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Biphasic Modulation of Mouse Macrophage Cell Growth by the *Salmonella choleraesuis* Cytotoxin P56

Kuo-Chieh Ho[1], Dai-Chang Kuo[1] and Gan-Nan Chang[2, 3]

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ABATRACT: P56, a cytotoxin with a molecular weight of 56 kDa and an isoelectric point of 6.2 was isolated and purified from the cell-free supernatant of *Salmonella choleraesuis* CH12440 culture. P56 was a secretory protein. It could be detected as early as 3 hr in the supernatant of bacterial culture, and the presence of calcium in the medium would increase the secretion by a factor of 3.2. P56 had dose-dependent effects on mouse macrophage cell line P388-D1 and mouse peritoneal macrophages: It caused death of both kinds of cells at high concentrations. In contrast, it had a mitogenic effect on the cells at lower doses. Western blot analysis showed that the antibody raised against P56 reacted to P56 and proteins of *S. choleraesuis* lysate. However, there was no cross-reaction between P56 and the antibody raised against the dead cells.

KEY WORDS: ***Salmonella choleraesuis*, Secretory cytotoxin, Cytotoxic effect, Mitogenic effect.**

INTRODUCTION

In the past decade, the cytotoxins produced by enteric pathogens have been increasingly investigated. These toxins were referred to as verotoxins because they are cytotoxic to Vero cells (Konowalchuk *et al*., 1977). Some cytotoxins are lethal to mammalian cells, such as Shiga toxin produced mainly by *Shigella dysenteriae* serotype 1 (Baloda *et al*., 1983) and the closely related Shiga-like toxin (SLT) produced by *Escherichia coli* (Cleary *et al*., 1985；Marques *et al*., 1986; O'Brien and Holmes, 1987). SLTs are divided into two groups: SLT type I (SLT-I) and SLT type II (SLT-II). However, only SLT-I can be neutralized by antibody against Shiga toxin (Marques *et al*., 1986). A similar cytotoxin is produced by *Campylobacter* sp. (Guerrant *et al*., 1987) and *Clostridium difficile* (Bartlett *et al*., 1986). Evidence shows that cytotoxins produced by toxigenic organisms play the critical role in the pathogenesis of particular diseases. However, very limited data are available concerning the cytotoxin produced by *Salmonella choleraesuis*. We previously isolated a cytotoxin, P65 from *S. choleraesuis* (It was changed to P56 based on re-determined molecular weight). Its cytotoxic effects to mouse peritoneal macrophages and mouse macrophage cell line P388-D1 were histopathologically investigated and reported (Chang and Tsai, 1996). In this communication, we present the studies on its cytotoxic and mitogenic effects on those cells *in vitro*.

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MATERIALS AND METHODS

Bacterium

S. choleraesuis CH12440 were locally isolated from the lymph node, lungs, liver, and spleen of pig suffering from systemic septicemic salmonellosis, and cultured on MacConkey's agar (Chang and Tsai, 1996；Rumeu *et al*., 1997).

Mammalian cells

Mouse macrophage cell line P388-D1 was purchased from American Type Culture Collection (ATCC TIB63). Mouse peritoneal macrophages were freshly prepared by the peritoneal injection (ip) with sterile saline into the peritoneal cavity of mouse, and then collected back into the syringe again. Both kinds of cells were cultured in RPMI 1640 containing 10 % fetal calf serum.

Purification of cytotoxin

S. choleraesuis were grown in BHI (Brain heart infusion) broth containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$, or 10 mM $CaCl_2$ only at 37 ºC for 15 hrs, and then centrifuged at 10,000x*g* for 30 min at 4 ºC. The supernatant was filtered through a 0.2 µm membrane and salted out with 35% saturated ammonium sulfate solution at 4 ºC for 1 hr. After centrifugation at 10,000 x*g* for 30 min at 4 ºC, the pellet was resuspended in PBS (phosphate-buffered saline, pH 7.0) and dialyzed against the same buffer for 30 hrs at 4 ºC. The dialyzed proteins were used as the crude cytotoxin. For further purification, crude cytotoxin was applied to a Sepharose CL6B column (60 cm x 1 cm). Proteins were eluted with PBS. The effluents were collected in 3 mL/fraction, and the fractions of the first peak were pooled together and concentrated by dialyzing against PBS containing 30% PEG (Polyethylene glycol, MW 20000, Sigma). The concentration of protein was assayed by using the Bio-Rad DC protein-assay system (Bio-Rad).

