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Monoclonal Antibody S2C4 Neutralizes The Toxicity of Shiga Toxin 2 and Its Variants*

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Abstract Shiga toxin 2 (Stx2) toxoid produced by formaldehyde treatment of purified toxin was used to immunize BALB/c mice for monoclonal antibody (MAb) production. The neutralizing activities of positive clones against Stx2 were screened by *in vitro* cytotoxicity assay. The isotype and specificity of resultant clone was determined, and its efficacy to neutralize the activity of purified Stx2 was evaluated by *in vitro* and *in vivo* toxicity model. Lastly, its spectrum of activity against Stx2 variants was also accessed by mouse toxicity model. It was demonstrated that one of the 12 positive MAb clones against Stx2, designating S2C4 had neutralizing activity. S2C4 belongs to the immunoglobulin G1 subclass and has a κ light chain, and it reacts with the A subunit of Stx2 and does not bind to Stx2 B subunit or to Stx1. S2C4 could efficiently neutralize the cytotoxicity of Stx2 to Vero cells and mice. It also protected mice against lethal doses of Stx2 variants challenge including Stx2c and Stx2vha. S2C4 is a promising candidate molecule in preventing the progression of hemolytic-uremic syndrome (HUS) mediated mainly by Stx2 in Stx-producing *Escherichia coli* (STEC) infection.

Key words monoclonal antibody (MAb) S2C4, Shiga toxin 2 (Stx2), neutralization, Stx-producing *Escherichia coli* (STEC)
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The outbreaks and sporadic cases of Shiga toxin (Stx)-producing *Escherichia coli* (STEC) infection world -wide have occurred with increasing frequency and the dominant sera type is O157∶H7[1]. In China, STEC O157∶H7 was initially isolated in 1986 in Xuzhou City, Jiangsu Province. In the following 1999～2000, two waves of large outbreaks of STEC O157∶H7 infections took place in three neighboring Provinces, Jiangsu, Anhui and Henan. The hospitalized patients, totaling thousands of cases, suffered from diarrhea and hemorrhagic colitis, and some developed hemolytic-uremic syndrome (HUS) characterized by acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, resulting in 208 deaths[2]. STEC secretes Stxs, which mediate STEC virulence. Stxs consists of an A-subunit monomer, which contains enzymatic RNA N-glycosidase activity that hydrolyzes the N-glycoside bond of adenosine of the 28 S rRNA of 60 S ribosomes and hence inhibits protein synthesis, and a B-subunit pentamer, which is involves in receptor binding[3,4]. There are two major types of Stx designated Stx1 and Stx2. Stx1 differs at a single amino acid in the A subunit from the Stx of *Shigella dysenteriae* 1[5]. Stx2 has approximately 56% amino acid homology with Stx1 and consists of several variants[6]. STEC isolates produce Stx1, Stx2 (or its variants), or both of these toxins. Although the mechanisms of action of Stxs are thought to be the same, Stx2 is much stronger than Stx1 in mediating HUS[7].

Currently, there is no effective therapy or prophylaxis for HUS other than clinical supportive measures. The use of certain antibiotics is controversial, which appears to increase the risk of HUS development[8]. As in many toxin-mediated diseases, such as tetanus and botulism, little endogenous serum antibody against Stxs is induced following STEC infection[9]. Nonetheless, passively administered toxin-specific antibodies have been

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shown to be highly effective at preventing toxin-mediated diseases. Several Stx2-specific monoclonal antibodies have been developed, and many have been shown to neutralize the activity of Stx2 *in vitro* and/or *in vivo* [10, 11]. Here we describe generation and characterization of a monoclonal antibody designated S2C4 against Stx2 which neutralizes Stx2 and its variants′ cytotoxicity. The availability of Stx2-specific MAb provides an opportunity to administer a safe, immunotherapeutic reagent and prevent development of HUS in susceptible individuals.

1 Materials and methods

1.1 Bacteria strains

The bacterial strains used in this study are described in Table 1. All STEC strains were isolated from the stools of patients and animals in our laboratory during 1999 ～2000 STEC outbreaks [2]. *E. coli* strains were routinely cultured at 37℃ in Luria-Bertani (LB) broth with shaking. The ability of each strain to produce Stxs was validated by using Duopath® verotoxins detection kit (Merck, Germany).

