The greater black krait (*Bungarus niger*), a newly recognized cause of neuro-myotoxic snake bite envenoming in Bangladesh

Md Abul Faiz,1 Aniruddha Ghose,2 Md Farid Ahsan,3 Md Ridwanur Rahman,4 Md Robed Amin,5 Md Mahtab Uddin Hassan,3 Md A. Wahed Chowdhury,3 Ulrich Kuch,6 Thalita Rocha,7 John B. Harris,7 R. David G. Theakston8 and David A. Warrell9

1 Department of Medicine, Sir Salimullah Medical College, Mitford, Dhaka 1100, Bangladesh
2 Department of Medicine, Chittagong Medical College, Chittagong 4000, Bangladesh
3 Department of Zoology, University of Chittagong, Chittagong 4331, Bangladesh
4 Department of Medicine, Shaheed Suhrawardy Medical College, Sher E Bangla Nagar, Dhaka 1207, Bangladesh
5 Department of Medicine, Dhaka Medical College, Dhaka 1000, Bangladesh
6 Biodiversity and Climate Research Centre (BiK-F), Senckenberganlage 25, 60325 Frankfurt am Main, Germany
7 Medical Toxicology Centre, Institute of Neuroscience, Newcastle University, Newcastle upon Tyne NE2 4HH, UK
8 Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK
9 Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, UK

Correspondence to: David Warrell,
University of Oxford,
Nuffield Department of Clinical Medicine,
John Radcliffe Hospital,
Oxford OX3 9DU,
UK
E-mail: david.warrell@ndm.ox.ac.uk

Prospective studies of snake bite patients in Chittagong, Bangladesh, included five cases of bites by greater black kraits (*Bungarus niger*), proven by examination of the snakes that had been responsible. This species was previously known only from India, Nepal, Bhutan and Burma. The index case presented with descending flaccid paralysis typical of neurotoxic envenoming by all *Bungarus* species, but later developed generalized rhabdomyolysis (peak serum creatine kinase concentration 29 960 units/l) with myoglobinuria and acute renal failure from which he succumbed. Among the other four patients, one died of respiratory paralysis in a peripheral hospital and three recovered after developing paralysis, requiring mechanical ventilation in one patient. One patient suffered severe generalized myalgia and odynophagia associated with a modest increase in serum creatine kinase concentration. These are the first cases of *Bungarus niger* envenoming to be reported from any country. Generalized rhabdomyolysis has not been previously recognized as a feature of envenoming by any terrestrial Asian elapid snake, but a review of the literature suggests that venoms of some populations of *Bungarus candidus* and *Bungarus multicinctus* in Thailand and Vietnam may also have this effect in human victims. To investigate this unexpected property of *Bungarus niger* venom, venom from the snake responsible for one of the human cases of neuro-myotoxic envenoming was injected into one hind limb of rats and saline into the other under buprenorphine analgesia. All animals developed paralysis of the venom-injected limb within two hours. Twenty-four hours later, the soleus muscles were compared histopathologically and cytochemically. Results indicated a predominantly pre-synaptic action (β-bungarotoxins) of *Bungarus niger* venom at neuromuscular junctions, causing loss of synaptophysin and the degeneration of the terminal components of the motor innervation of rat skeletal muscle. There was oedema and necrosis of extrafusal muscle fibres in envenomed rat soleus muscles confirming the myotoxic effect of...
Bungarus niger venom, attributable to phospholipases A2. This study has demonstrated that Bungarus niger is widely distributed in Bangladesh and confirms the risk of fatal neuro-myotoxic envenoming, especially as no specific antivenom is currently manufactured. The unexpected finding of rhabdomyolysis should prompt further investigation of the venom components responsible. The practical implications of having to treat patients with rhabdomyolysis and consequent acute renal failure, in addition to the more familiar respiratory failure associated with krait bite envenoming, should not be underestimated in a country that is poorly equipped to deal with such emergencies.

Keywords: snake bite; Bungarus niger; greater black krait; rhabdomyolysis; neurotoxicity
Abbreviations: BTX = bungarotoxin; TRITC = tetramethyl rhodamine isothiocyanate

Introduction

Neurotoxins and myotoxins from the venoms of elapid snakes (cobras, king cobras, kraits, mambas, coral snakes, sea snakes and Australasian terrestrial venomous snakes) have proved valuable tools for the study of neuromuscular transmission and muscle regeneration. From venoms of kraits (Bungarus species), β-bungarotoxin (BTX) acts pre-synaptically, α-BTX and γ-BTX antagonize binding of acetylcholine post-synaptically at peripheral neuromuscular junctions and κ-BTX blocks neuronal nicotinic receptors (Rowan, 2001; Nirthanan and Gwee, 2004; Doley and Kini, 2009). Throughout South Asia, life-threatening bulbar and respiratory muscle paralysis following snake bites is a common neurological emergency whose mortality may exceed 160/100,000 population/year in areas such as the south-eastern Terai region of Nepal (Sharma et al., 2004). In Thailand, 26 out of 46 patients who died of snake bite succumbed to respiratory paralysis after envenoming by kraits or cobras (Looareesuwan et al., 1988). The most common problems contributing to these deaths were related to the use of inappropriate antivenoms, failure to provide adequate mechanical ventilation and delay in reaching medical care. A recent nation-wide community-based survey in Bangladesh recorded ~700,000 snake bites/year with ~6000 fatalities (Rahman, et al., 2010).

