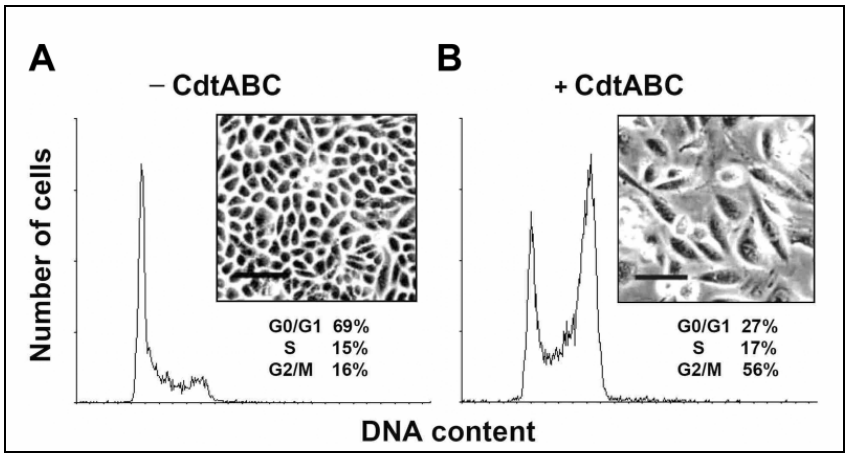

Figure 2. Cell-distending activity of recombinant *C. jejuni* Cdt subunits in CHO-K1 cells. CHO-K1 cells were untreated (A) or treated (B) with *C. jejuni* CDT holotoxin (CdtABC, 200 μM each subunit) for 48 h at 37°C. Cells were then examined under an inverted optical microscope for the effects of Cdt intoxication.

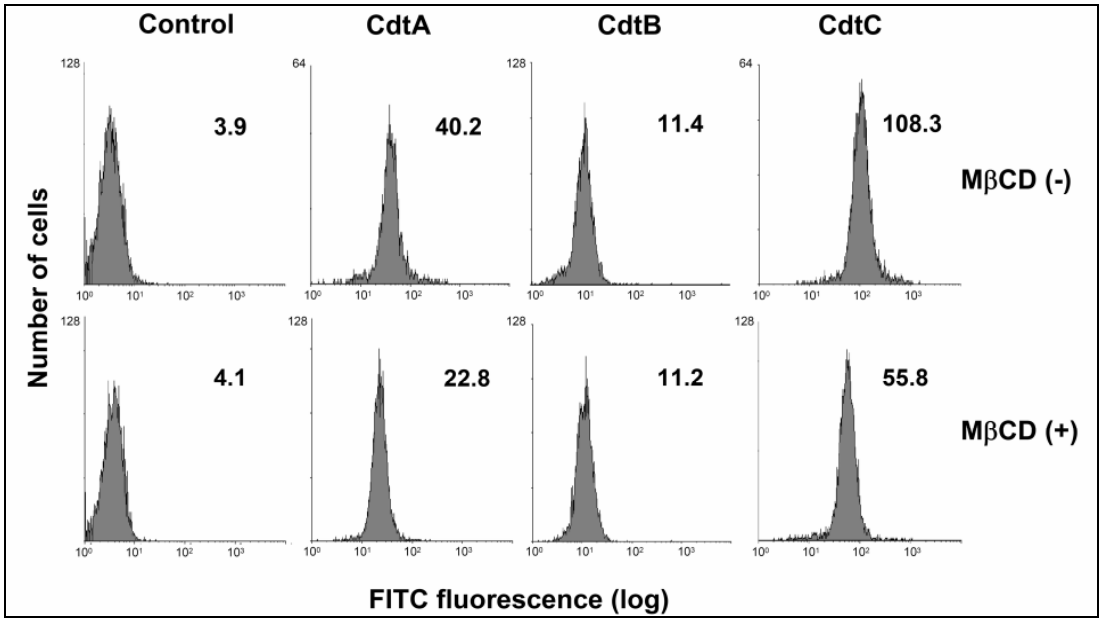

Figure 3. Sufficient cellular cholesterol is essential for CdtA and CdtC binding to CHO-K1 cells. Cells were untreated (upper panel) or treated (lower panel) with 10 mM MβCD for 1 h at 37°C followed by exposure to 200 μM of each recombinant *C. jejuni* CDT protein. After incubation with individual CDT proteins for 2 h at 4°C, cells were stained with control pre-immune serum or individual antiserum against each CDT subunit and stained with FITC-conjugated anti-mouse IgG. Binding activity was assessed by flow cytometry.