Table 1 Bacterial strains used for this study

Strain	Origin	Toxin type(s)	Serotype
99G143	Human	Stx1, Stx2	O157∶H7
99A008	Pig	Stx2c	O157∶H7
99A026	Goat	Stx1	O157∶H^-
00A086	Human	Stx2vha	O157∶H7

1.2 Stxs and toxoid

Stx2 was isolated, purified from STEC strain 99G143 by affinity chromatography. Briefly, liquid culture of 99G143 was grown in 2 liters of LB medium. After 4 h of incubation, 0.4 mg/L mitomycin C (Sigma-Aldrich, Germany) was added to enhance Stx release from the bacteria, followed by 20 h of incubation. The culture was then centrifuged for 30 min at 20 000 *g* at 4℃, and the supernatant was retained and sterile filtrated. The Stx2-containing culture supernatant was loaded to immno-affinity chromatography column packed by coupling Stx2-A subunit specific antibody S1D8[12] to cyanogen bromide-activated Sepharose 4B (GE Healthcare, Sweden) according to the manufacturer's protocol. The column was washed with 50 ml binding buffer (50 mmol/L Tris-HCl, pH 7.5), and toxin was eluted by elution buffer (100 mmol/L glycine-HCl, pH 2.7 containing 0.5 mol/L NaCl). Purity of Stx2 preparation is exemplified on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and its identity was confirmed by Western blot analysis [12]. Similarly, the culture supernatants of 99A008, 99A026 and 00A086 used as crude preparations of Stx2c, Stx1 and Stx2vha, respectively, were prepared described as above except chromatography process. Stx2 toxoid was prepared by formalin treatment of Stx2 as described previously[11].

1.3 Preparation of MAbs against Stx2

Hybridoma cell lines secreting antibodies to Stx2 were isolated from the fusion of myeloma cells with spleen cells from BALB/c mice immunized with Stx2 toxoid. Six-week old female mice were immunized with 10 μg Stx2 toxoid emulsified in Freund's complete (initial immunization) or incomplete (all subsequent immunizations) adjuvant intraperitoneally (ip) at biweekly intervals a minimum of three times. Four days before fusion, the mice were given a final boost injection with the toxoid intravenously without adjuvant. The spleens of mice with titers of ⩾1∶100 000 were fused to the murine myeloma SP2/0 by the method of Kohler and Milstein [13]. Supernatants from hypoxanthine-aminopterin-thymidine selected hybridomas were successively screened by ELISA on microtiter plates coated with 1.5 μg of Stx2 per ml and developed with HRP-labeled goat anti-mouse IgG (ZHGB-BIO, China). Stable, positive clones were selected by subcloning twice by limiting dilution. MAbs were prepared from the culture supernatant of hybridoma cells by separation on protein G column (GE Healthcare, Sweden) and dialysis against PBS (pH 7.3) and were stored at –20℃ until use.

1.4 Vero cell cytotoxicity assay

Cell viability was evaluated by using a 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA) reduction conversion assay [14]. Vero cells were plated at 3.0×10^4/well on 96-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Invitrogen, USA) and incubated overnight at 37℃ and 5% CO_2. Purified Stx2 was serially diluted, added to each well and incubated for 3 days. Then, 20 μl of MTT at 5 μg/L was added to each well, and incubation was continued for 2 h. The formazan crystals resulting from mitochondrial enzymatic activity on MTT substrate were solubilized with 100 μl of 20% (wt/vol) SDS/50% (vol/vol) N,N-dimethylformamide(pH 4.7), and incubated overnight.

Absorbance was measured at 590 nm by using a microplate reader. Result was expressed as percent viability compared with control culture viability from assays performed in the absence of Stx2. The cytotoxicity assay was done at least two times, and the average was used in the results.