Although the venomous snake fauna of Bangladesh remains poorly characterized, clinical reports from the regions of Chittagong and Cox’s Bazaar (Faiz et al., 1995, 1997, 1998, 1999), Khulna (Bakar and Amin, 2000), Rajshahi (Azhar et al., 1994) and Mymensingh (Bhuiyan, 1981) have described neurotoxic envenoming attributable to cobras (Naja species) and kraits (Bungarus species). Neurotoxic envenoming by these snakes is a life-threatening medical emergency that requires treatment with specific antivenoms and other measures, most notably urgent assisted ventilation in cases of respiratory paralysis (Warrell, 2010). In an effort to improve clinical management of these cases, we initiated a prospective hospital-based study in south-eastern Bangladesh. By identifying the species responsible for bites, we hoped to define clinical syndromes of envenoming and to provide a rational basis for designing the range of specificity of antivenoms intended for use in the region. During the course of the study, we admitted patients with severe neurotoxic snake bite who showed unusual clinical features. Here, we show that the venom of a species of venomous snake new to Bangladesh, possesses an unexpected activity of great clinical and scientific interest.

Materials and methods

Clinical studies

A prospective clinical study of snake bite was instigated in May 1999 at Chittagong Medical College Hospital, the major tertiary care referral hospital of south-eastern Bangladesh, which receives most admissions from Chittagong District, Cox’s Bazaar District and the three Chittagong Hill Tracts Districts (Harris et al., 2010). For species diagnosis we relied on the identification of snakes brought with bite victims, using morphological and molecular methods (Kuch et al., 2005a, b; Kuch and Mebs, 2007).

Ethical considerations

All patients, or a close relative where necessary, gave informed consent for the inclusion, in public academic presentations and medical publications, of personal, circumstantial, clinical and laboratory information including photographic and radiological, relating to the medical advice, diagnosis and treatment received by the patient at Chittagong Medical College Hospital.

Laboratory studies

Animals

Female Wistar rats, 128–141 g (n=11), were obtained from an accredited breeder and maintained under veterinary supervision according to the requirements of the Committee on Ethics (Newcastle University) and the Animals (Scientific Procedures) Act 1986. In five of them, snake venom (10 µg in 0.2 ml sterile normal saline) was injected subcutaneously into the anterolateral aspect of the left hind limb and sterile normal saline (0.2 ml) was injected into the right hind limb. In six non-envenomed controls, 0.2 ml sterile normal saline was similarly injected into the left hind limb. All animals received analgesic cover (buprenorphine 100 µg subcutaneously) at the time of inoculation. Twenty-four hours later the animals were euthanized and the soleus muscles from both hind limbs were removed, weighed and processed for histology and immunocytochemistry (Harris and Johnson, 1978; Dixon and Harris, 1999).
Venom and reagents

An adult female Bungarus niger (total length 910 mm) was caught in a shop on the campus of the University of Chittagong, and was later responsible for envenoming Case 5 (below). Whole venom was obtained by placing 50 μl microcapillary tubes on each fang, then dried over CaCl₂ and stored at 4 °C. Fluorescein isothiocyanate- and tetramethyl rhodamine isothiocyanate (TRITC)-conjugated α-BTX (catalogues F1176 and T1175, respectively) were obtained from Molecular Probes Inc., UK. Goat anti-acetylcholinesterase (catalogue SC64300/C15) and TRITC-conjugated donkey anti-goat IgG (catalogue C2094) were purchased from Santa Cruz Biotechnology Inc., USA. Rabbit anti-synaptophysin (catalogue RB1461) was from Dako, UK. Chicken anti-neurofilament protein (catalogue AB5539) was from Chemicon Internatio, USA. Fluorescein isothiocyanate-conjugated donkey anti-chicken IgG (catalogue 703 095155) was from Jacksons ImmunoResearch Europe Inc., UK and fluorescein isothiocyanate-conjugated swine anti-rabbit IgG (catalogue F0205) and TRITC-conjugated swine anti-rabbit IgG (catalogue R0156) were from Dako, UK. All secondary antibodies were incubated with rat serum (Dako, UK) and centrifuged to yield a clear supernatant with eosin (Harris and Johnson, 1978). To determine whether exposure to the venom impaired binding of α-BTX to the junctional acetylcholine receptor, we incubated (1 h, ambient temperature) transverse cryosections of muscle tissue dissected from naive rats with crude venom at concentrations ranging between 0 and 300 μg/ml in phosphate buffered saline. Neuronal junctions were identified by labelling junctional acetylcholinesterase first with anti-acetylcholinesterase primary antibodies diluted 1:25 and then with an appropriate secondary fluorescein isothiocyanate-conjugated IgG and α-BTX (2 h, ambient temperature). The percentage of acetylcholinesterase-positive sites labelled with α-BTX was calculated (Hart et al., 2008).