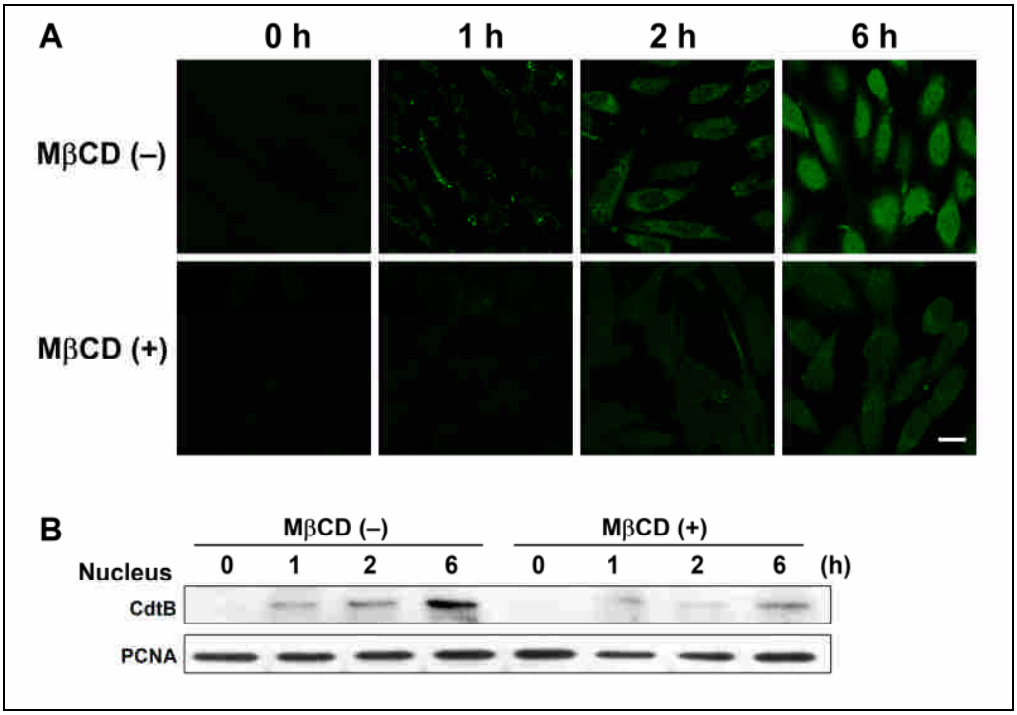

Figure 4. Depletion of cholesterol prevents *C. jejuni* CdtB localization in the nucleus. (A) CHO-K1 cells were untreated or treated with 10 mM MβCD for 1 h prior to expose to CDT holotoxin (200 μM each subunit) at 37°C for the indicated times. Cells were washed and stained with anti-CdtB antiserum followed by stained with FITC-conjugated anti-mouse IgG. The stained cells were then analyzed by confocal microscopy. (B) Nuclear fraction of cell lysates was prepared from CHO-K1 cells untreated or treated with 10 mM MβCD for 1 h followed by incubation at 37°C in the presence of CDT holotoxin for the indicated times. CdtB in the nucleus of cell lysates was detected by western blot.