1.5 Cytotoxin neutralization assay

The cytotoxin-neutralizing ability of MAbs was assayed on Vero cells. A 50 μl volume of toxin solution in DMEM containing 5 times the 50% cytotoxic dose (CD_{50}) of the toxin was preincubated with 50 μl of diluted MAbs solution for 1 h at 37℃, and then remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described above. Results were expressed as percent viability compared with control culture viability from assays performed with anti-Stx2 mouse sera without Stx2 (100% viability) and with only Stx2 (0% viability).

1.6 Characterization of MAb S2C4

The isotype of the MAb S2C4 was determined by using a mouse MAb isotyping kit (PIERCE, USA). The subunit specificity of S2C4 was determined by Western blot analysis. Crude Stx1 in 99A026 culture supernatant and purified Stx2 was electrophoresed by SDS-PAGE in 12% polyacrylamide gel and electrophoretically transferred to Nitrocellulose membrane (GE Healthcare, USA). Membrane was blocked with 5% fat-free milk powder in PBS for 0.5 h. The membrane was incubated with blocking buffer containing 10 μg of MAb S2C4 per ml for 2 h at room temperature, and then washed five times in PBS containing 0.05% Tween-20 for 5 min. The membrane was then incubated at room temperature with a 1∶2 000 dilution of HRP-conjugated anti-mouse immunoglobulin IgG (ZHGB-BIO, China) and washed five times as described above. Blot was developed with ECL plus Western blotting detection system kit (GE Healthcare, UK).

1.7 Murine toxin neutralization assay

Two variations of *in vivo* toxicity assays were used to evaluate the ability of S2C4 to neutralize the toxic effects of Stx2 and its variants. Assay Ⅰ involved examining the effect of limiting MAb in the presence of Stx2. Dose-response study was performed with groups of 10 6-week-old female BALB/c mice (Shanghai Slac Laboratory Animal Co. Ltd, China) to determine the amount of purified Stx2 required to induce 100% mortality in untreated animals. A minimum of 5 ng Stx2 was lethal to mouse within 12 d interval (data not shown). The MAb S2C4 was serially diluted 1∶2 from 30 to 0.5 μg/mouse in 200 μl of PBS or PBS alone (control) and administered intravenously (iv) to each group of mice at 16 h after ip administration of 5 ng Stx2. Assay Ⅱ involved examining spectrum of S2C4 activity against Stx2 variants. 4 groups of 10 6-week-old female BALB/c mice were administered iv with S2C4 at a dose of 3.8 μg/mouse or PBS as blank control at 16 h after ip administration of 100 μl per mouse crude preparation of Stx2c in the 99A008, or Stx2vha in 00A086 culture supernatant. Mice were observed twice daily for survival and both experiments were terminated at d12.

2 Results

2.1 Isolation of Stx2

Stx2 was successfully purified to homogeneity from STEC O157∶H7 strain 99G143 culture supernatant by affinity chromatography scheme. SDS-PAGE revealed two protein bands with molecular mass of 32 000 and 7 500 under reducing condition, and monoclonal antibodies against Stx2 A and B subunit can react the two bands in Western blot assay, respectively (Figure 1).

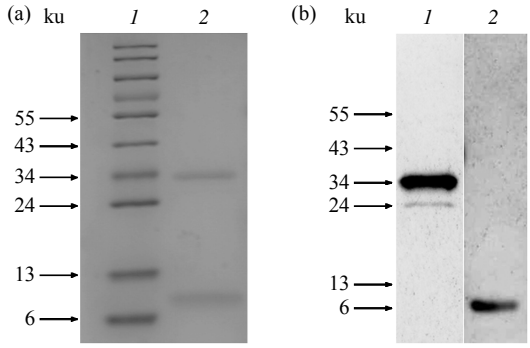

Fig. 1 SDS-PAGE and Western blotting of purified Stx2

Stx2 holotoxin was purified by affinity chromatography. (a) The cytotoxic A-subunit has a molecular mass of 32 ku and monomeric B-subunits are separated at 7.5 ku. *1*: Standard molecular-mass markers; *2*: Purified Stx2 under reducing condition. (b) A Western blot analysis of this toxin molecule, using anti-Stx2 A and B-subunit antibodies, respectively as described in **Materials and methods**. *1* and *2*: Stx2 reacted with anti-A and B- subunit antibodies, respectively.