Histology and cytochemistry

Transverse and longitudinal cryosections of muscle tissue were collected onto glass slides coated with gelatin and chromium potassium sulphate (i.e. subbed), stained with haematoxylin and counterstained with eosin (Harris and Johnson, 1978). To determine whether exposure to the venom impaired binding of α-BTX to the junctional acetylcholine receptor, we incubated (1 hom ambient temperature) transverse cryosections of muscle tissue dissected from naive rats with crude venom at concentrations ranging between 0 and 300 μg/ml in phosphate buffered saline. Neuronal junctions were identified by labelling junctional acetylcholinesterase first with anti-acetylcholinesterase primary antibodies diluted 1:25 and then with an appropriate secondary fluorescein isothiocyanate-conjugated IgG and α-BTX (2 h, ambient temperature). The percentage of acetylcholinesterase-positive sites labelled with α-BTX was calculated (Hart et al., 2008).

Immunocytochemistry

Transverse cryosections of muscle were mounted onto subbed glass slides, permeabilized in ethanol and methanol (−20 °C, 10 min) and then in 0.1% Triton X-100 in phosphate buffered saline (10 min, ambient temperature) and rinsed with phosphate buffered saline. Sections were incubated overnight in a closed moist chamber at 4°C with appropriate primary antibodies diluted to a final concentration of 1/100 (anti-acetylcholinesterase was used at a dilution of 1/25). They were then allowed to return to room temperature and washed in phosphate buffered saline before being counter-labelled with anti-synaptophysin primary antibodies overnight at 4°C. The following day, slides were allowed to return to room temperature, washed in phosphate buffered saline and incubated with a combination of the appropriate fluorescein isothiocyanate- or TRITC-conjugated secondary antibody and α-BTX for 2 h at room temperature and then mounted in Vectashield®.

To determine whether exposure to venom in vivo caused a loss of synaptic vesicles from the motor nerve terminals, we documented the loss of labelling of the surrogate marker, synaptophysin (a component of the membrane of the synaptic vesicle). Two techniques were used. A subjective measure was made by the visual examination of transverse cryosections of muscle tissue, incubated overnight with anti-synaptophysin primary antibodies and then with a combination of the appropriate fluorescein isothiocyanate-secondary antibody and TRITC-conjugated α-BTX. The percentage of sites labelled with α-BTX that were co-labelled with anti-synaptophysin was calculated. The tissue sections were examined using a Leica DMRA fluorescence microscope (Leica, Heidelberg, Germany) and data were collected independently by T.R. and J.H. The reported results represent our agreed interpretation of the data. An objective measure was made using the analysis of full ‘en face’ images of junctional regions collected from longitudinal cryosections labelled first with a combination of anti-synaptophysin and anti-neurofilament primary antibodies and then with a combination of the appropriate fluorescein isothiocyanate-secondary antibodies and TRITC-conjugated α-BTX as described above. Images were collected without further selection and analysed using a Leica TCS SP2 UV dual-channel confocal microscope controlled by the LCS Build 1537 software. The area occupied by TRITC-conjugated α-BTX was measured, as was the pixel intensity of the both the TRITC- and fluorescein isothiocyanate-images. The intensity level was set at the midpoint between threshold and saturation for a randomly selected TRITC-labelled image on a control section and remained unchanged. Fluorescein was excited at 488 nm and rhodamine at 543 nm. Emissions were collected at 552–626 nm for fluorescein and 496–551 nm for rhodamine. The number of optical slices collected for the final image was 10 in every case. The images were collected by a colleague (Dr Trevor Booth) who also measured the area occupied by acetylcholine receptor clusters and collected data on pixel intensity. He was blind to the origins of the tissue being examined and to the objectives of the investigation. All images were encoded and stored. Data were analysed by J.H.

Electron microscopy

Ultrastructural observations were made on segments of muscle (approximately 1 x 1 x 2 mm) collected from the end-plate region, fixed in Karnovsky’s fluid, post-fixed in OsO₄, dehydrated and embedded in TAAB resin (TAAB Laboratory Equipment, Berks, England). Sections were cut at 50–70 nm, stained with uranyl acetate followed by lead citrate and examined under a Philips EM500 microscope (FEI, Oregon, USA). Images were collected by a colleague (Tracey Scott-Davey) who was blind to the experimental status of the individual muscles.

Statistics

Data are generally expressed as mean ± SEM. Differences between means were analysed using a paired Student’s t-test. A difference was considered significant where P < 0.05.

Results

Clinical studies

Among 350 envenomed patients recruited to the study over a period of 42 months, 108 presented with or developed features
of neurotoxic envenoming. Only 26 brought the dead or living snake responsible for the bite. These included several non-venomous snakes (Harris et al., 2010), 12 monocellate cobras (Naja kaouthia), one banded krait (Bungarus fasciatus), and three greater black kraits (B. niger). Two additional patients with proven bites by B. niger were admitted in 2003 and 2007. The five cases of envenoming by this species are described.