Preparation of *Salmonella choleraesuis* lysate

S. choleraesuis were cultured in BHI broth at 37 ºC for 15 hrs and harvested at 10,000 x*g* for 30 min at 4 ºC. The pellet was resuspended in PBS and sonicated on ice for 10 sec each with cooling pause of 15 sec in between at 30 W (Heat systems ultrasonic processor, NY, USA, grade 5) until the lysate became transparent and yellowish. The bacterial debris was removed at 12,000 x*g* for 30 min at 4 ºC. The supernatant was saved as the native proteins and stored at –70 ºC if it was not used immediately.

Cytotoxic and mitogenic activity assay

Mouse macrophage cell line P388-D1 and mouse peritoneal macrophages were used for determining the cytotoxic and mitogenic activities of P56. Different concentrations of P56 were added to the culture media. After 48 hrs, MTT [methythiazoletetrazolium (sigma), 5 mg/mL in PBS] was added and the cells were incubated at 37 ºC for 3 hrs. The medium was aspirated carefully, then DMSO and Sorenson's glycine buffer (0.1 M glysine and 0.1 M NaCl) were added. The solution was mixed well to lyze the cells and dissolve formazan. The absorbance of the solution was measured at wavelength of 570 nm (OD_{570}). The cells survived in different concentrations of P56 were calculated (Mosmann, 1983; Pang *et al*., 1994).

Preparation of antibodies against P56, dead cells and *S. choleraesuis* lysate

Two New Zealand rabbits were subcutaneously immunized with 0.5 mL of the purified P56 (0.16 mg/mL) mixed with an equal volume of Freund's complete adjuvant. Two booster injections were given at a 10 day-interval with the same protein dose coupled with Freund's incomplete adjuvant instead of the complete adjuvant. Two weeks after the last booster immunization, the rabbits were bled for antisera. The complements of antisera were inactivated at 56 ºC for 30 min, and then the antisera were applied to a protein A-Sepharose CL4B column (10 cm X 1 cm, Amicon). The column was washed with PBS, and then the proteins (IgG) were eluted by 0.1M glycine, pH 2.5. The antibodies against proteins of lysate and dead cells of *S. choleraesuis* were prepared by the same procedure except that the antigens for immunization were the lysate and the 0.5 % formalin-treated cells, respectively.

Isoelectric focusing point (pI) determination

Isoelectric focusing point (pI) determination was carried out using a pharmacia IEF 3–9 phast gel (Pharmacia). Electrophoresis was performed at 2000V, 3.5W at 15ºC for 1 hr using the microprocessor program 600Vh. The reference used was the broad pI calibration kit containing trypsinogen (pI 8.45), lentil lectin-acidic band (pI 8.15), myoglobin-basic band (pI 7.35), myoglobin-acidic band (pI 6.85), human carbonic anhydrase B (pI 6.55), bovine carbonic anhydrase B (pI 5.85), β-lactoglobulin A (pI 5.20), soybean trypsin inhibitor (pI 4.55), amyloglucosidase (pI 3.50). After electrophoresis, the gel was stained with a solution containing 0.2 % Comassie blue, 40 % methanol and 7 % acetate, and then destained with 7 % acetate.

Western blot assay

The proteins on a 9 % SDS-polyacrylamide gel were transferred onto nitrocellulose paper (NC-paper) using the Bio-Rad transfer system at 1A for 1 hr. After rinsed with PBS, the NC-paper was incubated with specific antisera for 35 min at 37 ºC, and then the conjugate, goat-anti-rabbit-IgG-ALP (alkaline phosphatase) was added. The incubation was continued at 37 ºC for another 35 min. Chromatogen development was performed using a substrate solution (10 mL) containing 0.1 M Tris-base, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5, mixed with 60 μL BCIP [5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL)] and 60 μL NBT [nitro blue tetrazolium (50 mg/mL)] (Chang, 1995).

