Cloning and protein expression of *Aggregatibacter actinomycetemcomitans* cytolethal distending toxin C

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ABSTRACT

Purpose: *Aggregatibacter actinomycetemcomitans* was associated with localized aggressive periodontitis, endocarditis, meningitis, and osteomyelitis. The cytolethal distending toxin (CDT) of *A. actinomycetemcomitans* was considered as a key factor of these diseases is composed of five open reading frames (ORFs). Among of them, An enzymatic subunit of the CDT, CdtB has been known to be internalized into the host cell in order to induce its genotoxic effect. However, CdtB can not be localized in host cytoplasm without the help of a heterodimeric complex consisting of CdtA and CdtC. So, some studies suggested that CdtC functions as a ligand to interact with GM3 ganglioside of host cell surface. The precise role of the CdtC protein in the mechanism of action of the holotoxin is unknown at the present time. The aim of this study was to generate recombinant CdtC proteins expression from *A. actinomycetemcomitans*, through gene cloning and protein used to investigate the function of Cdt C protein in the bacterial pathogenesis

Materials and Methods: The genomic DNA of *A. actinomycetemcomitans* Y4 (ATCC29522) was isolated using the genomic DNA extraction kit and used as template to yield *cdtC* genes by PCR. The amplifed *cdtC* genes were cloned into T-vector and cloned *cdt C* gene was then subcloned to pET28a expression vector. The pET28a-cdtC plasmid expressed in BL21 (DE3) *Escherichia coli* system. Diverse conditons were tested to opitimize the expression and purification of functional CdtC protein in E. coli.

Results: In this study we reconstructed CdtC subunit of *A. actinomycetemcomitans* Y4 and comfirmed the recombinant CdtC expression by SDS-PAGE and Western Blotting. The expression level of the recombinant CdtC was about 2% of total bacterial proteins.

Conclusion: The lab condition of procedure for the purification of functionally active recombinant CdtC protein is established. The active recombinant CdtC protein will serve to examine the role of CdtC proteins in the host recognition and enzyme activity of CDT and investigate the pathological process of *A. actinomycetemcomitans* in periodontal disease.

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KEY WORDS: *Aggregatibacter actinomycetemcomitans;* localized aggressive periodontitis Cytolethal distending toxin(CDT); cloning; recombinant protein.

Introduction

Aggregatibacter actinomycetemcomitans, a small non-motile gram-negative coccobacillus, is highly associated with localized aggressive periodontitis (LAP)[1]. Although the pathogenic mechanism of *A. actinomycetemcomitans* is not known, it does produce several potential virulence factors capable of facilitating colonization, destroying host tissue, inhibiting tissue repair, and interfering with host defense. These virulence factors were included cytotoxic factors, chemotactic inhibitor, collagenases, lipopolysaccharide(LPS), and cytolethal distending toxin (CDT)[1].

The CDT activity was first described for cell extracts of *Camphylobacter* spp. and *E. coli* clinical isolates. CDTs are present in a large spectrum of gram-negative bacterial species, all known as potential mucosal pathogens in man and animals[2-7]. The term CDT was coined for activity that induced progressive cell distension and eventual cytotoxicity on cultured cells[8].

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Statistical analysis in Sweden indicated that 86% (43 of 50 strains) of *A. actinomycetemcomitans* from patients with periodontitis expressed a cytotoxic activity, and those strains were all positive for the three *cdt* gene[9]. Another report from the Republic Singapore revealed that among the 106 subgingival plaque samples, 13 sites (12%) were positive for the bacteria with the *cdt* genotype of *A. actinomycetemcomitans*. This may indicate a relatively low occurrence of CDT-producing strains. However, 10 of the 13 positive sites were obtained from patients diagnosed with aggressive periodontitis (77%), suggesting the strong association between CDT production and this severe type of periodontitis[10]. Epidemiological studies indicated a high frequency of CDT-positive *A. actinomycetemcomitans* in patients with periodontitis, suggesting its role in the onset and/or exacerbation of periodontitis, but the molecular pathophysiology of CDT in the disease still remains obscure.