2.2 Neutralization of Stx2-mediated Vero cell cytotoxicity

Of the 12 positive MAb clones against Stx2 toxoid, only one designated S2C4 showed neutralizing activity towards Stx2, and its ability to neutralize the activity of purified Stx2 was further explored with *in vitro* Vero cell cytotoxicity assay. S2C4 concentration was

varied in the presence of a constant amount of Stx2 (5 times of CD_{50}, 207 fg/L) and the percentage of Stx2 neutralized was determined (Figure 2). The antibody concentration required to obtain 100% and 50% neutralization were 1 250 μg/L and 320 μg/L, respectively.

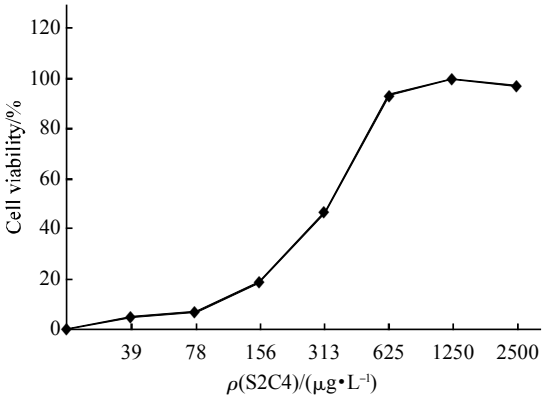

Fig. 2 Neutralizing activity of MAb S2C4 against Stx2 in cytotoxicity assay

Purified Stx2 with a cytotoxicity of 5 times of CD_{50} was incubated with diluted MAb S2C4 at 37℃ for 1 h, and the remaining unbound toxin cytotoxicity was measured by cytotoxicity assay as described in **Materials and methods**. Cell viabilities were calculated according to the following formula: (A_{590} of sample $-A_{590}$ obtained with only Stx2)/(A_{590} with only MAb S2C4$-A_{590}$ obtained with only Stx2)×100%[11].

2.3 Isotype and specificity of MAb S2C4

MAb S2C4 belonged to IgG1 subclass and had a κ light chain (result not shown). The subunit specificity of it was determined by Western blot. S2C4 bound A subunit of Stx2 only and showed no binding to the Stx2 B subunit or to Stx1 (Figure 3).

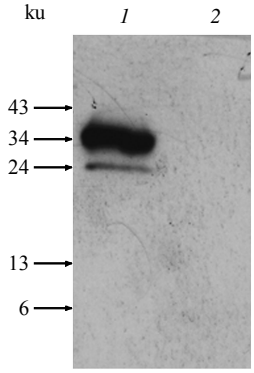

Fig. 3 Western blot analysis of S2C4 binding specificity with Stxs

Purified Stx2 and crude Stx1 were subjected to electrophoresis and blotted with MAb S2C4. *1*: Stx2; *2*: Stx1.

2.4 Neutralization of Stx2 and its variants

Considering the identical *in vitro* neutralization profile of MAb S2C4 against Stx2, a test was performed to assess its potency *in vivo* by using the mouse toxicity model, and its spectrum of activity against Stx2 variants including Stx2c and Stx2vha was also evaluated by this model. S2C4 could significantly prolonged mice survival (experiment was terminated

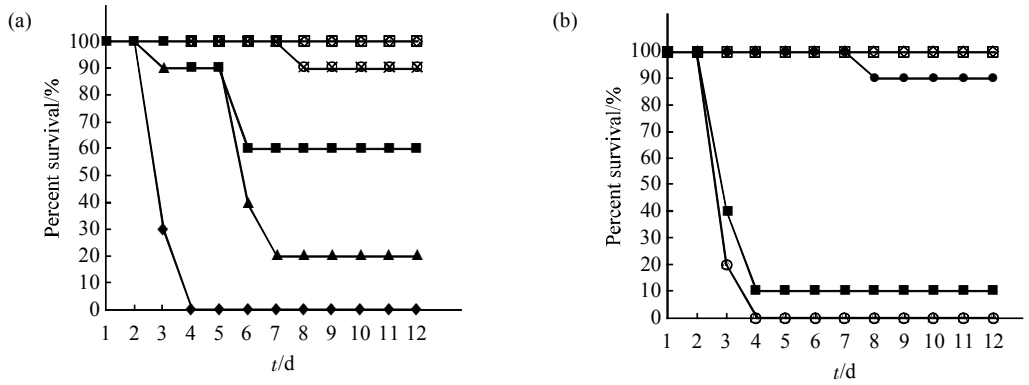

Fig. 4 Neutralization activity of the S2C4 MAb against Stx2 and its variants in the mouse toxicity model