RESULTS

The protein, P56 was purified from the cell-free supernatant of pathogenic *S. choleraesuis* CH12440 culture. P56 had a molecular weight of 56 kDa and a pI of 6.2. The protein could be detected as early as 3 hrs in the supernatant and its secretion increased by a factor of 3.2 when cells were grown in BHI containing 10 mM $CaCl_2$ compared to that in the medium without $CaCl_2$.

The cytotoxicity of P56 was assayed on mouse macrophage cell line P388-D1 and mouse peritoneal macrophages by MTT colorimetric test. Forty eight-hr old macrophage cells were grown in RPMI 1640 medium containing different amounts of P56. After 48 hrs, the effects of P56 were monitored. Results in Figures 1 and 2 displayed that P56 had a dose-dependent cytotoxic and mitogenic effects to both kinds of cells. It caused degeneration and necrotic

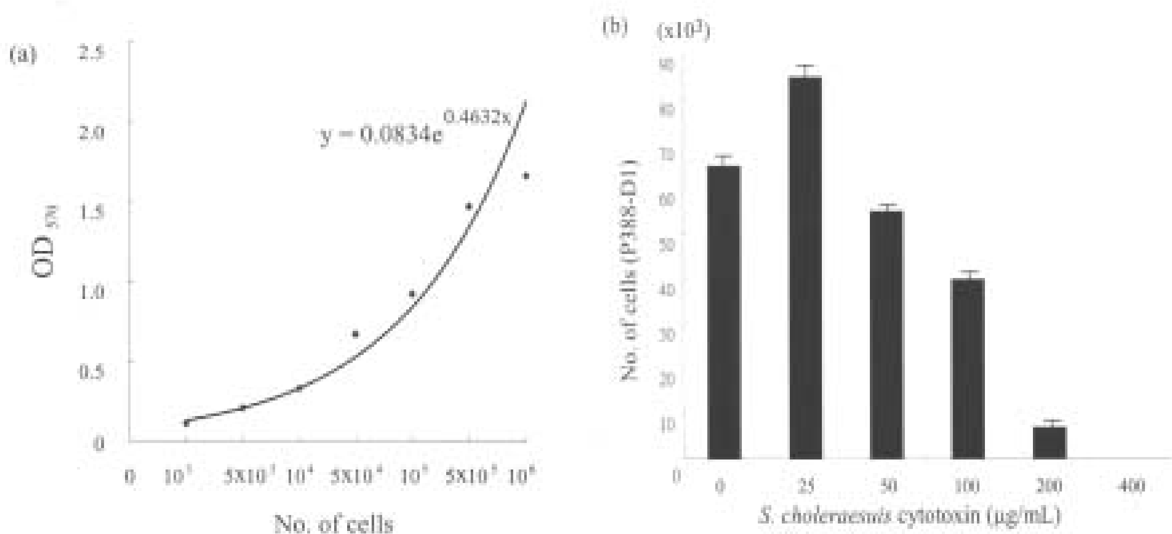

Fig. 1. The dose-dependent cytotoxic and mitogenic effects of P56 on mouse cell line P388-D1. (a) The standard curve showing cell numbers of P388-D1 vs optical density at 570 nm in MTT test. (b) The cytotoxic and mitogenic effects of P56 on P388-D1 cells. The concentrations higher than 50 μg/mL, P56 were toxic to the cells. The concentrations lower than 25 μg/mL, it was a mutagen and stimulated cell growth.