Case reports

Case 1
At ~09:00 h, a 40-year-old Bangladeshi day labourer was bitten on his right hand by a snake while cutting wood near Chandanaish, a forest-fringe area ~50 km from the Burmese border. He caught the snake and consulted a traditional healer who reassured him but gave no treatment. After 4–5 h, insidious weakness compelled him to see a local physician and, ~7 h after the bite, his consciousness became clouded. He was driven to Chittagong Medical College Hospital and admitted at 18:30 h, 9.5 h after the bite, bringing the live snake to hospital. The Glasgow Coma Scale was 7 (E1 M5 V1) and he was deeply cyanosed (oxygen saturation 65% by pulse oximetry). Both pupils were widely dilated and non-reacting. There was bilateral ptosis, paralysis of the neck flexors and deep tendon and plantar reflexes were absent. His blood pressure was 110/60 mmHg and pulse rate 114/min. After urgent intubation and manual ventilation with high flow oxygen via Ambu bag, oxygen saturation increased to 97%. He was then mechanically ventilated. Polyvalent antivenom (Haffkine, India) 260 ml was given by intravenous infusion without reaction. Neostigmine (50 µg/kg subcutaneous) and atropine (15 µg/kg intravenous) were given every 4 h but there was no detectable response. The next morning, 23 h after the bite, he was passing black urine (Fig. 1). His peripheral leucocyte count was 20 × 10⁹/l (82% polymorphs), platelet count 350 × 10⁹/l, haemoglobin concentration 13 g/dl, erythrocyte sedimentation rate 65 mm/h, blood urea nitrogen 19 mg/dl (6.8 mmol/l), K⁺ 8.5 mmol/l, Na⁺ 129 mmol/l, creatine kinase 29 960 units/l, blood glucose 229 mg/dl (12.7 mmol/l). Urine contained albumin 500 mg/dl, ketones 5 mg/dl. No erythrocytes or casts were present. The ECG showed tall T waves. To combat hyperkalaemia, he was given 10 ml of 10% calcium gluconate and 100 ml of 25% glucose intravenously. Despite treatment with dopamine, pulse and blood pressure became unrecordable over the next 6 h and he died 47 h after the bite (38 h after admission). The snake was preserved and later identified as an adult male B. niger, 975 mm in total length (Fig. 2).

Case 2
While sleeping on the floor of a hut near Khagrachhari in the Chittagong Hill Tracts, a 35-year-old male was bitten on the buttocks. He visited a traditional healer but developed progressive difficulty with breathing, keeping his eyes open and talking.

Figure 1 Case 1: forty-year-old male 23 h after being bitten by a greater black krait (B. niger, Fig. 2), showing generalized flaccid paralysis requiring mechanical ventilation and myoglobinuria.

Figure 2 Greater black krait (B. niger). Adult male 975 mm in total length responsible for biting and fatally envenoming Case 1 near Chandanaish, Bangladesh. (A) Dorsal view and (B) ventral view.
He was taken to a sub-district hospital at Fatikchari but died early the next day. The partially skeletonized snake measuring 575 mm in total length was later recovered and identified as a *B. niger* based on characters of vertebral morphology and scale colouration.

**Case 3**

A 12-year-old male was bitten on his left foot while sleeping on the floor of his home near Fatikchari at 04:00 h. The snake was killed and identified as a krait (‘kaal shap’ in the local language). There was minimal bleeding and pain at the bite site. For the next 4 h he received traditional treatments, including local incisions and application of ligatures to the left lower leg (one) and thigh (two). Two hours after the bite, he vomited; 3 h after the bite there was drooping of the upper eyelids, blurred and double vision and later difficulty in swallowing, weakness of the neck flexors and breathlessness. Eleven hours after the bite, he became weak and fainted. On admission to Chittagong Medical College Hospital, 17 h after the bite, his blood pressure was 90/70 mmHg and pulse rate 140/min. He was not cyanosed. He had bilateral ptosis and external ophthalmoplegia that persisted for 4 days, difficulty in eating, talking and opening and closing his mouth persisting for 2 days, pooling of secretions persisting for 1 day and weakness of the neck flexors and extensors persisting for 2 days. He was treated with polyvalent antivenom (Haffkine, India), two doses of 100 ml each, both of which were interrupted by severe early anaphylactic reactions despite prophylactic adrenaline. Although he did not respond to edrophonium, he was treated with neostigmine and atropine 4-hourly for 84 h. He was mechanically ventilated for 22 h. His leucocyte count was $10 \times 10^9/l$ (polymorphs 48%, lymphocytes 41%). The urine contained albumin (+). The ECG was normal on Days 1–2. He made a complete recovery. The snake was later identified as an adult female *B. niger*, 804 mm in total length.

