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Intravenous dose-lethality study of American pit viper venoms in mice using standardized methods

PAUL CONSROE, PhD, KEVIN GERRISH, MS, NED EGEN, PhD and FINDLAY E. RUSSELL, MD, PhD

Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, Tucson, AZ 85721, USA

An intravenous dose-lethality study of 14 clinically important North and South American pit viper venoms was carried out in mice using standardized, rigorous protocols developed to meet international (WHO) guidelines and US government (FDA) regulations for the development of new treatment products. The study was undertaken in order to establish baseline LD_{50} values for the ongoing testing of a new crotalid antivenin F_{AB} prepared from sheep immunoglobulin (IgG). The venoms (lyophilized and pooled from juvenile and adult snakes of both sexes) were taken from 10 rattlesnake (nine *Crotalus* and one *Sistrurus*) species, two *Agkistrodon* species, and two *Bothrops* species. The dose-response data were subjected to probit analysis. Median lethal dose (LD_{50}) values and their 95% confidence limits (CL) were calculated and statistical comparisons between the median potencies were made. The intravenous LD_{50}s (and 95% CL) ranged from a low of 0.13 (0.10–0.15) mg kg^{-1} for *Crotalus durissus terrificus*, to a high of 6.32 (5.76–6.94) mg kg^{-1} for *Crotalus horridus horridus*. Significant positive rank-order correlations were also found for comparisons between the present LD_{50}s and published LD_{50}s for intravenous, intraperitoneal, and subcutaneous administration.

Key words: American pit vipers, venoms, LD_{50}, mice, snakes, antivenin

Introduction

Studies have been carried out by various groups to assess the lethal effects in mice of venoms from North and South American snakes [1–4]. Because of varying methods in such studies, including the sources and handling of venoms and their routes of administration, published values of lethal potencies vary widely [5]. The World Health Organization (WHO) addressed these concerns [6,7] and concluded that assay methods for assessing the biological activity of medically important venoms and the neutralizing capacity of antivenins required standardization. A study evaluating the standardization of methods was carried out by Theakston and Reid [8]. Venoms from 30 different species of snake of clinical importance throughout the world were used to investigate several bioassays, including dose-lethality in mice. In the LD_{50} (median lethal dose) test, the techniques were standardized for sex, strain, weight, and number of mice, route and volume of venom injection, time of evaluation of lethality, and a statistical evaluation of the dose-lethality data. Further suggestions on standardization of the LD_{50} assay for venoms and antivenins have been given by Aird and Kaiser [9], Russell [10], and Gutierrez and colleagues [11].

The present study was initiated to determine the dose-lethality index in mice of 14 clinically important North and South American snake venoms, using techniques similar to those described above. Additional considerations includes the use of pooled venoms from juvenile and adult snakes of both sexes over the reptiles' geographical range, which

also addresses the possibility of venom potency differences due to sex, age, or environmental factors [5,13]. The present study was undertaken to obtain preliminary data required for the preparation of a novel antivenin for the treatment of snakebite in the US [12]. The use of 'good laboratory practice for nonclinical laboratory studies' was also documented. The latter is prescribed by the US FDA for assuring the quality and integrity of the data obtained, and is intended to support subsequent application for development of new medical products [14].

Materials and methods

Venoms

Fourteen lyophilized pooled venoms from juvenile and adult male and female snakes were used. The snake venoms were representative of the snakes' geographical distribution. However, no attempt was made to standardize the numbers of males and females or adults and juveniles for different geographical areas. The venoms of *Crotalus durissus terrificus* (tropical rattlesnake), *C. scutulatus scutulatus* (Mojave rattlesnake) venom 'A' [13], *C. horridus atricaudatus* [canebrake rattlesnake], *C. adamanteus* (eastern diamondback rattlesnake), *C. viridis helleri* (southern Pacific rattlesnake), *C. atrox* (western diamondback rattlesnake), *C. molossus molossus* (northern black-tailed rattlesnake), *C. horridus horridus* (timber rattlesnake), *Sistrurus miliarius barbouri* (southeastern pygmy rattlesnake), *Bothrops atrox* (fer-de-lance), *B. asper* (terciopelo), and *Agkistrodon piscivorus piscivorus* (eastern cottonmouth) were purchased from a herpetologist (J. Glenn) at the Western Institute of Biomedical Research at the Utah Medical Center (Salt Lake City, UT). The above venom pools were supplemented with venom pools from our own collection, and with venom pools from selected herpetologists. The venoms of *C. durissus durissus* (central American rattlesnake) and *A. contortrix contortrix* (southern copperhead) were purchased from the Miami Serpentarium (Miami, FL). All venoms were lyophilized and stored at 4° C prior to use.