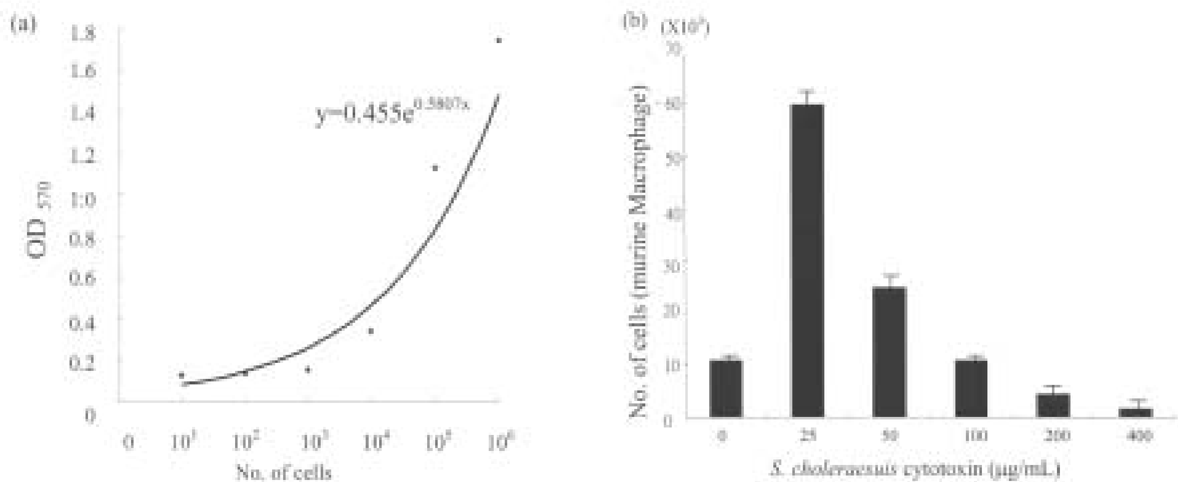

Fig. 2. The dose-dependent cytotoxic and mitogenic effects of P56 on mouse peritoneal macrophage. (a) The standard curve showing cell numbers of mouse peritoneal macrophage vs optical density at 570 nm in MTT test. (b) The cytotoxic and mitogenic effects of P56 on mouse peritoneal macrophage. The concentrations higher than 100 μg/mL, P56 were toxic to the cells. The concentrations lower than 25-50 μg/mL, it was a mutagen and stimulated cell growth.

changes at higher concentration, 50 μg/mL for P388-D1and 100 μg/mL for peritoneal macrophages. However, at lower concentration, 25 μg/mL for P388-D1and 25-50 μg/mL for peritoneal macrophages P56 displayed a mitogenic effect and stimulated the growth of cells.

Three kinds of antisera were prepared from rabbits immunized with P56, dead cells and the proteins of *S choleraesuis* lysate, respectively. The immunological properties of P56 were investigated by the western blot analysis. The antiserum raised against the proteins of lysate reacted to the proteins of lysate themselves and P56. The antiserum against P56 also showed a cross reactivity with the proteins of lysate. However, the antiserum against dead bacteria reacted only to the proteins of lysate, not to P56 (Fig. 3).

DISCUSSION

Traditionally, the endotoxin is a major virulence factor of *Salmonella* spp. and is the cause of death in people with systemic infections. Enterotoxin production has also been reported for a large number of *Salmonella* serotypes and, at least in part, responsible for the loss of electrolytes and fluid from small intestine (Guianella *et al*., 1975; Jiwa, 1981; Peterson *et al*., 1981；Caprioli *et al*., 1982；Singh *et al*., 1985). In fact, a *Salmonella* cytotoxin has long been suspected based on the pathogenesis of *Salmonella* infections. In 1986, Reitmeyer *et al*. reported the isolation of the membrane-bound cytotoxin from *Salmonella typhimurium*. This cytotoxin could cause the tissue damage. Kita *et al*. (1993) also isolated a cytotoxic protein of 32 kDa from L-form *Salmonella typhimurium,* with an isoelectric point (pI) of 6.4. It had a cytolytic effect at concentrations higher than 0.7 μg/mL. In 1997, Rumeu *et al*. reported another outer membrane-bound, heat-stable cytotoxin produced by *Salmonella enteritidis* and *S*. *typhimurium* that inhibits protein synthesis in culture cells as well as in the intestinal mucosa. Recently, Ho and Chou (2001) reported the detection of a cytotoxin in *S. choleraesuis* lysate.

