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Full Length Research Paper

Cloning and expression of *C-terminal of Clostridium perfringens* type A enterotoxin and its biological activity

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Pathogenic clostridia produce exocellular toxins that resemble lipoteichoic acid and are described as super antigens. These toxins stimulate T-cell receptor-carrying lymphocytes in peripheral blood and have been used to study immunodeficiency diseases and cancers. The CPE C -terminal region from one of the local type A strain was cloned in the pET32a vector its expression induced with IPTG. The expressed protein was purified by Ni-NTA affinity chromatography and tested for biological activity with Vero cells assay. This region of *Clostridium perfringens* enterotoxin (CPE) has a predominant ligand-binding activity. In the present study, the biological activity of the C-terminal region of local purified CPE came under study with Vero cell assay, guinea pig skin test and mouse test to evaluate for future use as a therapeutic purpose. The result of this study showed that, the study's local purified C -CPE had cytotoxic activity in Vero cells even at the minimum dilution of 0.625 ng after a 4-h incubation period. It caused transient increase in capillary permeability in guinea pigs. C- CPE did not have systemic effect on Balb/c mice. The use of the C-CPE peptide may provide a novel way to target drugs to Claudine-expressing cells.

Key words: Cloning, gene expression, *Clostridium perfringens* enterotoxin (CPE), vero cells, nigrosin, guinea pig skin test, mouse test, Claudine.

INTRODUCTION

Clostridium perfringens isolates are commonly classified into types A-E on the basis of their ability to produce alpha, beta, iota, and epsilon toxins (MacClane et al., 2000; MacClane and Rood, 2001). Approximately 1 - 5% of all *C. perfringens* isolates, mostly belonging to type A (MacClane et al., 2000; MacClane and Rood, 2001), produce the *C. perfringens* enterotoxin (CPE), which is a bio-medically important toxin. CPE is a single polypeptide with a molecular mass of 35 kDa that causes food poisoning associated with most human food-borne illnesses (McClane and Chakrabarti, 2004), and its production does not require sporulation (Czeczulin et al., 1993). It has a heat-and pH-labile biological activity (MacClane et al., 2001). CPE is made up of two functionally distinct domains: An approximately 22-kDa N -terminal domain that mediates cytotoxicity and an approximately 13-kDa C-terminal domain (C-CPE) that mediates binding (McClane and Chakrabarti, 2004).

The *cpe* gene is chromosomally located in the type A strain that commonly causes food poisoning. However, this gene has been documented to be located on a plasmid in type A veterinary isolates as well as in the type A non-food borne strain that causes human gastro-intestinal (GI) disease (MacClane et al., 2001).

CPE is being investigated as a candidate marker in cancer diagnostics and as a potential chemotherapeutic agent to treat various cancers. The Claudine family of

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proteins (encompassing Claudines 1 – 18) is characterrized by the presence of four transmembrane/cytoplasmic domains and two extracellular loops that serve to link ligands on the surface of the cell. CPE was shown to bind claudine-3 through its second extracellular loop (Fujita et al., 2000). Recent studies have documented a dramatic up regulation of the CPE receptors claudine-3 and claudine-4 in pancreatic, ovarian, breast, and uterine cancers (Morin et al., 2005). For example, injection of CPE into pancreatic tumors induced in nude mice resulted in tumor necrosis and significant reduction in tumor growth (Michl et al., 2001).

Claudine-3 and -4 are the receptors for CPE (Katahira et al., 1997; Sonoda et al., 1999), and it has been shown that they bind to CPE via the C-CPE domain (Katahira et al., 1997; Sonoda et al., 1999; Fujita et al., 2000; Kondoh et al., 2005). These findings suggest that CPE could be used for the targeting of Claudines on epithelial carcinoma cells. Indeed, CPE has been successfully used to treat human ovarian and pancreatic cancers, both of which express high levels of claudine-3 or -4 (Michl et al., 2001; Santin et al., 2005). Claudine-3 and -4 have been evaluated as potential targets for CPE toxin-mediated therapy for prostate cancer (Long et al., 2001).

Injection of CPE into the peritoneum of mice seeded with human ovarian cancer cells caused elimination of the malignant cells (Santin et al., 2005). Intracranial administration of CPE inhibited tumor growth and increased survival in two murine models of breast cancer brain metastasis (Kominsky et al., 2007).