LD_{50} determinations

Venoms were dissolved in physiological saline at the highest concentration of venom that would be used for injection. This solution was incubated at 37° C in a water bath for 30 min and placed on ice. Dilutions of this solution with saline were made to obtain five additional concentrations used in the study. All solutions were stored at 0° C and warmed to 37° C just before use. The lethal toxicity was determined by injecting 0.20 (±0.02) ml of venom into a tail vein of a 21–23 g female mouse (ICR stock; Harlan-Sprague Dawley Company, Indianapolis, IN). The injections were performed using a 0.25 ml Hamilton glass syringe fitted with a 27 gauge, ½ inch stainless steel needle. Six mice were used at each of the six dose levels of the venom [15]. The LD_{50} and 95% confidence limits (CL) were calculated by the method of Litchfield and Wilcoxon [16] as incorporated into a computer program by Tallarida and Murray [17]. The quantal endpoint of lethality or survival of the mice was determined at 48 h for calculation of the LD_{50} [18].

Correlation analysis

The rank-order of the LD_{50} values were compared with the rank-order of LD_{50} values from previously published studies in mice [2–5] using the intravenous (iv), intraperi-

toneal (ip), subcutaneous (sc), or intramuscular (im) routes of administration. For these comparisons, Spearman's rank-order correlation coefficients (p) were calculated, and were assessed for statistical significance [19].

Results

Table 1 presents the iv LD_{50}s and 95% CL of the 14 venoms. A rank-order comparison and statistical evaluation of these LD_{50}s showed the following relative potency relationships: *C. durissus terrificus* = *C. scutulatus scutulatus* > *C. horridus atricaudatus* = *C. adamanteus* = *B. asper* = *C. durissus durissus* > *A. piscivorus piscivorus* = *C. viridis helleri* = *C. atrox* = *C. molossus molossus* = *S. miliarius barbouri* = *A. contortrix contortrix* = *B. atrox* = *C. horridus horridus.* The vehicle injection (0.20 ml isotonic saline) was innocuous.

Also included in Table 1 are the iv, ip, sc, and im LD_{50} values of venoms as previously published. Comparisons of the rank-ordered iv LD_{50} values with the published iv ($n = 13$), ip ($n = 13$), and sc ($n = 10$) rank-ordered LD_{50} values yielded ρ values of 0.79, 0.75, and 0.65, respectively. Each of these correlations was significant ($p < 0.05$). Comparison of the present rank-ordered iv LD_{50} values with the published rank-ordered im LD_{50} values yielded a ρ of 0.50, which was not significant ($p > 0.05$). The latter

Table 1. A comparison of the lethal potencies of American pit viper venoms in mice

Species of venom	LD_{50} *iv*[a]	*95% CL iv*[a]	LD_{50} *iv*[b]	LD_{50} *ip*[c]	LD_{50} *sc*[d]	LD_{50} *im*[e]
C. durissus terrificus	0.13	0.10–0.15	0.19	0.25	1.40	--
C. scutulatus scutulatus 'A'	0.17	0.12–0.23	0.17	0.24	0.31	0.75
C. horridus atricaudatus	0.92	0.75–1.14	--	2.04	4.61	--
C. adamanteus	1.35	1.14–1.60	1.84	2.12	12.98	10.70
B. asper	1.72	1.22–2.45	1.11	3.69	--	--
C. durissus durissus	1.79	1.4 –2.3	1.43	0.71	--	--
A. piscivorus piscivorus	3.38	2.81–4.07	4.00	5.11	25.10	--
C. viridis helleri	3.48	2.51–4.78	1.50	2.00	3.56	5.10
C. atrox	3.79	3.18–4.53	3.75	5.71	18.80	--
C. molossus molossus	4.42	3.78–5.16	2.04	--	--	--
S. miliarius barbouri	4.87	4.15–5.71	3.64	6.84	24.25	--
A. contortrix contortrix	4.99	3.52–7.07	10.92	10.50	26.10	--
B. atrox	5.41	3.39–8.64	4.27	3.80	--	--
C. horridus horridus	6.32	5.76–6.94	2.11	4.42	12.40	--