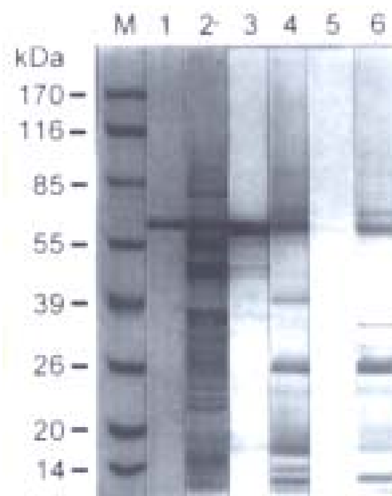

Fig. 3. Immunological properties of P56. Lane M, protein size markers; lane 1, purified P56; lane 2, sonicated-cell lysate of *S. choleraesuis;* lanes 3 and 4, the antibody raised against P56 reacting to P56 (lane 3) and proteins of cell lysate (lane 4); lanes 5 and 6, the antibody raised against dead cells of *S. choleraesuis* reacting to the proteins of cell lysate (lane 6), but not to p56 (lane 5). Lanes M, 1 and 2 were Coomassie blue-stained. Lanes 3, 4, 5 and 6 were Western blot analysis.

In a previous communication, we reported the isolation of a cytotoxin, P56 from the supernatant of *S. choleraesuis* culture and the histopathological studies in ICR mice and mouse macrophage cell line P388-D1. It caused hepatomegaly, splenomegaly and pneumonia in ICR mice following intravenous injection at a dose of 40 – 60 μg per mouse, and degeneration and necrosis in the cell line (Chang and Tsai, 1996). In this study, we further characterized this cytotoxic protein. The protein had a pI of 6.2. Although the sensitivities of mouse macrophage cell line P388-D1 and mouse peritoneal macrophages to P56 were different, P56 showed a dose-dependent cytotoxicity to both kinds of cells. It resulted in degeneration and necrotic changes at high concentrations. However, at low concentrations, P56 had a mitogenic effect and stimulated the growth of cells. A same phenomenon was reported for the 32 kDa-cytotoxin of *Salmonella typhimurium* (Kita *et al*., 1993). P56 was a type of secretory or excretory protein since its amount in medium could be increased by 3.2 times in the presence of divalent ions such as Ca^{+2} or Mg^{+2}. The protein identity of P56 was further confirmed by its sensitivity to trypsin cleavage and a negative Shwartzman's reaction (data not shown). P56 might be considered as an exotoxin.

The immunological properties of P56 were investigated by the Western blot analysis. The antiserum raised against the formalin-treated cells of *S. choleraesuis* reacted to the proteins of cell lysate, but not to P56. However, the antiserum raised against P56 reacted not only to P56 but also to the proteins of cell lysate. The data suggested that a secretory or an

extracellular protein such as P56 had a much more potent antigenicity than that of dead bacteria and might be potential for the development of a subunit vaccine of *S. choleraesuis* in the near future. This phenomenon might explain the reason why the killed bacteria were not a very effective vaccine developed in the last decades. It might also be true for other secretory or extracellular proteins of bacteria being more potential for the development of the subunit vaccine.

The gene encoding P56 have cloned from a genomic library of *S. choleraesuis* and being characterized.

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霍亂沙氏桿菌細胞毒素 P56 雙相調節老鼠巨噬細胞的生長

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摘　　要

P56 是一種由霍亂沙氏桿菌 CH12440 之培養基上清液分離到的細胞毒素。它的分子量 56 千道爾頓，等焦點 6.2，是一種分泌型的蛋白質。在細菌培養 3 小時即可於上清液中偵測到它的存在，而且在培養基中有鈣離子時，其存在量為沒有鈣離子存在時的 3.2 倍。P56 對老鼠巨噬細胞株 P388-D1 及腹膜巨噬細胞的影響視其計量而定：在高濃度下，它會引起這兩種細胞的死亡；在低濃度下，它反而會促進細胞的分裂。在西方轉漬分析中，P56 的抗體可與 P56 蛋白質本身及沙氏桿菌的細胞溶解物反應，但是 P56 與利用死菌製備的抗體沒有交叉反應產生。