(a) Percent survival of mice given 30, 15, 7.5, 3.8, 1.9, 0.9, 0.5 μg of MAb S2C4 or PBS control in 200 μl volume at 16 h after ip administration of a 100% lethal dose of the Stx2. S2C4 protected 90% of the mice at the doses ranging from 1.9 to 30 μg/mouse (average survival 11.8 days) compared to PBS control (average survival, 2.3 days), but prolonged survival was not observed at the two lower doses of 0.9 and 0.5 μg/mouse. Experiments were terminated on day 12. Mice survived throuth day 12 were euthanized and were given a survival score of 12 days. □—□: 30 μg/mouse; △—△: 15 μg/mouse; ◇—◇: 7.5 μg/mouse; ×—×: 3.8 μg/mouse; ○—○: 1.9 μg/mouse; ■—■: 0.9 μg/mouse; ▲—▲: 0.5 μg/mouse; ◆—◆: Blank. (b)Percent survival of mice given 3.8 μg of MAb S2C4 or PBS control at 16 h after ip administration of 100 μl per mouse crude preparation of Stx2c or Stx2vha culture supernatant. Similar to the neutralization profile to Stx2, S2C4 with 3.8 μg/mouse protected mice against the lethal challenge of both Stx2c and Stx2vha variants, demonstrating its wide protective spectrum. □—□: Stx2c+S2C4; △—△: Stx2c+PBS; ◇—◇: Stx2vha+S2C4; ■—■: Stx2vha+PBS; ●—●: Stx2+S2C4; ○—○: Stx2+PBS.

on day 12) compared to blank control (2.3 days). At each dose ranging from 1.9 to 30 μg/mouse, S2C4 protected up to 90% of the mice with an average survival of 11.8 days, and didn't show dose dependency. Prolonged survival was not observed at the two lower doses of 0.9 and 0.5 μg/mouse (Figure 4a). S2C4 also showed effective protection when administered to mice challenged lethal dose of Stx2c and Stx2vha, respectively, the most prevalent Stx2 variants associated with HUS[2, 15], indicating a broad spectrum of activity against Stx2 variants (Figure 4b).

3 Discussion

In this study we developed and functionally characterized Stx2-specific antibody designated S2C4. Most lethal infection of STEC always starts with colonizing the bacteria to intestinal mucosa with a characteristic "attaching and effacing" mechanism, and terminates with HUS mainly mediated by Stx2, so it is reasonable to directly target the Stx2 molecule for therapeutic strategies in the course of the acute disease.

Initially each MAbs was screened *in vitro* by using a cytotoxicity assay as a first filter to determine the relative ability to neutralize Stx2. Of the 12 positive MAbs clones against Stx2, only one designated S2C4 showed neutralizing activity towards Stx2. We were unsuccessful in adapting the streptomycin-treated mouse infection model [16] for strain 99G143, which produces both Stx1 and Stx2. Consequently, its potential protective efficacy was confirmed by an *in vivo* murine assay. The ability to neutralize Stx2 *in vivo* correlated with the ability to neutralize Stx2 *in vitro* well. At antibody dose of 1.9 μg per mouse or more, S2C4 could protect up to 90% of the mice from the toxic effects of Stx2 in a 12-day experiment interval, and showed similar dose-response curve.