4. Depletion of cholesterol prevents CDT-induced cell-cycle arrest

To determine whether *C. jejuni* CDT-induced cell cycle arrest is dependent on membrane rafts, we further investigated whether the integrity of cholesterol-rich microdomains is essential for CDT holotoxin-induced cell-cycle arrest. Only 17% of CHO-K1 control cells incubated without CDT holotoxin were arrested in G2/M (Fig. 5A). Upon exposure to 2 μg/ml ICRF-193, a DNA topoisomerase II inhibitor (27), 60% of cells were arrested in G2/M (Fig. 5B). Cells were treated with 10mM MβCD at 37°C for 1 h, the treatment alone did not alter the CHO-K1 cell cycle. CHO-K1 cells were then pretreated with 0, 5, or 10 mM MβCD at 37°C for 1 h. After removing MβCD, cells were incubated for 24 h in the presence of CDT holotoxin. A significantly higher number of cells exposed to CDT holotoxin were arrested in G2/M compared with cells not exposed to CDT holotoxin (Fig. 5A and C). When cells exposed to CDT holotoxin were pretreated with 0, 5, or 10 mM MβCD, the number of cells arrested in G2/M decreased in a dose-dependent manner (Fig. 5C–E). Thus, cholesterol depletion by MβCD, which disrupts the integrity of rafts, also interrupted CDT action, leading to the reduction of G2/M arrest. Upon replenishment of cholesterol, the inhibitory effect of MβCD on CDT-induced G2/M arrest was reversed (Fig. 5F). Together, these results suggest that the presence of sufficient cholesterol in lipid raft microdomains is required for the activities of *C. jejuni* CDT.

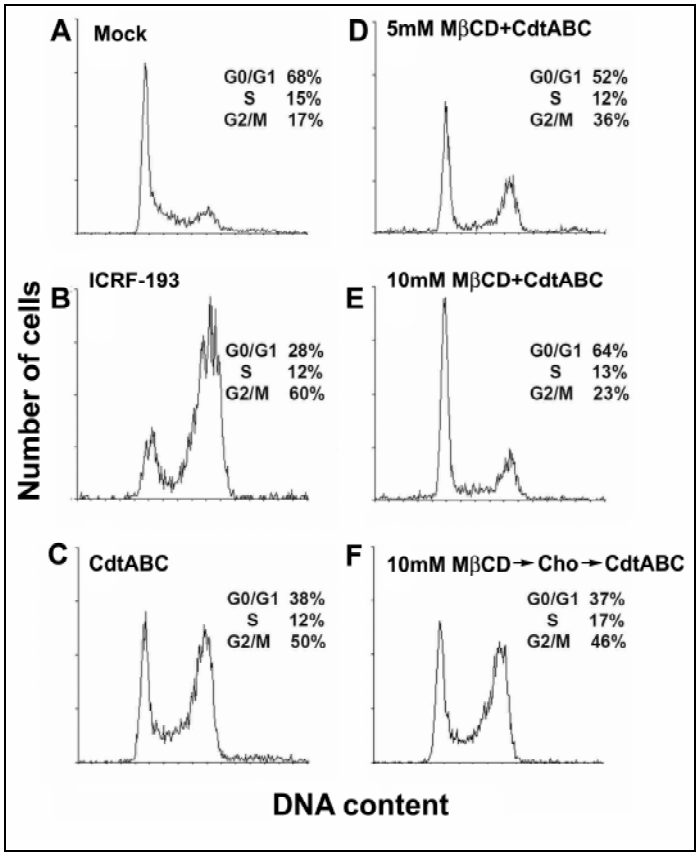

Figure 6. Sufficient cellular cholesterol is essential for *C. jejuni* Cdt-induced cell-cycle arrest. CHO-K1 cells were pre-exposed to medium alone (A–C), 5 mM MβCD (D), 10 mM MβCD (E), or treated with 10 mM MβCD and replenished with cholesterol (400 μg/ml) (F) for 1 h at 37°C. Cells were then incubated for 24 h at 37°C in the presence of medium (A), ICRF-193 (B), and *C. jejuni* CDT holotoxin (C–F). Cell-cycle distribution was based on the DNA content analyzed using flow cytometry. The percentage of cells in the G0/G1, S, and G2/M phases of the cell cycle are indicated at right. Results represent one of three independent experiments.