The CDT gene of *A. actinomycetemcomitans* is composed of five open reading frames (ORFs): orf1, orf2, cdtA, cdtB, and cdtC[8]. The orf1 and orf2 located upstream of the cdtA gene are probably transcribed together with the three cdt genes. Still, it is not known whether orf1 and orf2 are actually translated in vivo. A growing amount of evidence supports a model of CDT intoxication by which CdtB delivery into the cells is mediated by CdtA and CdtC[11-13]. Once inside the cell, CdtB can translocate to the nucleus and induce DNA lesions, then will activate DNA damage response cascades and cause cell cycle arrest[12-14]. Very limited data are available about the function of CdtA and especially of CdtC. Many studies find that both CdtA and CdtC are capable of binding to the cellular membrane and some suggested that they might share the same receptor[15-18]. Reports indicating that antibodies raised against CdtC were protective against CDT toxicity corroborated these findings. Also, crystal structure of the CDT holotoxin from *Haemophilus ducreyi* revealed that the N terminus of CdtC created a steric block that seems to be a self-regulatory mechanism for CDT, possibly to prevent indiscriminate DNase activity.

Therefore, the aim of this study was to generate active recombinant CdtC proteins from *A. actinomycetemcomitans*, through gene cloning and protein expression. In fllowing study, the recombinant CdtC proteins was utilized used to generate anti-CdtC antibody and investigate the function of Cdt C protein in the bacterial pathogenesis

Materials and Methods

1. Bacterial strains and culture conditions

A. actinomycetemcomitans Y4 (ATCC 29522) was cultured in Brain heart infusion (BHI) broth at 37°C in anaerobic chamber containing 85% N_2, 10% H_2, and 5% CO_2[19]. Growth was monitored by optical density at 600nm, with all batch cultures having an initial O.D. 600nm of approximately 0.01 after sub-culturing from overnight growth. For the expression of recombinant CdtC subunit, *Escherichia coli* strain, BL21 (DE3), was cultured in Luria-Bertari (LB) medium at 37° C with shaking at 200 rpm[2].

2. PCR amplification of *cdtC* gene

The extracted genomic DNA was used as templates for amplifying *cdtC* gene. The specific oligonucleotide primer for *cdtC* gene was designed synthesized as described in Table 1. PCR was performed in 50μl reaction mixtures containing DNA template, *Taq* DNA polymerase, each deoxyribonucleotide triphosphate (dNTP), primers, 10×PCR buffer. The genomic DNA was denatured by 4 min incubation at 95°C and then *cdtC* gene was amplified for 20 cycles of PCR reaction (denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s). Then, the PCR products was extended for 5 min at 72°C and stored at 4°C. The PCR products were visualized by gel electrophoresis on 1% agarose gel followed by staining with ethidium bromide and illumination with UV light.

Table 1 Oligonucleotides primers used to amplify *cdtC* gene

	sequence
cdtC f	Cgggatcc ACT ACT TAT CCT GAT GTA GAG
cdtC r	Ccgctcgag TTA GCT ACC CTG ATT TCT CCC

The underline sequences are the location of restriction enzyme sites.-

3. Cloning of *cdtC* gene

Amplified PCR products were ligated into pGM-T vector. The ligation mixtures were transformed into *E. coli* TOP10 competent cells. The transformants were cultured on LB agar containing ampicillin. The resulting ampicillin resistant bacteria were selected from LB agar and screened by checking the plasmid from each bacterial colony. The plasmids containing expected insert size were then sequenced to confirm its integrity by Xenotech (Korea, Sungnam). The DNA sequence of cloned *cdtC* gene was analyzed by comparing with the previously known sequences. The known sequence from *A. actinomycetemcomitans* Y4 (GenBank GI9955924) was used as standard to generate sequence alignment between two genes.

4. Subcloning of *cdtC* gene

The gene fragments of *cdtC* were prepared by digesting with restriction enzyme Xho Ⅰ, and BamH Ⅰ. The digested fragments were then subcloned to the corresponding sites of pET28a expression vector. The ligation mixtures were transformed into *E. coli* TOP10 competent cells. The transformants were cultured on LB agar containing kanamycin. The resulting kanamycin resistant bacteria were selected from LB agar and then transformed into *E. coli* BL21 (DE3) competent cells.