[a]The intravenous (iv) median lethal doses (LD_{50}) and 95% confidence limits (CL) of the venoms are in mg kg^{-1}; all these data are from the present study (see Methods section for further experimental details).
[b]The iv LD_{50} values (mg kg^{-1}) are taken from Glenn and Straight [3] and references therein, Russell and Puffer [2] and Bolanos [4].
[c]The intraperitoneal (ip) LD_{50} values (mg kg^{-1}) are taken from Glenn and Straight [3] and references therein and Bolanos [4].
[d]The subcutaneous (sc) LD_{50} values (mg kg^{-1}) are taken from Glenn and Straight [3] and references therein and Russell [5].
[e]The intramuscular (im) LD_{50} values (mg kg^{-1}) are taken from Glenn and Straight [3] and references therein.
[b–e]A dashed line indicates the absence (to our knowledge) of published lethality values.

number of comparisons was only three, and thus the lack of a significant relationship appears to reflect the limited amount of data available for analysis.

Discussion

The purpose of this paper is to report the dose lethality data in mice of 14 clinically important pit viper venoms using standardized methods developed in our laboratory. These data were obtained as part of a continuing investigation of a novel crotalid antivenin F_{AB} prepared from sheep immunoglobulin (IgG), as well as to further study the venoms.

It is well known that snake venoms are complex mixtures of many, often very potent, toxins. Even within a single species of snake, venom composition and pharmacological activity are known to vary, reflecting various environmental and constitutional factors [5,13]. In our ongoing project, pooled venoms from juvenile and adult snakes of both sexes representing their geographical distribution were used for immunization and lethality testing. A venom pool should tend to average out any individual antigenic or lethality difference. As no attempt was made to measure the LD_{50} of venoms from individual snakes, the venom pools were not normalized with regard to lethality. Also, the venom pools were not normalized with regard to age, sex, or geographical location. Thus, it is conceivable that if the pools were prepared with venom from different individuals, the lethality of the resulting pools could differ from the present results.

Since its development [20], the LD_{50} test in rodents has remained a standard quantitative method for the safety evaluation of individual chemical substances and complex natural products [15]. Further, regulatory requirements of numerous governmental agencies have continued to dictate the use of the LD_{50} test in decisions on the marketing of new drugs and on the evaluation of hazardous chemicals. In more recent years, however, the LD_{50} test has come under some criticism with respect to the value of the determinations and the importance of the number of animals necessary to determine the 'exact' LD_{50} [21]. Accordingly, at least one group [22] has recommended that the precise determination of LD_{50} values be limited to special cases, e.g. where it is scientifically necessary for the comparison of the potencies of different batches of a drug, or for the direct comparison of a new drug with a standard drug in use.

Although the value of using the LD_{50} test in toxinology research is well accepted, the standardization of biological and chemical assays for testing the activity of venoms and the neutralizing capacity of antivenins continues to be a point of issue. It has become obvious that venom assay techniques should include the same gender and strain or stock of mice, the same injection volume and route, pooled stocks of venom obtained from snakes of both sexes, diverse ages and representative geographical distribution, and the same incubation conditions as those to be used in venom-antivenin neutralization studies. These issues were addressed in the present study and the data provide the basis for testing of antivenin developed in sheep for the treatment of snakebite, as well as for the study of the venoms themselves.

In summary, the iv LD_{50}s of 14 clinically important North and South American snake venoms were determined in mice and their rank-order potencies were positively correlated with the rank-order potencies previously published. The findings indicate the general appropriateness of the data of the study, and suggest that these specific standardized techniques will provide an accurate basis for determining LD_{50} values in continuing

antivenin production. The methods used were compatible with WHO guidelines and FDA regulations currently required for the development of new treatment drugs. These data and methods are being applied to studies in our laboratory on additional snake venoms and new antivenin products. These studies will be reported at a later date.

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