**Case 4**

An 18-year-old day labourer was bitten on the dorsum of his right hand at 07:00 h, while handling a fishing net at home at Dighinala in Khagrachhari Hill Tract District. Ligatures were applied to his right forearm (one) and upper arm (two) for 7.5 h. He spent 3 h with a traditional healer, who made an incision at the site of the bite. He was admitted to Chittagong Medical College Hospital 8 h after the bite. There was pain and slight bleeding at the bite site. He had first noticed drooping of the eyelids, blurred double vision and weakness 5 h after the bite. He had difficulty in swallowing and speaking and felt burning in his throat and chest tightness. On admission, his blood pressure was 220/80 mmHg, later dropping to 120/70 mmHg, and pulse rate 100/min. No fang marks or local swelling were detectable. ECG, complete blood count and results of urine examination were normal. He was treated with two doses of polyvalent antivenom (Haffkine, India; 100 ml each) and neostigmine and atropine 4-hourly for 32 h. He did not require assisted ventilation but dysphagia, dysphonia and inability to protrude the tongue persisted for 3 days, and bilateral ptosis, external ophthalmoplegia, difficulty in opening his mouth and weakness of neck flexors and extensors persisted for 5 days. He recovered and was discharged after 7 days. The snake, described as ‘kaal shap’, was later identified as an adult female *B. niger*, 850 mm in total length.

**Case 5**

While a captive adult female *B. niger*, 910 mm in total length, was being force-fed with a fish at 16:00 h, one of its fangs scratched the pulp of a 27-year-old snake handler’s right index finger (Fig. 3). He tried to suck the blood from the wound and an elastic pressure bandage was applied from hand to upper arm. He was rushed to Chittagong Medical College Hospital, arriving within 2 h of the bite. On admission there were no features of envenoming and the bandage was removed. About 5 h after the bite, he began to develop increasing generalized myalgia, which had become agonizing 9 h after the bite and was accompanied by a headache. He found swallowing painful but not difficult (odynophagia).

Generalized muscle tenderness was most severe around his neck but there was no abdominal pain. He had slight ptosis (Fig. 4) and minimal weakness of the bilateral lateral rectus muscles on extreme lateral gaze (Fig. 5A and B). Neurological examination was otherwise unremarkable. ECG and urine were normal. Pain was treated with diclofenac by suppository. Four hours later myalgia and odynophagia had increased. Posis and lateral rectus weakness were more marked but there was no respiratory or pharyngeal weakness. Treatment with neostigmine and atropine produced minimal improvements in ptosis and lateral rectus involvement but caused generalized fasciculations, slurred nasal speech and restlessness that settled after 2–3 h.

Blood sampled 18 and 24 h after the bite showed minimal increases in creatine kinase (309–442 units/l) and lactic dehydrogenase (254–318 units/l). There was a mild leucocytosis (13.5 and 12.0 $\times 10^9/l$). K+, HCO$_3^-$, creatinine, prothrombin time, alanine and aspartate transaminases and alkaline phosphatase remained normal.

His symptoms slowly improved and by 31 h after the bite, muscle tenderness, ptosis and lateral gaze had improved but odynophagia persisted. This was treated with warm saline gargles, hot compresses over the neck, rectal diclofenac and intravenous midazolam. Forty hours after the bite, his symptoms had subsided, the only remaining sign being slight bilateral lateral rectus palsy. Serum creatine kinase peaked at this time (640 units/l) and declined to normal over the next 36 h.

**Figure 3** Case 5: site of envenoming. Scratch made by a fang of *B. niger*.
Laboratory studies of *Bungarus niger* venom

**Signs of envenoming**

After a delay of \( \sim 2 \) h, animals inoculated with *B. niger* venom exhibited paralysis of the ipsilateral hind limb and a loss of the hind limb and toe extensor reflexes when lifted by the tail. The paralysis affected the inoculated limb until 24 h when the animals were euthanized. At no time was there any sign of weakness in the contralateral limb. There was no loss of ambulation or exploratory behaviour and no aversion to handling or palpation of the inoculated limb. There was no lacrimation, salivation or rhinitis and no sign of anxiety or restlessness. The animals ate and drank normally. Defecation and urination appeared normal. The urine was typically clear and straw coloured.

**Skeletal muscle**

Soleus muscles from the envenomed limbs were oedematous and pale in colour and exhibited a statistically significant increase in wet weight \(+41 \pm 5.6\%\), \(n=5\), cf. \(+7 \pm 2.4\%\), \(n=6\) in control muscles following the inoculation of an equivalent volume of normal saline). Histological examination confirmed oedematous separation of extrafusal muscle fibres, invasion of the extracellular space by inflammatory cells and the absence of haemorrhage. Swollen, hyaline and necrotic extrafusal muscle fibres were widely distributed throughout the muscles. Intrafusal fibres of the muscle spindles were unaffected. The microcirculation including intramuscular veins and arterioles were typically dilated but undamaged. Typical images are shown in Fig. 6A and B.

**Immunocytochemical labelling**

Neuromuscular junctions on transverse cryosections of soleus muscles were labelled with anti-acetylcholinesterase antibodies and co-labelled with TRITC-conjugated \( \alpha \)-BTX. The percentage of junctions labelled with anti-acetylcholinesterase antibodies that were co-labelled with \( \alpha \)-BTX averaged \( 94.7 \pm 3.21\%\) \((n=6\) sections). There was no significant reduction in the percentage of junctions labelled with \( \alpha \)-BTX after pre-incubation of six sections of muscle with concentrations of *B. niger* venom of \(100 \mu g/ml\) or...
more (91.5 ± 4.98%). Pre-incubation with unlabelled α-BTX (10 μg/ml) resulted in a loss of co-labelling with TRITC-conjugated α-bungarotoxin.