5. Expression of recombinant CdtC protein

The transformed BL21 (DE3) bacterial colonies were inoculated and cultured into 5ml LB broth containing 50ug/ml kanamycine and incubated at 37°C for overnight with shaking at 200rpm. When all batch cultures have an initial O.D. 600nm of approximately 0.5, they were transferred into the 500 ml LB broth containing 50ug/ml kanamycine and grown at 37°C with vigorously shaking until an optical density of 0.8 was reached. In order to induce expression of His6-tagged recombinant CdtC protein, isopropyl-b-D-thiogalactopyranoside (IPTG) was added to a final concentration 1mM and further cultured for 4 h at 37°C.

6. Isolation of recombinant CdtC protein

Induced bacteria were harvested using centrifugation at 6,000rpm for 10min, and then the pellet was suspended in phosphate buffer saline (PBS: 137mM NaCl, 2.7mM KCl, 8.1mM Na_2HPO_4, and 1.5mM KH_2PO_4, pH 7.3). The suspended bacteria were sonicated for 30 s bursts on ice. The sonicated samples were centrifuged at 13,000rpm for 10min and then the supernatant was collected as the soluble fractions. The remaining pellets were washed with PBS containing 0.01% Triton X-100 and then re-suspended in PBS to obtain the inclusion body.

7. SDS-PAGE and Western blots analysis of *CdtC* protein

The fractions containing recombinant protein (His6-tagged protein) were dialyzed and then resolved by SDS-PAGE. Recombinant CdtC proteins were mixed with 100㎕ of gel loading buffer and heated in a boiling water bath for 5 min. The samples were applied to 10~20% polyacrylamide gel. After electrophoresis, protein bands were transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA in

TBS containing 0.1% Tween-20 (TBST) buffer for 1h at room temperature with shaking. The blocked membrane was incubated with Ni-NTA tagged with AP (alkaline phosphate) and then washed in TBST. The membrane was treated with BCIP/NBT to induce developing reaction and incubated in the dark. The membrane was rinsed with H_2O to stop the developing reaction.

Results

1. PCR products of *cdtC* gene

The gene for CdtC was amplified by PCR using the genomic DNA. By used primer, PCR reaction yielded 483 bp of the *cdtC* gene (Fig. 1). The PCR band was observed at the expected position (around 500 bp) of the *cdtC* gene.

2. Cloning into T vector of *cdtC* gene

The T-*cdtC*, were transformed into *E. coli* TOP10 plasmids were prepared from ampicillin-resistant colonies and separated in agarose gel (Fig. 2). In an agarose gel of plasmids from the transformed bacteria, the first and fourth lane bands are higher than the others because it includes insert DNA. The first and fourth lane showed the corresponding size of T vector plus *cdtC* gene, while others were not.

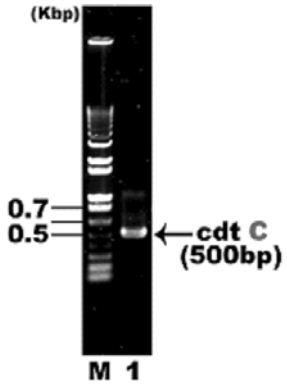

Figure 1. PCR amplification of the cdtC gene of A. actinomycetemcomitans Y4
The gene for cdtC was amplified by PCR using the genomic DNA. The PCR band (Lane 1) was observed on 500 bp position of the amplified cdtC gene.

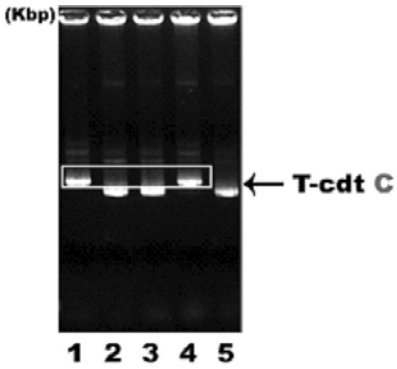

Figure 2. Screening of plasmids cloned with cdtC gene
The genes encoding CdtC were cloned into T vector and the bacterial transformants were screened for the presence of insert DNA. Arrow indicated the position of plasmids with insert DNA.

3. Sequence6 analysis of cloned genes.

The representative sequence alignment was shown in Fig. 3. In this 483bp sequence, there was no miss match at all.