Alessandro D. Santin (2007) evaluated the expression levels of claudine-3 and claudine-4, the low- and high-affinity receptors, respectively, for CPE in uterine carcino sarcomas and explored the potential for targeting these recaptors in the treatment of this aggressive uterine tumor. Claudine-3 and claudine-4 receptors are highly over expressed in carcino sarcoma. These proteins may offer promising targets for the use of CPE as a novel type-specific therapy against this biologically aggressive variant of endometrial cancer.

The aims of this study were (1) To clone and express the C-terminal region of local purified CPE to evaluate its biological activity with Vero cell assay and animal assay and (2) To determine whether the C-terminal region has cytotoxic activity in addition to its ligand-binding function to prepare it for its therapeutic characteristic in our future plan.

MATERIALS AND METHODS

Bacteria, cells, vectors and animals

The *C. perfringens* strain obtained from the veterinary organization of the Ahvaz University, Iran, was used as a template to amplify the *cpe* gene. Bacteria were grown anaerobically on the enriched blood agar at 37°C for 48 h. Cloning vector pTZ57R/T (2.8 kb) and expression vector pET32a (5.9 kb) were obtained from Fermentas, Lithuania and Pasteur Institute, Iran, respectively.

Vero cells NCBI C1O1 9(Pasteur Institute, Tehran, Iran) were routinely cultured at 37°C in RPMI supplemented with 10% FBS (Gibco, Germany) and 100 U penicillin (Gibco) and 100 g streptomycin (Gibco) in a humi-dified incubator containing 5% CO_2. Animals including guinea pigs and BALB/C's were obtained from Pasteur Institute, Iran.

PCR and cloning procedures

Purified bacterial DNA was prepared using the High Pure PCR template preparation kit (Roche, Germany). The 380-bp C- terminal region of the genome was amplified. PCR cycling conditions were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 51°C for 30 s, and 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR products were analyzed by separation on a 1.5% agarose gel and staining with cyber green. The appropriate DNA fragments were excised from the gel, purified using a gel extraction kit (Fermentas, Lithuania), and cloned into pTZ57R/T. *Escherichia coli* cells were transformed with ligation products as described previously (Hanahan et al., 1983), and recombinants were selected with IPTG (Sigma) and X-Gal.

Recombinant clones were confirmed by universal PCR and restriction enzyme digestion analysis. The *cpe* gene was released by *Bam*H1/*Sac*1 digestion (Fermentas, Lithuania) and sub cloned into the pET32a expression vector.

Gene expression

Expression of CPE was induced as follows, overnight cultures of positive clones were induced with 1 mM IPTG, incubated with shaking at 37°C, and cells were collected at different time points post-induction (2, 3, and 5 h) . The cells were harvested and prepared for electrophoresis and the protein was purified by Ni-NTA affinity chromatography (Novagen, USA) as described previously (Bandehpour et al., 2006).

Western blot

Protein expression in cell-free lysates was confirmed by western blotting. Proteins resolved on a 15% SDS-PAGE were transferred to nitrocellulose membranes. The membranes were then blocked in 3% BSA for 1 h and incubated at room temperature with anti-His-Tag antibodies, 1:500 dilution (Novagen, USA). The membranes were washed extensively and incubated with anti-mouse antibodies, 1:500 dilution (Novagen, USA) before visualizing the protein with DAB (Sigma, Germany).

Biological assay for enterotoxin

Vero cell assay

Vero cells were seeded at a density of 5×10^4 cells/well in a micro titer plate. Two-fold dilutions of the CPE C-terminal toxin were prepared in RPMI and added to each well. Cells were incubated for different periods of time (1, 3, and 4 h) at 37°C in a CO_2 incubator. Plates were centrifuged at 1500 rpm for 10 min, and the supernatant was aspirated with a Pasteur pipette. Adherent cells in each well were stained with 100 l of 0.3% nigrosin for 20 min at 37°C to evaluate cell death. The ratio of stained cells to total cells was calculated. Before working on animals, the absence of endotoxin was confirmed by LAL (Limulus amebocyte lysate).

