Biologicals 42 (2014) 8–21

Contents lists available at ScienceDirect

Biologicals

journal homepage: www.elsevier.com/locate/biologicals

Neutralization of cobra venom by cocktail antiserum against venom proteins of cobra (*Naja naja naja*)

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ARTICLE INFO

Article history:
Received 27 May 2013
Received in revised form
6 September 2013
Accepted 7 September 2013

Keywords:
ELISA
MTT
Neutral red
Naja naja
Cocktail antiserum
Cobra

ABSTRACT

Naja naja venom was characterized by its immunochemical properties and electrophoretic pattern which revealed eight protein bands (14 kDa, 24 kDa, 29 kDa, 45 kDa, 48 kDa, 65 kDa, 72 kDa and 99 kDa) by SDS-PAGE in reducing condition after staining with Coomassie Brilliant Blue. The results showed that *Naja* venom presented high lethal activity. Whole venom antiserum or individual venom protein antiserum (14 kDa, 29 kDa, 65 kDa, 72 kDa and 99 kDa) of venom could recognize *N. naja* venom by Western blotting and ELISA, and *N. naja* venom presented antibody titer when assayed by ELISA. The neutralization tests showed that the polyvalent antiserum neutralized lethal activities by both *in vivo* and *in vitro* studies using mice and Vero cells. The antiserum could neutralize the lethal activities in *in-vivo* and antivenom administered after injection of cobra venom through intraperitoneal route in mice. The cocktail antiserum also could neutralize the cytotoxic activities in Vero cell line by MTT and Neutral red assays. The results of the present study suggest that cocktail antiserum neutralizes the lethal activities in both *in vitro* and *in vivo* models using the antiserum against cobra venom and its individual venom proteins serum produced in rabbits.

1. Introduction

Snakebite is a serious problem in tropical and subtropical countries and responsible for morbidity and mortality. Global incidence is estimated to be about 5.4 million snakebites per year [1]. Snakebite is also a major public health problem and claims a large number of lives in the Indian subcontinent. In India, approximately 15,000–20,000 people are affected every year by snake envenomation [2]. The four major poisonous such as snakes cobra, krait, Russell's viper and saw-scaled viper are responsible for fatality in India. The venoms of cobra and krait of Elapidae family are neurotoxic in nature, considered to attack the victim's central nervous system and usually results in heart failure. Immunotherapy using polyvalent antivenom is the only effective treatment against snake venom poisoning. Antivenoms are usually prepared by immunizing large animals, usually horses, with individual venom or a range of different venoms obtained from several snakes to eliminate intraspecific variation [3].

Anti-snake venom therapy may cause various side effects such as anaphylactic shock, pyrogen reaction and serum sickness [4]. These side effects may be due to the presence of high concentrations of non-immunoglobulin proteins in many commercially available antivenoms [5]. Moreover, antivenom production in animals is time consuming, expensive and requires ideal storage conditions. In view of the above facts, an alternate technology for production of antivenom has to be developed or a suitable snake venom neutralizing agent has to be found [6].

In the present study, an attempt was made to develop an alternate technology to neutralize the venom of Indian cobra using cocktail antiserum prepared by mixing of antisera raised against individual proteins of venom of cobra and we report the production of species specific antiserum, neutralization of its lethal actions by species specific antiserum raised against *Naja naja* venom using rabbit. The results of this study will form the basis for development and application of monoclonal antibodies to treat envenomation.

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1045-1056/$36.00
http://dx.doi.org/10.1016/j.biologicals.2013.09.002

2. Materials and methods

2.1. Collection of snake venom

The lyophilized snake venom was collected from cobra, and obtained in Irula Snake Catcher's Society, Chennai, Tamil Nadu, India with proper permission (No.WL1/7/2009, dt 13.11.2008) and the sample preserved in desiccator at 4 °C for further use. It was dissolved in 0.9% saline and centrifuged at 2000 rpm for 10 min. The supernatant was collected and kept at 4 °C until further use. Venom concentration was expressed in terms of dry weight and protein concentration determined by Lowry method [7]. Commercial polyvalent antiserum was purchased from King Institute of Preventive Medicine, Chennai, Tamil Nadu, India.

2.2. Collection and maintenance of experimental animals

New Zealand rabbit (2–3 kg), adult Swiss mice (20–22 g) and Balb/c mice (18–22 g) obtained from the Institute of Veterinary Preventive Medicine (IVPM), Ranipet, Tamil Nadu, India were used in this study. They were kept in animal cages with sawdust as bedding under conditions of 12:12 h light and dark cycle and fed with standard diet. Equal numbers of male and female mice were used in each experimental group, keeping their mean weight as near as possible. The animal studies were conducted with the prior permission of the Institutional Animal Ethics Committee, C.Abdul Hakeem College, Melvisharam, Tamil Nadu, India (No.1011/c/06/CPCSEA, dt 19.12.2006).