All neuromuscular junctions seen in transverse cryosections of control muscle fibres (n = 139) were consistently labelled with both α-BTX and anti-synaptophysin antibody (Fig. 7A). In muscles exposed in vivo to the venom of B. niger, 401 junctions were identified by labelling with α-BTX. Only 36% were labelled fully with anti-synaptophysin antibodies, 40% were only patchily labelled and 34% were unlabelled (Fig. 7B).

The pattern of innervation of individual muscle fibres was further determined by labelling longitudinal sections with α-BTX and a combination of anti-synaptophysin and anti-neurofilament antibodies. In sections of control muscles, the terminal axon and
Combined labelling of synaptophysin and neurofilament (green) and junctional acetylcholine receptor (red) at neuromuscular junctions in longitudinal cryosections of soleus muscles of rats. In control muscles (A) the terminal axon and its arborizations could be clearly identified and was closely associated with the acetylcholine receptor clusters. In muscles exposed in vivo to the venom of *B. niger*

(Continued)
its junctional ramifications were easily identified in every junction examined \((n = 36, \text{ Fig. 8A})\). In the muscles exposed \textit{in vivo} to venom, however, the normal pattern of innervations was seen in only 3 of the 28 junctions identified (Fig. 8B). In each case, those junctions were located on a muscle fibre that had not been destroyed by exposure to the venom. In the remaining junctions, all of which were located on a severely damaged muscle fibre, there was either a residual labelling of a degenerating axon with remnants of terminal labelling (Fig. 8C) or remnants of labelling in terminal boutons of \(\sim 1 \mu m\) diameter located within a gutter of acetylcholine receptors (Fig. 8D). In two cases the junctions appeared to be completely denervated (Fig. 8E). The mean area occupied by acetylcholine receptor clusters and the mean fluorescent intensity of those clusters in the envenomed muscles was not significantly different from the controls, but there was a significant fall in the fluorescence intensity of the fluorescein label, representing neurofilament and synaptophysin (Table 1).

Electron microscopy

Ultrastructural studies were very difficult because the tissue was so severely damaged following exposure to the venom. The principal findings were that intra-axonal myelin figures and the loss of integrity of myelin were common (Fig. 9A), muscle fibres were devoid of internal architecture or hypercontracted but that post-junctional specializations at the neuromuscular junctions (folding and high density at the tops of junctional folds) were preserved (Fig. 9B–D). Plasma membranes of nerve terminal boutons were indistinct and the boutons were filled with dense mats of disorganized neurofilamentous material. Vesicles of widely varying size were present in the terminal boutons but appeared to be sparse (Fig. 9B–D). Mitochondria were clearly damaged.

Unfortunately we do not have enough data to provide a more quantitative analysis of the ultrastructural findings.

**Discussion**

Thirteen species of \textit{Bungarus} are currently recognized (Slowinski, 1994; Kuch \textit{et al.}, 2005a). First described in 1908 by physician-herpetologist Frank Wall, \textit{B. niger} (Fig. 10) is now known to be widely distributed in areas of high humidity and rainfall in northern India, Nepal, Bhutan and Burma (Wall, 1908; Leviton \textit{et al.}, 2008; Theophilus \textit{et al.}, 2008). Prior to our prospective study at Chittagong Medical College Hospital, the presence of this species in Bangladesh was not documented (Montaquim \textit{et al.}, 1980). However, the specimens brought by our patients and another collected in Sundarban National Park (Bagerhat District, Khulna Division, Bangladesh) in April 2004 by M. Anisuzzaman Khan and M. Sazedul Islam (personal communication) demonstrate that \textit{B. niger} is wide-ranging in Bangladesh and that its habitat extends from mangroves at sea level to at least \(1450 m\) in the Himalayas (Tillack and Grossmann, 2001). The largest \textit{B. niger} on record measured 1250–1300 mm (Tillack and Grossmann, 2001). The few living specimens of \textit{B. niger} that we observed were all fast-moving, nervous snakes that hissed and attempted to escape when disturbed during the day. Compared with common kraits (\textit{Bungarus caeruleus}), these snakes move swiftly and respond more rapidly and vigorously when disturbed or restrained. Bites by \textit{B. caeruleus} in Sri Lanka and India are inflicted almost exclusively on people sleeping on the floor of their homes (Ariaratnam \textit{et al.}, 2008), an epidemiological feature so consistent as partly to define the syndrome of krait bite in these countries (Ariaratnam \textit{et al.}, 2009; Warrell, 2010). These were

**Table 1** Properties of nerve-muscle junctions on muscle fibres of soleus muscles of rats 24 h after the inoculation of the venom of \textit{Bungarus niger} (20 \(\mu g\) subcutaneously into the anterolateral aspect of one hind limb)