Guinea pig skin test

Erythemal activity of enterotoxin was assayed in the skin of six

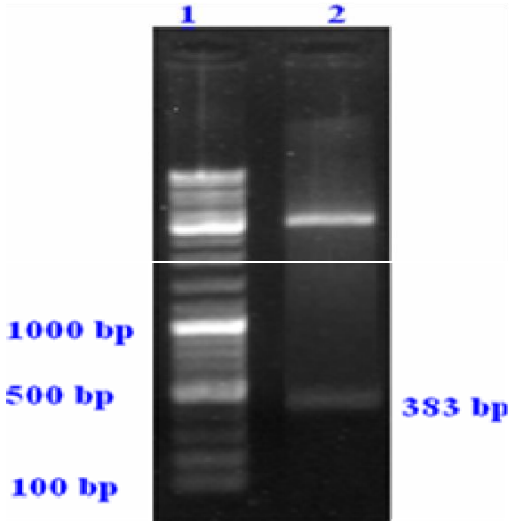

Figure 1. Agarose gel electrophoresis. Lane 1: 100 bp DNA ladder marker. Lane 2: recombinant plasmid digested with BamH1 and Sac1 and released 383 bp fragment.

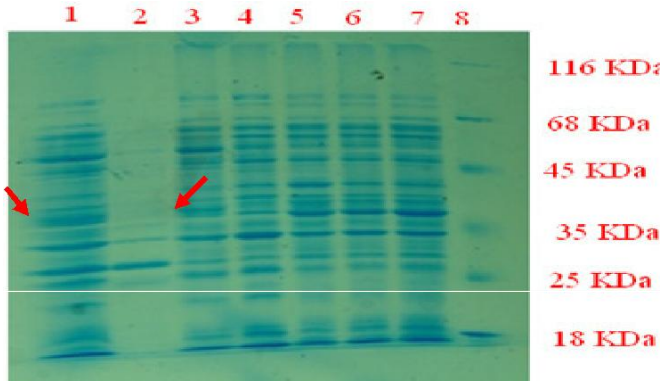

Figure 2. SDS PAGE electrophoresis. Lane 1: the pellet of 5h induced cell contained recombinant plasmid (without preparation). Lane 2: the pellet of 5 h induced cell contained recombinant plasmid (after preparation). Lane 3: supernatant of 5 h induced cell contained recombinant plasmid. Lane 4: The pellet of 0h cell contained recombinant plasmid. Lane 5: The pellet of 5h cell contained plasmid vector. Lane 6: The pellet of 0 h uninduced plasmid vector. Lane 7: The pellet of 0 h uninduced cell. Lane 8: Protein marker.

albino guinea pigs weighted 600 - 700 g. Serially diluted enterotoxin was injected intradermaly into the back of depilated guinea pigs. After 20 min each animal was injected intracardially with 1 ml of 1% Evans blue dye. The diameter of the resulting blue areas around the sites of intradermal injections were measured after 60 min.

Mouse lethality test

Groups of eight BALB/c mice weighing 18 - 20 g (almost six weeks old) were injected intraperitoneally with 1 ml of doubling dilutions of enterotoxin in phosphate buffered saline.

Figure 3. Western blot analysis. Lane 1: Cell contained recombinant pET32a 0 h after induction. Lane 2: The supernatant of cell contained recombinant pET32a 5 h after induction. Lane 3: The pellet of cell contained recombinant pET32a 5 h after induction.

RESULTS

The 383-bp from the end of *cpe* gene was cloned into the PTZ57R vector and sub cloned into the pET32a vector (Weinrauc and Zychlinsky, 1999) (Figure 1). The expressed recombinant protein was analyzed by SDSPAGE. The data for the present study demonstrated (Figure 2, lanes 1 and 3) that the 14.5-kDa enterotoxin was bound to TrxA (thioredoxin) and migrated with an apparent molecular weight of 40 kDa. This band was not seen in the control lanes. Western blotting (Figure 3) confirmed the expression of the protein in cell pellets at 5 h post-induction. The resultant protein was purified with Ni-affinity chromatography.