關鍵詞：霍亂沙氏桿菌、分泌型細胞毒素、細胞死亡、細胞分裂。

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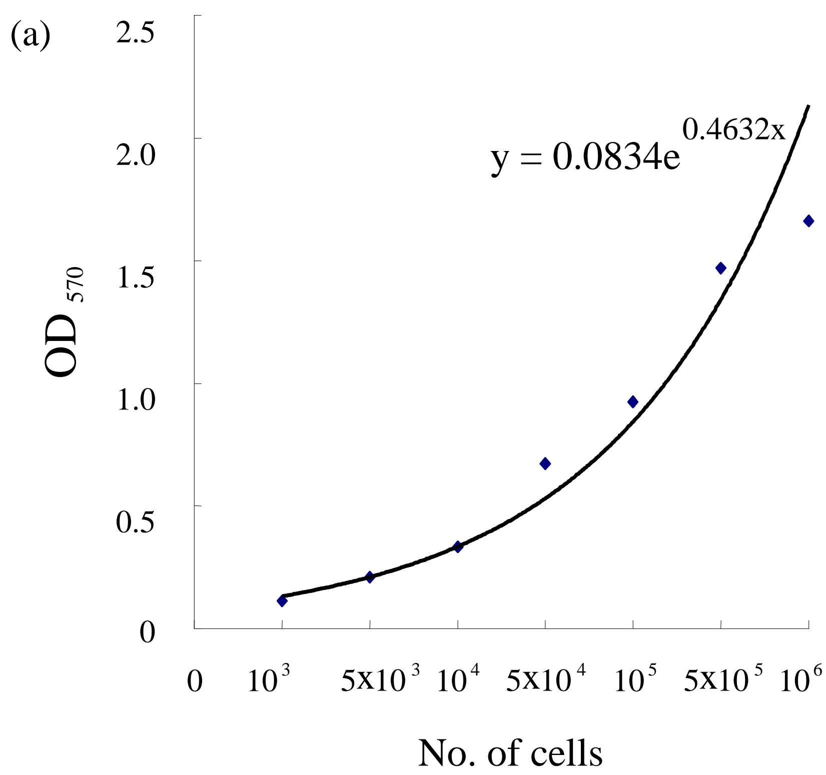
(a)