4. Subcloning into pET28 expression vector.

The bacteria containing the expected *cdtC* gene were screened by kanamycin-resistancy and the isolated plasmids were separated in agarose gel (Fig. 4). Two colonies containing pET-*cdtC* plasmid were obtained (lanes 2 and 5).

Figure 3. *Sequence alignment between cloned cdtC and the corresponding region in reported cdtC gene*
The sequence of the cloned cdtC gene was compared with that of the known cdtC gene (GenBank GI9955924).

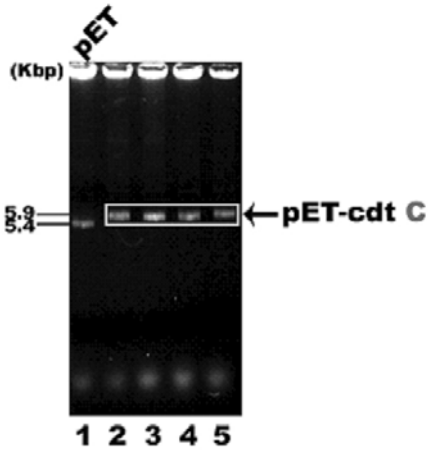

Figure 4. *Screening of plasmids of cdtC cloned in pET28*
The pET-cdtC plasmid was transformed in BL21 (DE3) bacterial strains, and their transformants were screened for the presence of the cdtC gene. Arrow (5.9kbp) indicated the position of plasmids with insert DNA.

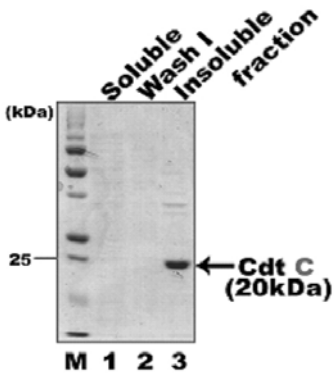

Figure 5. *SDS-PAGE of recombinant CdtC*
Insoluble fractions (Lane 3) contained the recombinant CdtC protein.

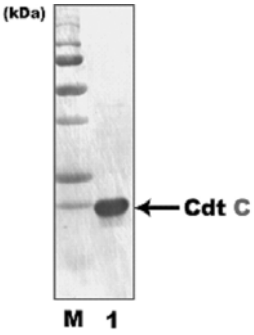

Figure 6. *Western blot of His6-tagged CdtC protein*
The colored region on Lane 1 indicated the presence of His6-tagged CdtC protein.

5. Expression and isolation of recombinant CdtC

After isolation of recombinant CdtC protein, the fractions prepared during the isolation procedure were analyzed by SDS-PAGE (Fig. 5). CdtC band (20kDa) was clearly shown in the SDS-PAGE gel. Western blotting with anti- His6-tagged antibody also confirmed that, this band was the His6-tagged CdtC protein (Fig. 6). The expression level of the recombinant CdtC was about 2% of total bacterial proteins.

Discussion

A. actinomycetemcomitans has served as a model system for studying the contribution of pathogenic bacterial species and their products to the initiation and progression of oral infectious disease. This bacterium has a strong historical association with localized aggressive periodontitis (LAP)[1].

In this study, *cdtC* genes from *A. actinomycetemcomitans* were cloned and sequenced. Sequence analyses of the cloned cdtC gene showed that the nucleotides sequence of cloned genes had perfect homology to the sequences previously reported from *A. actinomycetemcomitans* Y4. Recombinant CdtC proteins were expressed and purified. These recombinant CdtC proteins are inactive form, thus these should be changed to active form by refolding. Diverse conditons were tested to opitimize the expression and purification of functional CdtC protein in E. coli system. The lab condition of procedure for the purification of functionally active recombinant CdtC protein is established.

The various cell populations in the periodontium respond differently to the CDT of *A. actinomycetemcomitans*. Gingival fibroblast and periodontal ligament cells become distended and dually arrested in G1 and G2/M[20], oral epithelial cells become distended and arrested solely in G2/M[21]. whereas T-lymphocytes do not un-

dergo morphological changes but are arrested in G2/M and become rapidly apoptotic[22,23]. These differences in response to the toxin may reflect different roles of the cells in the pathogenesis of localized aggressive periodontitis (LAP), in which *A. actinomycetemcomitans* is highly implicated. Although it remains unclear if there is an association between the presence of CDT and the occurrence of this disease several hypotheses have been raised con concerning the putative role of the toxin in the pathogenesis of the disease[10]. By inducing growth arrest in periodontal fibroblasts and oral epithelial cells, the toxin may interfere with the normal periodontal connective tissue remodeling equilibrium or may facilitate the invasion and multiplication of *A. actinomycetemcomitans* in the deeper periodontal tissues[21].