Biological activity

By staining Vero cells harvested at different incubation periods; under microscopic observation, the Vero cells stained with nigrosin were easily distinguished from un-stained ones (Figure 4). When Vero cells were previously incubated with the complete enterotoxin gene, the ratio of stained cells to the log dose of purified toxin ranging from 25 - 400 ng/ml was shown to be nearly linear (Uemura et al., 1984). The biological activity of the CPE C-terminal region was tested in Vero cells at a concentration of 0.625, 5, 10, 15, 20 ng. As shown in Figure 5, the data demonstrated that at concentrations ranging from 5 to 20 ng of CPE, cell death was directly proportional to the in-cubation time. Significant viscosity of CPE at these dilutions was observed, followed by decrease in cell death at higher CPE dilutions. The study data indicated that the effective dose of the toxin was between 5 and 20 ng and that Vero cells are less sensitive to higher dilu-tions of the toxin. The present study findings agreed with a previous study (Mahony et al., 1989) in which Vero cells were stained with neutral red followed by extraction of the dye. This process allowed toxin levels to be determined either visually or by optical density measurement. An eightfold increase in enterotoxin titer was observed when the number of cells per well was decreased from 4×10^5 to 25×10^3.

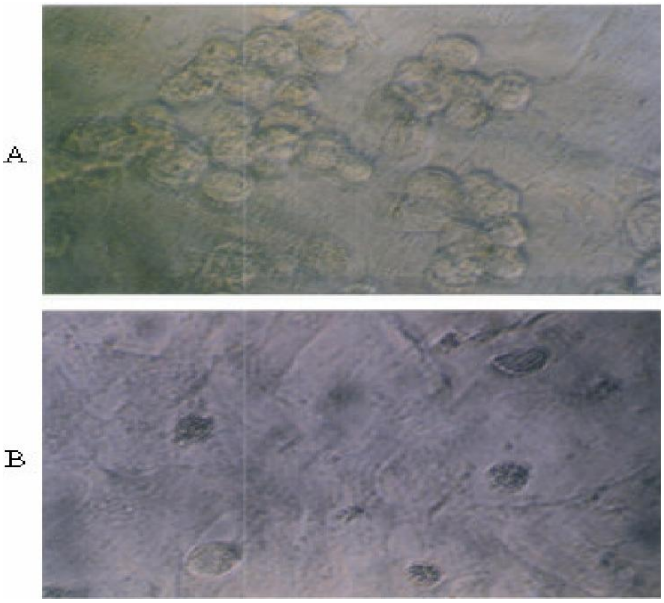

Figure 4. *Vero* cell staining assay ,before treating with entertoxin (A)trasnparent alive cells and after treating with enterotoxin, satined by nigrosin(B) dead cells appeared in dark blue.

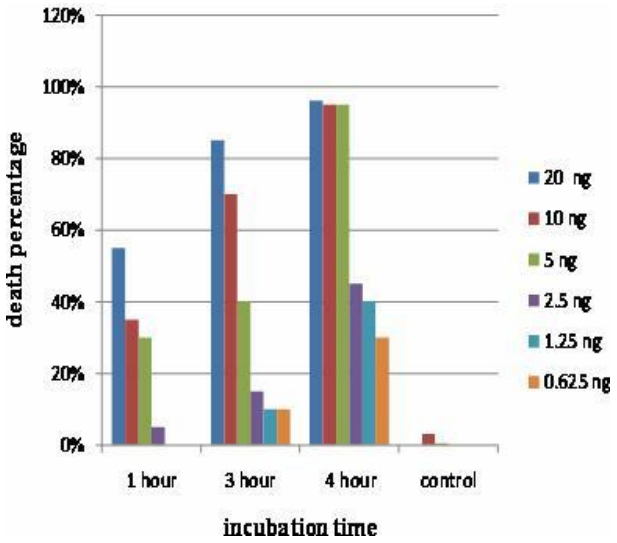

Figure 5. Staining response of vero cells (5× 10^4) to two fold dilutions *clostridium perfringens* enterotoxin at various incubation time.