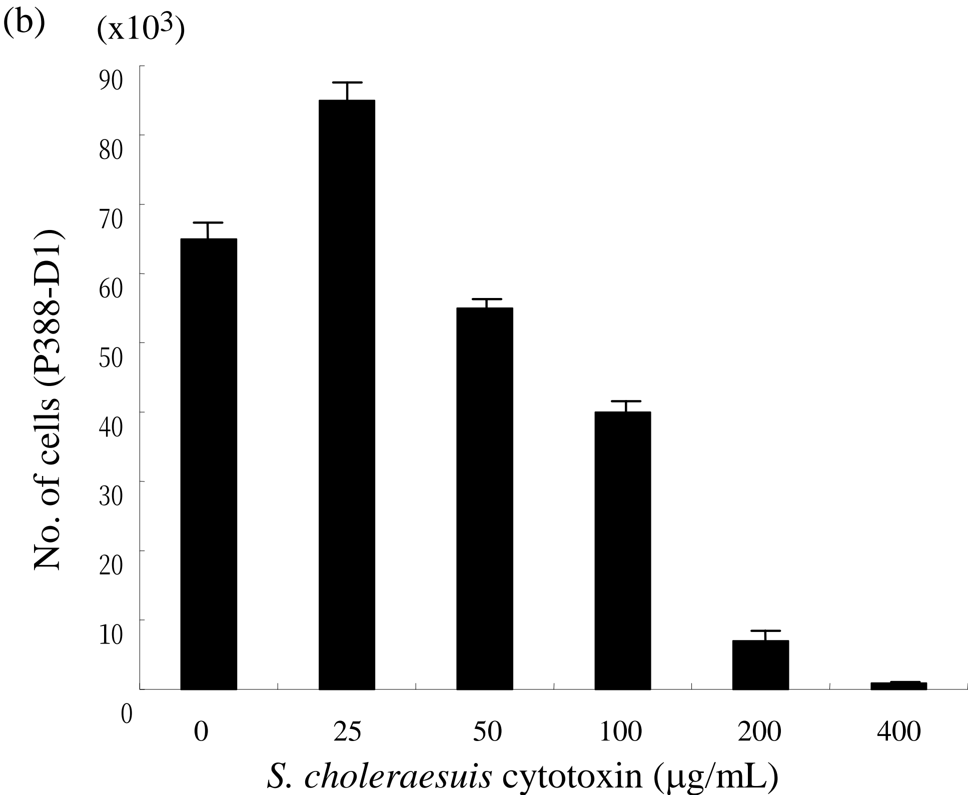


Fig. 1

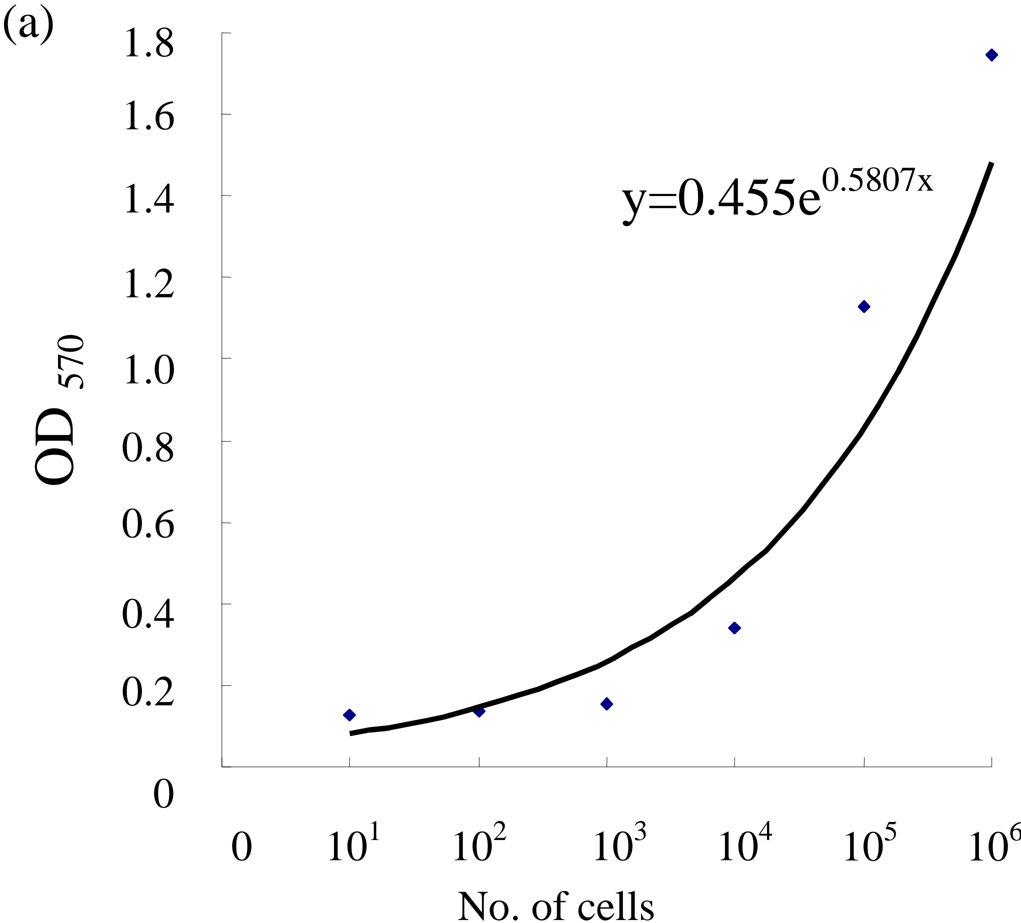


Fig. 2