Alternatively, growth inhibited T-lymphocytes in the periodontal environment may result in impaired local immunity to bacterial challenge[22,23]. The proliferation and turnover rate of the periodontal connective tissues in adolescents is higher than in adults. Therefore, potential inhibition of proliferation may be more detrimental in young than in adult periodontal tissues, mirroring the nature of aggressive periodontitis[2].

In consideration of signification of CDT in cell cycle, active recombinant CdtC protein will serve to investigate the pathological process of *A. actinomycetemcomitans* in periodontal disease. In the future study, the anti-CdtC antibody will be generated to examine the role of CdtC proteins in the host recognition and enzyme activity of CDT. Also, Recombinant CDT toxin is good vaccine material and antibody against CDT can be used for neutralization or for a detection kit.

References

1. Henderson B, Nair SP, Ward JM, Wilson M.: Molecular pathogenicity of the oral opportunistic pathogen *Actinobacillus actinomycetemcomitans*. Annu Rev Microbiol. 2003; 57:29-55.
2. Pickett CL, Cottle DL, Pesci EC, Bikah G.: Cloning, sequencing, and expression of the Escherichia coli cytolethal distending toxin genes. Infect Immun. 1994;62:1046-51.
3. Okuda J, Kurazono H, Takeda Y.: Distribution of the cytolethal distending toxin A gene (cdtA) among species of Shigella and Vibrio, and cloning and sequencing of the cdt gene from Shigella dysenteriae. Microb Pathog. 1995;18:167-72.
4. Pickett CL, Pesci EC, Cottle DL, *et al*.: Prevalence of cytolethal distending toxin production in Campylobacter jejuni and relatedness of Campylobacter sp. cdtB gene. Infect Immun. 1996;64:2070-8.
5. Young VB, Knox KA, Schauer DB.: Cytolethal distending toxin sequence and activity in the enterohepatic pathogen Helicobacter hepaticus. Infect Immun. 2000;68:184-91.
6. Purdy D, Buswell CM, Hodgson AE *et al*.: Characterization of cytolethal distending toxin (CDT) mutants of Campylobacter jejuni. J Med Microbiol 2000;49:473-9.
7. Mayer MP, Bueno LC, Hansen EJ, DiRienzo JM.: Identification of a cytolethal distending toxin gene locus and features of a virulence-associated region in *Actinobacillus actinomycetemcomitans*. Infect Immun 1999;67:1227-37.
8. Sugai M, Kawamoto T, Peres SY *et al*.: The cell cycle-specific growth-inhibitory factor produced by *Actinobacillus actinomycetemcomitans* is a cytolethal distending toxin. Infect Immun 1998;66:5008-19.
9. Ahmed HJ, Svensson LA, Cope LD *et al*.: Prevalence of cdtABC genes encoding cytolethal distending toxin among *Haemophilus ducreyi* and *Actinobacillus actinomycetemcomitans* strains. J Med Microbiol 2001;50:860-4.
10. Tan KS, Song KP, Ong G.: Cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. Occurrence and association with periodontal disease. J Periodontal Res 2002;37:268-72.
11. Deng K, Latimer JL, Lewis DA, Hansen EJ.: Investigation of the interaction among the components of the cytolethal distending toxin of *Haemophilus ducreyi*. Biochem Biophys Res Commun 2001;285:609-15.
12. Lara-Tejero M, Galan JE.: CdtA, CdtB, and CdtC form a tripartite complex that is required for cytolethal distending toxin activity. Infect Immun 2001;69:4358-65.
13. Cortes-Bratti X, Chaves-Olarte E, Lagergard T, Thelestam M.: Cellular internalization of cytolethal distending toxin from *Haemophilus ducreyi*. Infect Immun 2000;68:6903-11.

