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LETTERS TO THE EDITOR

STANDARDIZATION OF ASSAYS FOR TESTING THE NEUTRALIZING ABILITY OF ANTIVENOMS

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EVALUATION of the neutralizing ability of antivenoms has been traditionally assessed by studying neutralization of lethality (WHO, 1981). However, most venoms are complex secretions that induce a wide variety of pharmacological and pathological effects. Thus, an adequate evaluation of antivenoms must include not only neutralization of lethality, but also neutralization of other relevant effects. In the case of viperine and crotaline snake venoms, it is necessary to evaluate neutralization of lethal, hemorrhagic, defibrinating, coagulant, myotoxic, hypotensive, nephrotoxic and edema-forming effects.

Several groups of investigators have addressed this problem in recent years, and a more comprehensive view of the neutralizing potential of antivenoms is being developed (see for example the works of: YAMAKAWA, 1981; OWNBY *et al.*, 1984, 1985; GUTIÉRREZ *et al.*, 1985, 1987; MEBS *et al.*, 1988; GENÉ *et al.*, 1989; KORNALIK and TABORSKA, 1989; CLAUS and MEBS, 1989). However, these studies have used highly different methodologies and experimental strategies, thereby making the comparison and evaluation of results difficult. Due to the urgent need to standardize a methodology to test the neutralizing ability of antivenoms, we would like to propose some basic principles to be used.

1. SELECTION OF THE VENOM DOSE

Prior to any neutralization assay, it is necessary to select a fixed dose of venom ("challenge dose") for each particular test. To do this, it is necessary to study the dose–response relationship for a given effect in a particular test system. Then, in the majority of cases, a dose of venom giving a submaximal response, and located in the linear portion of the dose–response curve, is selected. This basic principle holds for the study of hemorrhagic, myotoxic, edema-forming, cytotoxic, defibrinating, hypotensive and neuromuscular-blocking effects, as well as when studying neutralization of enzymatic activities of venoms. In contrast, in the case of lethality, the "challenge dose" must give a maximal response, since it is necessary to use a dose of venom corresponding to a number of LD_{50} known to kill all animals injected. Many neutralization studies have been performed by using a venom dose selected arbitrarily, without performing dose–response studies. Under these circumstances, doses used are not necessarily located in a linear portion of the dose–response curve.

2. INCUBATION OF VENOM AND ANTIVENOM

Once the "challenge dose" of venom has been selected for a particular effect, then this constant amount of venom is mixed with various dilutions of antivenom, in a constant

final volume. Different antivenom/venom ratios are obtained in this way. Controls must include mixtures containing venom alone and antivenom alone. Mixtures are then incubated at 37°C for 30 min, before testing the effect in a particular system. It is relevant to mention that experiments in which antivenom is kept constant and venom concentration is varied must be avoided, since there will be doses of venom located in regions of the dose–response curves where linearity of response is lost. On the other hand, centrifugation of antivenom–venom mixtures after incubation is not recommended, since there is a possibility that toxins in insoluble antigen–antibody complexes may have their active pharmacological sites free and, therefore, have not been neutralized.

A different approach has been followed in experiments with independent injection of venom and antivenom (see for example GUTIÉRREZ *et al.*, 1985, 1987; OWNBY *et al.*, 1986). In these cases, preincubation of venom and antivenom is avoided, in an attempt to simulate the real conditions of an envenomation. Thus, the challenge dose of venom is injected and, at different time periods after envenomation, various volumes of antivenom are administered, usually by the intravenous route, and neutralization is assessed. However, when standardizing the neutralizing ability of an antivenom, preincubation type of experiments are used more extensively, since results do not depend on pharmacokinetics of venom and antivenom, but instead on concentration and neutralizing ability of antibodies present in the antivenom.

3. EXPRESSING THE NEUTRALIZING ABILITY OF ANTIVENOMS

After incubation, the activity of each antivenom/venom mixture is tested in the appropriate system. In the majority of assays, results can be expressed as a percentage, taking as 100% the response induced by the control of venom incubated with no antivenom. In these cases, we suggest that neutralizing ability of antivenom be expressed as Effective Dose 50% (ED_{50}), defined as the antivenom/venom ratio in which the activity of venom is reduced to 50% when compared to the effect induced by venom alone (see for example GUTIÉRREZ *et al.*, 1985). This value is more precise than Effective Dose 100% in the characterization of neutralizing ability of antivenoms, since ED_{50} is located in the linear portion of the neutralization curve, in contrast to ED_{100}. There are exceptions to this general principle, due to the particular characteristics of some test systems. For instance, in the case of neutralization of coagulant activity of venoms on plasma or fibrinogen, neutralization ability has been expressed as Effective Dose, defined as the antivenom/venom ratio in which coagulation time was prolonged three times when compared with coagulation time of plasma incubated with venom alone (GENÉ *et al.*, 1989).