Skin test

An area of erythema developed at the site of injection. Most reactions became visible in 1.5 to 2 h and measured 5 to 8 mm in diameter as it shown in Figure 6. The lesions became darker in color, but did not increase in size after 4 h. Maximum darkening of the reaction was sometimes attained in 24 h. After slight induration, these

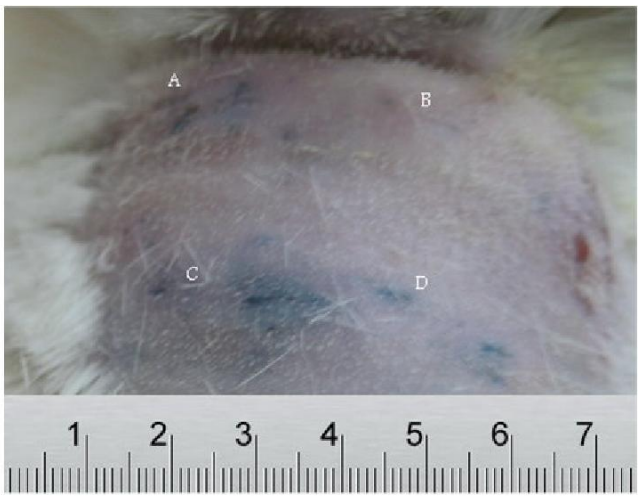

Figure 6. Intradermal injection in a guinea pig to test capillary permeability. Photograph taken 20 min after administration of Evans blue. In each dilution four spot were injected. A, C, D is shown for 1/32 diluted enterotoxin and B is control, only injected with PBS, no blue ring.

reactions resolved themselves without abscessation or necrosis within the following 2 to 3 days (Niilo, 1971).

Histological examination of the skin sections taken from lesions 24 h old showed that the initial erythematous reactions were located in the dermis followed by disruptions of hair follicle (Figure 7). Control sites injected with PBS formed a small non spreading blue spot at the needle puncture immediately after inoculation, indicating trauma.

Mouse lethality test

For toxicity in mice, serial dilutions of purified enterotoxin were prepared as for erythema test. One of them was taken as a control which only was injected by PBS and the other one was taken as Sham. The mice were weighed and enterotoxin preparations were injected on the basis of body weight in volume of 250 µl intraperitoneally. Mice were observed for 24 – 48 h. Usually four groups of mice would be sufficient to determine the median lethal dose (LD_{50}), which could be calculated by the method of Reed and Muench. While in experiment of the oresent study all mice were alive, therefore the purified C region of CPE did not have any systemic effect on mice.

DISCUSSION

The Vero cell assay is more sensitive and rapid to perform than the guinea pig skin test. Many investigators have employed Vero cell to analyze enterotoxin for the biological activity. Thus Vero cell staining assay may be a

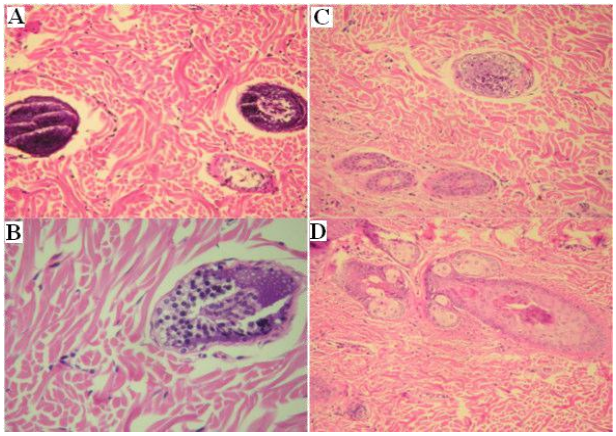

Figure 7. Histological examination of the Guinea pig skin sections that were kept in formalin. These photographs taken from lesions 24 h after injection. The a, b and c (different concentration of diluted enterotoxin) showed erythematous reactions in the dermis and disruptions of hair follicle. Hair follicles in a and b appeared in dark purple as compare to the (d) control which is injected with PBS only.

useful method for quantization of the biological activity in the studies on the structure and function of *C. perfringens* (Giugliano et al., 1983). Vero-cell plating efficiency assay for enterotoxin developed by McDonel and McClane (1981) is reproducible and comparable in the sensitivity to such immunological methods as reversed passive hemagglutination (Umeura et al., 1984), RPLA (Sugiyama et al., 1983), ELISA (Olsvik et al., 1982), and radio immunoassay (Skjelkvale., 1980) . By Vero cell assay presented in this paper, however, the enterotoxin contents of so many samples can easily be assayed within 4 h without laborious procedure for sterilization. The result of the present study agreed with Umeura et al. (1984) report pointing out that the reproducibility of the test increased by fixing the incubation period for nigrosin-Vero cell mixture to 60 min or a little longer at 37˚C.