C. RESEARCH DESIGN AND METHODS

Although CdtA and CdtC were found to have the ability on the interaction of membrane cholesterol-enriched microdomains, the exact binding sites on the amino acid sequence of CdtA and/or CdtC remained unclear. Whether the structure and orientation of cholesterol-recognition motifs are important for the interaction of CdtA/CdtC and cholesterol needed further explored.

Specific aim 1. Determination of cholesterol-recognition sites of CdtA/CdtC. The cholesterol recognition amino acid consensus (CRAC) region contains a conserved pattern $L/V(X)_{1-5}Y(X)_{1-5}R/K$, which is present in all proteins that associate with cholesterol (28). A recent report showed that the *A. actinomycetemcomitans* CdtC subunit contains a CRAC region, which contributes to the interaction between CdtC and cholesterol (25). Our present data indicated that both CdtA and CdtC were mainly localized in the cholesterol-rich microdomains. Thus, these evidences indicated that *C. jejuni* CdtA and CdtC may contain a hypothetical CRAC region, which contribute to cholesterol-binding activity. However, whether the CRAC-like motif is the most important region for the interaction of *C. jejuni* CdtA and CdtC with cholesterol requires further investigation.

***Approach 1*. Prediction of cholesterol-binding sites on CdtA/CdtC.** Analysis of the motif within the *C. jejuni* CdtC subunit also revealed a CRAC-like region (^{77}LPFGYVQFTNPK^{88}). This analysis suggests that the CRAC region, essential for the association of CdtC with cholesterol, is commonly preserved in these bacterial species. Moreover, our analysis of the *C. jejuni* CdtA amino acid sequence also revealed a CRAC-like region (^{17}LYACSSK^{23}). These sequence analyses indicate that

although CdtA and CdtC have diverged, both have a CRAC-like region that may contribute to cholesterol-binding activity.

***Approach 2*. Construction of CdtA/CdtC CRAC mutants.** To determine whether the CRAC region was required for cell surface interaction and the downstream toxic effects of CDT, we will generate a single-point mutant of CdtA and CdtC. Amino acid substitution will be introduced into the *cdtA* or *cdtC* gene by *in vitro* site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange II site-directed mutagenesis kit (Stratagene) according to the manufacturer's directions. Amplification of the mutant plasmid was carried out using PfuUltra HF DNA polymerase (Stratagene). The mutation was verified by DNA sequencing.

***Approach 3*. Analysis of the biological activity of CdtA/CdtC CRAC mutants.** CHO-K1 cells were incubated for 2 h in the presence of medium or wild-type or mutant CdtA or CdtC at 4°C. Cells were washed, exposed to normal mouse IgG and then stained for cell surface CdtA/C peptides with each anti-CDT subunit monoclonal antibody conjugated to Alexa fluor 488 (Invitrogen) according to the manufacturer's directions. After washing, the cells were fixed in 4% paraformaldehyde and analyzed by flow cytometry or confocal microscopy.

***Possible problems and alternatives*.** Since the presence of a putative CRAC domain predicted based on the consensus "L/V(X)1-5Y(X)1-5K/R" is highly hypothetical, a detailed analysis of other candidates for cholesterol binding sites is required. Not all proteins that bind cholesterol have CRAC domains. For instance, in a very recent report revealed that cholesterol dependent cytolysin family of toxins containing a threonine-leucine pair mediates its recognition and binding to membrane cholesterol (29). Thus, the search for cholesterol recognition motif required more extensive scanning.

Anticipated outcome. The results from this proposal will reveal that membrane cholesterol serves as an essential ligand for CdtA or CdtC and that this association can be blocked by mutation of the cholesterol-recognition sites.

Specific aim 2. Structural analysis of cholesterol-recognition motif in CdtA and CdtC. If motif analysis of CdtA or CdtC identified CRAC site within each CDT subunit, the location of the CRAC site may be at the surface of the molecules and accessible to the membrane. The data from this proposal may revealed that binding of cholesterol by the CRAC region contained in the CdtA and/or CdtC subunits respond for the association of the CDT holotoxin with membrane lipid rafts.