2.3. Collection and maintenance of Vero cell line

The Vero cell line (Ethiopian kidney green monkey cells from American type culture collection CCL81) was obtained from King Institute of Preventive Medicine, Chennai and was used to study the neutralization of cytotoxic effects of *N. naja* venom. The cells were cultured in 25 cm^2 culture flasks in DMEM, with 10% fetal bovine serum (FBS) and 2% of antibiotic solution (100 μg/ml streptomycin and 100 IU/ml penicillin) incubated in a CO_2 incubator with 5% of CO_2 at 37 °C.

2.4. SDS-PAGE analysis of snake venom

Snake venom protein is medicinally important and analyzed by 12% SDS-PAGE under reducing conditions [8]. Prior to electrophoresis, venom samples (10 μg) were mixed 1:3 (v/v) with Laemmli sample buffer (10% SDS, 10% w/v β-mercaptoethanol, 50% sucrose, 0.02% bromophenol blue), boiled for 5 min, and electrophoresed at a constant current of 30 mA. After electrophoresis the gels were stained with Coomassie Brilliant Blue. Molecular weight standards were co-electrophoresed. Phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), soyabean trypsin inhibitor (92 kDa) and lysozyme (14.3 kDa) were used as colored molecular weight markers.

2.5. Electroelution of different proteins of N. naja venom

The major venom proteins were electro-eluted on the basic method [9]. A preparative SDS-PAGE was run with proteins of cobra venom. After the run, the gel was soaked in prechilled KCl (0.4 M). The prominent venom protein bands were excised and the gel slices were minced into small pieces (~1 mm) using a sterile razor blade. The gel pieces were transferred into a dialysis bag with TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and the bag was kept in a horizontal electrophoretic tank filled with TE buffer. Constant power supply (50 mA) was set and run for 6 h. After elution the sample was dialyzed and concentrated by a Speed vac evaporator. The purified venom proteins were estimated and confirmed on SDS-PAGE.

2.6. Detoxification and production of antiserum

Indian cobra venom (1 mg/ml) was dissolved in physiological saline. The dissolved venom was detoxified by heating at 60 °C for 60 min and was immediately followed by placing the sample in ice-cold (0–2 °C) water for 10 min [10]. This treatment was repeated twice. Male healthy New Zealand white rabbits (2–3 kg body mass) were chosen for the production of polyclonal antibodies. Detoxified cobra venom and individual venom proteins (200 μg/kg of body mass) were emulsified with an equal volume of Freund's complete adjuvant and injected intramuscularly at multiple sites. First booster dose (200 μg/kg of body mass) was given along with Freund's incomplete adjuvant intramuscularly, after 4 weeks of first dose [11]. After first booster dose, injections i.e. second and third booster doses (200 μg/kg of body mass) were administered intramuscularly along with Freund's incomplete adjuvant at an interval of 2 weeks [12]. The blood was collected from the marginal ear vein. After coagulation, the blood was centrifuged at 2000× *g* for 10 min and the serum was collected for determination by ELISA and Western blot analysis [13,14].

2.7. Western blot analysis

Cobra venom (20 μg) was first fractionated by SDS-PAGE, as described above. The gel was placed in the electroblotting apparatus adjacent to nitrocellulose paper in buffer, as described by Towbin and his co-workers [15]. After transfer, the nitrocellulose paper (NCP) was blocked for 1 h with 3% skimmed milk in PBS (20 nM sodium phosphate containing 0.9% NaCl, pH 7.2). The NCP was washed in PBS for 5 min and then incubated with 1:10,000 dilution of rabbit anticobra antiserum (or) rabbit antiindividual venom proteins antiserum for 1 h. This membrane was washed three times in PBS containing 0.05% Tween-20 (PBS/T) followed by PBS was three times for 20 min each. The membrane was incubated with 1:30,000 dilutions of alkaline phosphatase conjugated goat anti-rabbit IgG for 2 h. The membrane was washed as described above and developed with the substrate nitroblue tetrazolium and 5-bromo-4-chloro indolyly phosphate in substrate buffer 10% 1 M Tris pH 9.5, 2.5% 4 M NaCl and 0.5% 1 M $MgCl_2$. Same molecular weight markers were used in the gel electrophoresis.

2.8. Titer determination using ELISA

ELISA was done in the samples of cobra venom using antiserum produced against and individual proteins. The flat-bottomed ELISA plates were coated with venom sample in PBS for overnight at 4 °C. The plates were washed thoroughly with PBS and blocked with 2% BSA in PBS for 1 h at 37 °C. Subsequently, the plates were washed thoroughly with PBS and incubated with antiserum raised against cobra venom and individual venom proteins at 37 °C for 2 h. The plates were washed with PBS/T and PBS three times each for 2 min and further incubated with 100 μl of rabbit anti-mouse IgG conjugated with alkaline phosphatase for 1 h. The plates were washed with PB/T and PBS three times each for 2 min and developed with the substrate *p*-nitrophenyl phosphate in substrate buffer. The optical density was measured at 405 nm using an automated ELISA reader and the titers were determined [16].

2.9. Median lethal dose (LD_{50}) determination

The lethal toxicity was determined in male Swiss strain mice. Groups of six animals were injected i.p. with 0.5 ml of 0.85% NaCl containing increasing concentrations of cobra venom by the