Although enterotoxin N-terminal sequences are known to be responsible for cytotoxicity and the C-terminal sequences for binding activity, it was suggested that additional sequences may be required for cytotoxicity (Hanna et al., 1989). It was postulated that lack of CPE cytotoxicity did not necessarily imply the absence of the cytotoxic domain(s) in the C-terminal region of CPE, (Horiguchi et al., 1987; Sugii and Horiguchi, 1988). Lack of CPE cytotoxicity could arise due to the fact that (1) several domains are required for cytotoxicity or (2) that the cytotoxic domain is partially present in the C-terminus region of CPE.

The results of the present study showed that the C-terminal region of our local purified CPE had cytotoxic activity in Vero cells even at the minimum concentration of 0.625 ng after a 4-h incubation period.

Among various biological assay methods, the Guinea pig skin test has most often been employed. Hauschild in 1970 reported that the assay for detection of CPE by the skin test is rapid, reproducible, accurate, and about 1000 times as sensitive as the intestinal loop technique in rabbits. Skin test in guinea pigs showed that C -CPE caused an immediate brief increase in capillary permeability and subsequent erythema without fluid exudation.

There is a recent study that evaluated whether a potent claudine-4-binding C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) would allow targeting to Claudine- 4-expressing cells (Ebihara et al., 2005) fused C-CPE to the protein synthesis inhibitory factor (PSIF), which lacks the cell binding domain of *Pseudomonas* exotoxin. This fusion protein, C-CPE-PSIF, was cytotoxic to MCF-7 human breast cancer cells; which express endogenous claudine-4, but it was not toxic to mouse fibroblast L cells, which lack endogenous claudine-4. The cytotoxicity of C-CPE-PSIF was attenuated by pre treating the MCF-7 cells with C-CPE but not bovine serum albumin. Also, deletion of the claudine-4-binding region of C-CPE reduced the cytotoxicity of C- CPE-PSIF. Finally, they found that C-CPE- PSIF is toxic to L cells expressing claudin-4 but not to normal L cells or cells expressing claudine-1, -2, or -5. These results indicated that use of the C-CPE peptide may provide a novel way to target drugs to Claudine-expressing cells. Claudines are over expressed in some tumor cells. Administration of CPE has been shown to reduce the growth of claudin-4-overexpressing human ovarian and pancreatic tumors (Michl et al., 2001; Rangel et al., 2003; Santin et al., 2005). CPE contains not only a claudine-4-binding domain but also a cytotoxic domain (McClane and Chakrabarti, 2004). Therefore, it is hard to use CPE in itself as a targeting molecule to claudine-4. Offner et al. (2005) reported that antibodies for Claudines bind to Claudine-expressing carcinomas, suggesting that anti-Claudine antibodies or their Fv domains could be used to target antitumor agents to Claudine-positive tumors. However, targeting of an antitumor agent to cells via a Claudine has never been achieved. In this point, C-CPE is a useful claudine-4-targeting molecule, and C-CPE could target not only antitumor agents but also liposomes to claudine- 4-overexpressing tumor cells. Although claudine-4 is also distributed in normal tissues such as normal colon epithelium and several glands, the expression in normal tissues is weaker than in tumors (Long et al., 2001; Michl and Gress, 2004). Therefore, detailed analysis for the mechanism of interaction between C-CPE and claudine-4 is needed for a future application of antitumor therapy using C-CPE.

Because the plasmid encoding C -CPE-PSIF is a phagemid vector, its binding specificity can be easily manipulated using phage display. Scientists are currently attemptting to identify the precise claudine- 4 binding region of C-CPE and to use a phage display library to prepare versions of C-CPE that can bind other claudines.

In summary, Ebihara et al. (2005) showed that the C-CPE domain of C-CPEPSIF targets claudine-4. This is

the first report that C-CPE can allow the targeting of a drug to claudin-4. Because of these results, the authors are looking for a way of developing a Claudine-targeting drug delivery system for the authors purified local C-CPE which had biological effect in Vero cells, caused erythema in guinea pig skin with no systemic effect on mice.

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