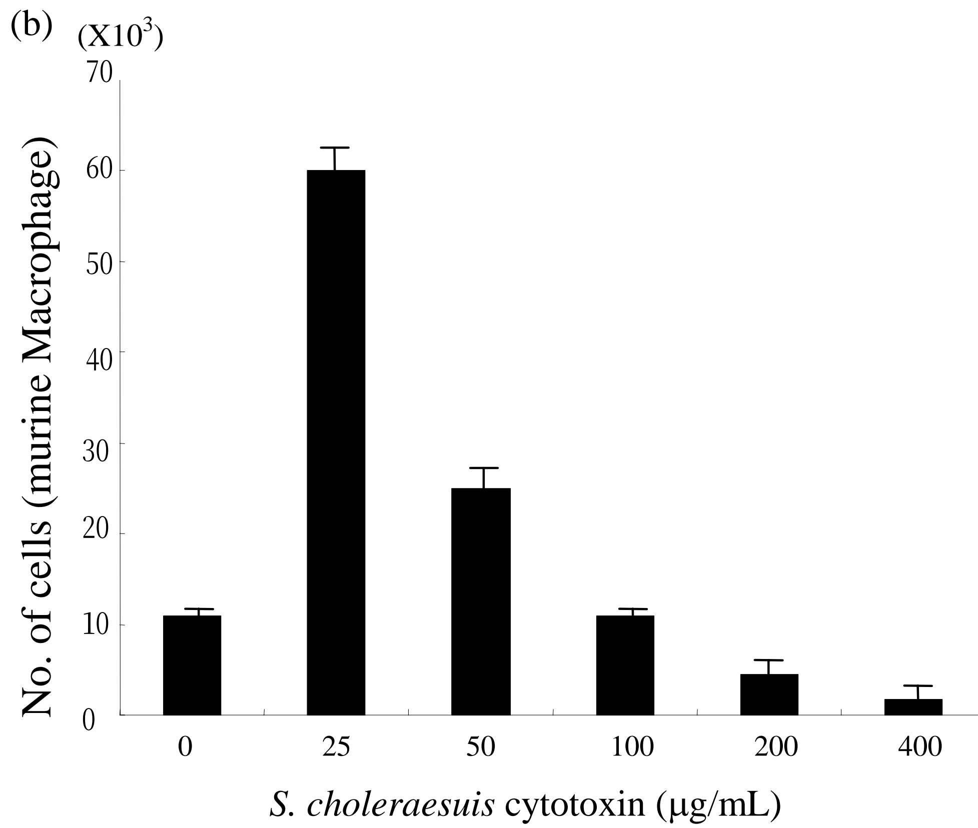


Fig. 2

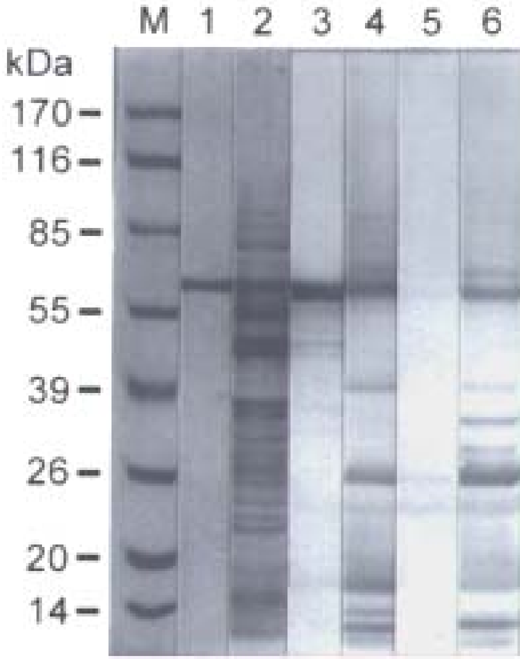


Fig. 3