***Approach 1*. Motif analysis of CdtA/C binding to cholesterol.** We next extended our studies to consider the possibility that binding subunits, CdtA and CdtC, contain a cholesterol recognition motif. Motif analysis using homologous modeling will be subjected of each CDT identified a CRAC site within the CdtA and/or CdtC subunits.

***Approach 2*. Cholesterol dot blot analysis.** To further assess the binding activity of each CDT or their mutants to cholesterol, a cholesterol dot blot analysis will be employed. A PVDF membrane was placed into a dot blot apparatus to which water-soluble cholesterol were seriously diluted from 1 μg/ml to 200 μg/ml in each set of 12-well. After the cholesterol was pulled through the membrane

by vaccum, the membrane was then blocking in skim milk. Each set of 12-well was cut into strip and incubated with wild-type or mutant CdtA/C. The membranes were incubated with primary antibodies against CdtA or CdtC, and then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen). The proteins of interest were visualized using the ECL Detection Reagents (GE Healthcare) and detected using X-ray film (Kodak).

***Approach 3*. Homologous competitive binding assay.** The wild-type or mutant CdtA or CdtC were labeled with biotin and allowed to compete with unlabelled toxin for binding sites liposome and their dissociation constant (Kd) will be determined. An artificial cholesterol-containing liposome for assessing CDT binding activity was prepared as described previously (29). Fixed concentration of biotinylated CDT and increasing concentrations (0 nM to 200 nM) of unlabelled CDT were added to liposome at 37°C for 1 h. All unbound toxins were removed by centrifugating at 1200 rpm for 10 min at room temperature and the supernatant removed. Detection of the biotinylated CDT was by the streptoavidin method. The dissociation constant was calculated by determining the IC50 (dose at which 50% displacement of the biotinylated CDT occurred).

***Approach 4*. Immunofluorescence labeling of CDT-treated cells.** To visualize localization of wild-type or mutant CdtA/CdtC in cells, CHO-K1 cells were seeded on coverslips in six-well plates and incubated for 20 h. Cells were cultured with each CDT subunit and then washed three times with PBS and fixed with paraformaldehyde (Sigma-Aldrich). The cells were then permeabilized and stained with Alexa Fluor 647–conjugated CTX-B (Molecular Probes). To label the individual wild-type or mutant CDT subunit, samples were incubated for 30 min with anti-CdtA, anti-CdtB, or anti-CdtC antiserum followed by fluorescein isothiocyanate (FITC)-conjugated AffiniPure goat anti-mouse IgG (Jackson ImmunoResearch). Samples were analyzed under a confocal laser scanning microscope (Zeiss LSM 510, Carl Zeiss) with a 100× objective (oil immersion, aperture 1.3).

Anticipated outcome. The results from this proposal will provide significant evidence that *C. jejuni* CdtA and CdtC may contain cholesterol-recognition motifs, which contribute to cholesterol-binding activity and thus assist CdtB delivery into host cells.

D. CONCLUDING REMARKS

The main focus of this grant proposal is to reveal molecular basis of *Campylobacter* CDT in the interaction of cholesterol-enriched microdomains and induced pathogenesis of host. Here, we will develop novel technology to apply the bacterial toxin in the cancer therapy. We plan to establish and consolidate a program with international quality of science, integrate experimental and theoretical means of multi-disciplinary research into a four-year road map. We will address bacterial toxin-cell interactions at the subcellular level. Based on our previous results from this peculiar microbe as well as understanding of biological functions of CDT, we are confident in executing this proposal.

This project will emphasize not only basic research with novel approaches but also translational research with potential clinical applications. In addition, we also anticipate to convey our outcomes

for start-up molecule-based biotech industries. The advanced methodology from multi-disciplinary areas will also be developed. The benefits in executing this proposal are:

(1) We will address scientific issues related to the molecular mechanisms of *Campylobacter* CDT pathogenesis.

(2) Additionally, we will train graduate students and research assistants to be independent and skillful biologists who will be the future human resource for the biologic research and biotech industry in Taiwan.

(3) We expect to have one or two high-quality publications, which contain cutting edge technology, tools and new information of microbiology.

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