<table>
<thead>
<tr>
<th></th>
<th>Junctions on control muscle fibres</th>
<th>Junctions on envenomed muscle fibres</th>
<th>Significance of difference</th>
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<tbody>
<tr>
<td>(n)</td>
<td>36</td>
<td>28</td>
<td></td>
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<tr>
<td>Motor axon and terminal innervation intact (%)</td>
<td>100</td>
<td>12</td>
<td></td>
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<tr>
<td>Terminal innervation partially lost (%)</td>
<td>0</td>
<td>81</td>
<td></td>
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<tr>
<td>Terminal innervation absent (%)</td>
<td>0</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Area of AChR clusters ((\mu m^2))</td>
<td>149 ± 8</td>
<td>170 ± 18</td>
<td>Not significant</td>
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<tr>
<td>Pixel intensity of red channel</td>
<td>119 ± 9</td>
<td>145 ± 16</td>
<td>Not significant</td>
</tr>
<tr>
<td>Pixel intensity of green channel</td>
<td>32 ± 5</td>
<td>8 ± 2</td>
<td>Significant ((P &lt; 0.05))</td>
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</tbody>
</table>

Control data were from the contralateral soleus muscles. Acetylcholine receptors (AChR) were labelled with TRITC-conjugated \(\alpha\)-BGX (red channel) and synaptophysin and neurofilament with the appropriate primary antibody followed by a fluorescein isothiocyanate-conjugated secondary antibody (green). Images generated by confocal laser microscopy under standardized conditions.
also the circumstances under which our Cases 2 and 3 were bitten by *B. niger*, but two were bitten outdoors in the morning, one while wood cutting and the other while handling a fishing net. These cases suggest that bites by *B. niger* might occur under a greater variety of circumstances than is usually the case with *B. caeruleus*, also a common snake in many parts of Bangladesh, complicating attempts to infer the identity of the biting species from circumstantial evidence.

**Neuropathological correlates of *B. niger* envenoming**

**Neurotoxicity**

The venoms of kraits contain two principal classes of neurotoxin that might be implicated in neuromuscular paralysis: the post-synaptically active \( \alpha \)-BTXs and the pre-synaptically active \( \beta \)-BTXs. \( \alpha \)-BTXs bind to the acetylcholine binding sites at the...
interfaces between the α/δ and the α/δ subunits of the acetylcholine receptor, thereby preventing the interaction between acetylcholine and the receptor to cause a rapidly developing neuromuscular paralysis. Neither in cryosections of the soleus muscle of naïve rats exposed in vitro to the venom of B. niger, nor in similar sections of muscles of rats previously inoculated with the venom in vivo, was there any reduction in the labelling of acetylcholine receptors by fluorescein isothiocyanate-conjugated α-BTX. Thus we conclude that α-BTXs were not involved in the neuromuscular paralysis seen in the hind limbs of rats inoculated with the venom of B. niger. We suggest that this may also be true in the human cases of envenoming by B. niger reported above.

β-BTXs are heterodimeric neurotoxins consisting of a phospholipase A₂ chain and a Kunitz-type protease inhibitor chain. Many neurotoxic phospholipases A₂ have been shown to cause a significant loss of labelling by anti-synaptophysin (reflecting a loss of synaptic vesicles) at the mammalian neuromuscular junction and degeneration of intramuscular branches of the terminal motor axon. They have been implicated in the resistance of the neuromuscular weakness of krait bite victims to antivenom and anti-cholinesterases (Dixon and Harris, 1999; Prasarnpun et al., 2005). Here, we have shown unequivocally that the inoculation of the venom of B. niger results in the loss of synaptophysin and the degeneration of the terminal components of the motor innervation of rat skeletal muscle. Thus, we suggest that β-BTXs contribute to the life-threatening neuromuscular weakness seen in the victims of envenoming bites by B. niger. It was noted that although the venom caused the degeneration of both skeletal muscle and the terminal components of the motor axon, the organization of the post-synaptic junctional membrane remained intact even in severely damaged muscle fibres and acetylcholine receptor clustering was unaffected. This has been reported for other myotoxic elapid venoms and for the myo-neurotoxic phospholipases A₂ isolated from the venom, and is usually ascribed to the complex of stabilizing proteins involved in the formation of junctional folds and the integration of the acetylcholine receptors (Slater and Allen, 1985; Harris et al., 2000). It would have been interesting to support our morphological findings in the rat soleus muscle preparation with detailed physiological studies of neuromuscular transmission, to determine involvement of post- and pre-synaptic components of the neuromuscular junction in the muscles that we removed. However, this was not possible because of the destruction of both nerve and muscle by this unusually neuro-myotoxic venom. In the future, it will be informative to confirm the absence of post-synaptic toxicity, to determine the cycle of events leading to nerve terminal degeneration and to document the repair and regeneration of the junction in studies on tissue removed from animals at various stages after the inoculation of the venom. More detailed studies of the effects of both whole venom and its individual toxins on isolated neuromuscular preparations would also be valuable. These studies await access to further supplies of venom from the elusive greater black krait.