14. Lewis DA, Stevens MK, Latimer JL *et al*.: Characterization of *Haemophilus ducreyi* cdtA, cdtB, and cdtC mutants in vitro and in vivo systems. Infect Immun 2001;69:5626-34.
15. Deng K, Hansen EJ. : A CdtA-CdtC complex can block killing of HeLa cells by *Haemophilus ducreyi* cytolethal distending toxin. Infect Immun 2003;71:6633-40.
16. Lee RB, Hassane DC, Cottle DL, Pickett CL.: Interactions of Campylobacter jejuni cytolethal distending toxin subunits CdtA and CdtC with HeLa cells. Infect Immun 2003; 71:4883-90.
17. McSweeney LA, Dreyfus LA.: Carbohydrate-binding specificity of the *Escherichia coli* cytolethal distending toxin CdtA-II and CdtC-II subunits. Infect Immun. 2005;73: 2051-60.
18. Akifusa S, Heywood W, Nair SP, Stenbeck G, Henderson B.: Mechanism of internalization of the cytolethal distending toxin of Actinobacillus actinomycetemcomitans. Microbiology 2005;151:1395-402.
19. Saiki K, Konishi K, Gomi T, Nishihara T, Yoshikawa M.: Reconstitution and purification of cytolethal distending toxin of *Actinobacillus actinomycetemcomitans*. Microbiol Immunol 2001;45:497-506.
20. Belibasakis G, Johansson A, Wang Y *et al*.: Inhibited proliferation of human periodontal ligament cells and gingival fibroblasts by *Actinobacillus actinomycetemcomitans*: involvement of the cytolethal distending toxin. Eur J Oral Sci 2002;110:366-73.
21. DiRienzo JM, Song M, Wan LS, Ellen RP.: Kinetics of KB and HEp-2 cell responses to an invasive, cytolethal distending toxin-producing strain of *Actinobacillus actinomycetemcomitans*. Oral Microbiol Immunol. 2002;17: 245-51.
22. Shenker BJ, McKay T, Datar S *et al*.: *Actinobacillus actinomycetemcomitans* immunosuppressive protein is a member of the family of cytolethal distending toxins capable of causing a G2 arrest in human T cells. J Immunol 1999;162:4773-80.
23. Nalbant A, Chen C, Wang Y, Zadeh HH.: Induction of T-cell apoptosis by *Actinobacillus actinomycetemcomitans* mutants with deletion of ltxA and cdtABC genes: possible activity of GroEL-like molecule. Oral Microbiol Immunol 2003;18:339-49.
24. Belibasakis GN, Johansson A, Wang Y *et al*.: The cytolethal distending toxin induces receptor activator of NF-kappaB ligand expression in human gingival fibroblasts and periodontal ligament cells. Infect Immun 2005;7:342-51.
25. Belibasakis GN, Mattsson A, Wang Y, Chen C, Johansson A.: Cell cycle arrest of human gingival fibroblasts and periodontal ligament cells by *Actinobacillus actinomycetemcomitans*: involvement of the cytolethal distending toxin. APMIS 2004;112:674-85.
26. Ueno Y, Ohara M, Kawamoto T *et al*.: Biogenesis of the *Actinobacillus actinomycetemcomitans* cytolethal distending toxin holotoxin. Infect Immun 2006;74:3480-7.
27. Shenker BJ, Demuth DR, Zekavat A.: Exposure of lymphocytes to high doses of *Actinobacillus actinomycetemcomitans* cytolethal distending toxin induces rapid onset of apoptosis-mediated DNA fragmentation. Infect Immun 2006;74: 2080-92.
28. Kang P, Korostoff J, Volgina A, Grzesik W, DiRienzo JM.: Differential effect of the cytolethal distending toxin of *Actinobacillus actinomycetemcomitans* on co-cultures of human oral cells. J Med Microbiol 2005;54:785-94.
29. Leung WK, Ngai VK, Yau JY *et al*.: Characterization of *Actinobacillus actinomycetemcomitans* isolated from young Chinese aggressive periodontitis patients. J Periodontal Res 2005;40:258-68.
30. Shenker BJ, Besack D, McKay T *et al*.: Induction of cell cycle arrest in lymphocytes by *Actinobacillus actinomycetemcomitans* cytolethal distending toxin requires three subunits for maximum activity. J Immunol 2005;174:2228-34.