In opposition to Stx1, which is structurally conserved, Stx2 has approximately 10 variants, such as Stx2, Stx2c, Stx2d, Stx2e, Stx2vha and Stx2f, *etc*[17~19]. Stx2 is the most prevalent Stx genotype identified in STEC isolated from patients with HUS, and Stx2c is the most common Stx2 variant associated with HUS. In addition to Stx2c, Stx2vha variant was found to the dominant genotype in the STEC outbreak during 1999~2000 in China. Pierard *et al*[20] had divided Stx2 and its variants into two groups according it's A subunit genes nucleotide sequence homology and the strains associated with diarrhea and HUS were always clustered in group 1 which harbors Stx2, Stx2vha and Stx2vhb, whereas group 2 containing Stx2d-O111, Stx2d-OX3a and Stx2d-Ount were less frequently associated with diarrhea and HUS. Additionally, the A subunit sequence homology among Stx2 variants is much greater than that of the B subunit. In amino acid sequence identity of A subunit of Stx2 variants, Stx2c and Stx2vha share 100% and 99% homology with that of Stx2, respectively. For the B subunit the amino acid sequence homologies of Stx2c and Stx2vha with Stx2, are both 96% only[21]. All these are consistent with our result that S2C4 has an A-subunit specificity and can neutralize Stx2 and its two variants, Stx2c and Stx2vha activity. In term of antagonist effect of neutralizing antibody, S2C4, binding Stx2 A subunit, perhaps either prevents Stx2 ribosomal inactivation or sterically hinders binding of the toxin to its target. The fact that S2C4 has a wider spectrum and reacts A subunit of Stx2 in Western blot assay in denatured form indicates conceivably that S2C4 bind a linear epitope in A subunit and this epitope be conservative and immunodominant among Stx2 and its variants.

The *stx* genes in most STEC strains are carried by lysogenic bacteriophages of the lambdoid family integrated in the bacterial genome[22] and the lysogenic-lytic cycle conversion is controlled by a regulatory cascade network mediated mainly by protein CI [23]. Some antimicrobial agents, particularly the quinolones, trimethoprim and furazolidone, can induce bacterial SOS response and activate the lytic cycle of Stx-encoding bacteriophages, resulting in increased toxin gene copy number, transcription and Stx production *in vivo* [24, 25]. So certain antibiotics with potential life-threatening complications in STEC infections should be avoided in clinical practice. Currently, there are two therapeutic approaches targeting Stx are being tested, one is the utilization of poly-trisaccharides[26] or recombinant bacteria displaying Stx-specific glycolipid[27] as toxin absorber in the gut, and the other involves of systemic administration of monoclonal antibody to neutralize Stx in the bloodstream [1]. In theory, an anti-toxin neutralizing antibody would have a wider therapeutic time window than a toxin absorber, because a toxin absorber would have no opportunity to exert its efficacy if administered after toxin has entered circulation from the gut [28]. However, S2C4 derives from mouse and clinical problems as a result of formation of human antimurine antibodies and other pharmacodynamic effects have hampered its efficacy in human body, so

efforts to humanize this murine antibody through recombinant techniques are now underway in our laboratory. In conclusion, S2C4 is a promising candidate for immunotherapy against HUS.

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单克隆抗体 S2C4 对 2 型志贺毒素及其亚型毒性的中和作用 *

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摘要 纯化的 2 型志贺毒素(Shiga toxin 2，Stx2)经福尔马林脱毒后免疫 BALB/c 小鼠制备 Stx2 单克隆抗体，用体外中和试验对具有中和活性的阳性抗体克隆进行初筛，对所获得的中和抗体的重、轻链同种型及结合特异性进行鉴定，其中和保护作用通过体内、体外中和试验加以验证，最后，中和抗体对 Stx2 亚型 Stx2c 和 Stx2vha 的中和谱用体内中和试验验证．结果显示，12 株抗 Stx2 的阳性抗体克隆中，只有 1 株具有中和活性，命名为 S2C4，其重、轻链同种型为 G_1/κ，其靶分子为 Stx2 的 A 亚单位，与 Stx2 的 B 亚单位或 Stx1 不结合．在体外中和试验中 S2C4 可有效中和 Stx2 对 Vero 细胞的杀伤作用，同样，S2C4 可中和致死量的 Stx2 及其亚型 Stx2c 和 Stx2vha 对小鼠的毒性作用．该抗体有望成为治疗产志贺毒素大肠杆菌感染的候选分子．

关键词 单克隆抗体 S2C4，2 型志贺毒素，中和作用，产志贺毒素大肠杆菌

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