Myotoxicity

Although the venoms of B. candidus and B. fasciatus, but not of B. caeruleus and B. multicinctus, caused dose-dependent muscle necrosis in rats (Summers and Harris, 1987), rhabdomyolysis has not previously been fully or convincingly documented in patients envenomed by terrestrial elapids in Asia. However, myalgia associated with modest increases in serum enzyme concentrations suggesting mild rhabdomyolysis was reported in victims of B. caeruleus bites in Sri Lanka (Theakston et al., 1990). Recently, in Nongbualamphu Province, north-eastern Thailand, a 42-year-old female developed neurotoxic symptoms and began to pass ‘coca-cola-coloured’ urine 14 h after being bitten by a B. candidus. She died in Udonthani Hospital 4 days later with acute renal failure (Chaiyabutr et al., 2008; Lawan Chanhome and Wirat Leeprasert, personal communication). In a series of patients envenomed by B. candidus in southern Vietnam, elevated serum creatine kinase levels suggested rhabdomyolysis (Trinh et al., 2010) and in northern Vietnam, 68% of a series of 60 patients presumed to have been bitten by B. multicinctus complained of generalized myalgia (Hung et al., 2009).

In contrast, devastating rhabdomyolysis, hyperkalaemia and acute renal failure are familiar features of envenoming by several species of sea snakes (Reid, 1961). Outside Asia, it has been described occasionally following bites by terrestrial elapid. Thus, rhabdomyolysis has been reported after bites by tiger snakes (Notechis species), broad-headed snakes (Hoplocephalus bungaroides), mulga snakes (Pseudechis australis), Collett’s snake (Pseudechis colletti), Papuan taipans (Oxyuranus scutellatus canni), Australian small-eyed snake (Rhinecephalus nigrescens), New Guinea small-eyed snakes (Micropechis ikaheka) (Warrell et al., 1996) and Papua New Guinean death adders (Acanthophis species) (Lalloo et al., 1996). In the Americas, generalized myalgia with evidence of gross rhabdomyolysis (serum creatine kinase levels of up to 18000 units/l) has been described after human envenoming by coral snakes [genus Micrurus: M. fulvius (Kitchens and Van Meirop, 1987), M. laticollaris (Pettigrew and Glass, 1985) and M. lemniscatus helleri (Manock et al., 2008), and in mice injected with the venoms of several Central and South American Micrurus species (Gutiérrez et al., 1983).

The most potent myotoxic components of elapid snake venoms are phospholipases A₂, many of which are also neurotoxic (Harris, 1991). We suggest that myotoxic phospholipases A₂ and/or phospholipases A₂ with both myo- and neurotoxic activity are present in the venom of B. niger. Our experimental data have validity only if the relative doses of venom used in these experiments (calculated as μg/kg) equate with the ‘dose’ that may be inoculated during a bite. In the rats, an average weight of 136 g, 10μg was inoculated equating to a dose of 73 μg/kg. The yield of venom on milking healthy, full-grown captive male kraits can exceed 30 mg and a single contraction of the venom gland may yields more than 5 mg (U. Kuch, personal information). Studies on venom release during defensive biting by Australian elapids show that ~20% of the stored venom is inoculated into the victim (Morrison et al., 1983), suggesting that envenoming by a quick bite involves a single contraction of the muscles of the venom glands. If we assume that ~5 mg of venom is inoculated into a human during a bite by an adult B. niger, then the average Bangladeshi male weighing 60 kg would receive a ‘dose’ of 83 μg/kg. Calculations of this kind are associated with numerous caveats. For example, the pharmacokinetics of uptake of venom
are unlikely to be similar in rats and humans and there is great variation in the amount of venom inoculated by a biting snake (Morrison et al., 1982, 1983; Young et al., 2002). Accepting these caveats, we consider that our experimental model equates to an acceptable degree with the clinical condition.

Conclusions

This is the first formally published report of (i) B. niger being found in Bangladesh, (ii) envenoming by this species in any country and (iii) generalized rhabdomyolysis attributable to envenoming by any Asian or African terrestrial elapid snake.

Our data show that B. niger is widely distributed in Bangladesh and confirm that it is capable of fatal neuro-myotoxic envenoming. Its venom should therefore be considered when antivenoms are designed for this region. The unexpected finding of rhabdomyolysis should prompt further investigation of the venom components responsible for this life-threatening effect of envenoming by the greater black krait. The practical implications of having to treat patients with rhabdomyolysis and consequent acute renal failure, in addition to the more familiar respiratory failure associated with krait bite envenoming, should not be underestimated in a country that is poorly equipped to deal with such emergencies.

Funding

The Wellcome Trust, London, UK (grants 052708/Z/97/Z and 079027/2/06/Z). The Association of Physicians of Great Britain and Ireland, the Association of British Neurologists, the Deutsche Forschungsgemeinschaft (DFG; grant KU 2328/1-1) and the LOEWE Programme of the Ministry of Higher Education, Research and the Arts of the State Government of Hessen, Germany, provided assistance with the cost of travel.

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