Due to the great number of antivenom/venom systems and to the variety of pharmacological and pathological effects induced by venoms, these general principles must be adapted to each particular situation. However, we believe that keeping these studies within the general methodological framework suggested here would greatly help in the standardization of assays and, consequently, in a better knowledge on the neutralizing ability of antivenoms.

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COMMENTS ON LETTER TO THE EDITOR— STANDARDIZATION OF ASSAYS FOR TESTING THE NEUTRALIZING ABILITY OF ANTIVENOMS

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IN GENERAL I agree with the points made in the Letter to the Editor by GUTIÉRREZ *et al.* It is true that an adequate evaluation of the neutralization ability of antivenoms should include its ability to neutralize biological activities other than just lethality. This point has been made over the years by many investigators including SCHOTTLER (1952), KONDO *et al.* (1965*a* and *b*), CHRISTENSEN (1966). Thus, this issue has been recognized for almost 40 years. As GUTIÉRREZ *et al.* indicate, this point has been addressed by several laboratories. It has also been recognized by the World Health Organization (WHO). In fact, the WHO

Program for the standardization of venoms and antivenoms includes assaying each standard antivenom for its ability to neutralize several pharmacological properties of each reference venom, i.e. lethality, coagulant, hemorrhagic, necrotizing, defibrinogenating, neuromuscular paralytic and systemic myotoxic activities (WHO Offset Publication No. 58, 1981; THEAKSTON and REID, 1983). There is little doubt that most investigators will agree that the best evaluation of any antivenom is one in which its ability to neutralize each individual toxin is determined separately. This has been done with some of the neurotoxins, myotoxins and hemorrhagic toxins and will be done as more toxins are isolated in the future.

The method proposed by GUTIÉRREZ *et al.* for evaluation of the neutralizing ability of antivenoms has merit and should be seriously considered by those of us working in this field. It seems reasonable that the "challenge dose" should be selected from the linear portion of the dose–response curve, that the venom and antivenom be mixed at 37°C for 30 min prior to injection and that these mixtures not be centrifuged after incubation. Most investigators have been using very similar approaches.

I think, however, that one additional point should be made on this subject. This concerns the expression of the neutralizing ability of antivenoms. It is tempting to extrapolate from experimental findings to the real situation of a snakebite case. In doing so, the amount of antivenom needed for therapy is often estimated by simply multiplying the amount of antivenom (in μl or ml) needed to neutralize 1 mg of venom by the number of milligrams of venom suspected to have been injected. It was shown many years ago (SCHOTTLER, 1952; CHRISTENSEN, 1966) that the neutralization of venom by antivenom does not follow the "Law of multiple proportions". The amount of antivenom required to neutralize twice as much venom as tested is not necessarily twice the amount of antivenom used. Thus, we should refrain from making such generalizations about the neutralizing ability of antivenoms until we can demonstrate that a specific venom (toxin)/antivenom (antitoxin) system does follow the law of multiple proportions.

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COMMENTS ON LETTER OF GUTIÉRREZ *ET AL.* ON STANDARDIZATION OF ASSAYS FOR TESTING THE NEUTRALIZING ACTIVITY OF ANTIVENOMS

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THE SUBJECT of standardization of the neutralizing activity of antivenoms is a very important one, and the development of simple, reliable procedures for objective assessment of antivenom neutralizing activity is long overdue. As an adjunct to the excellent letter of GUTIÉRREZ *et al.*, I would like to add the following comments.

1. WHO (1981) discusses not only the neutralization of lethality by antivenoms, but also the neutralization of other basic biological activities of venoms such as the neutralization of hemorrhagic, defibrinogenating and necrotizing activities of venoms of medical significance. The importance of developing such assays, as well as those for neutralization of lethality, is fully discussed in this document and also in papers by THEAKSTON and REID (1983) and THEAKSTON (1986). These workers also stress the importance of the development of assay systems for determination of the neutralizing activity of antivenoms against procoagulant, neuromuscular paralytic and systemic myotoxic activity of antivenoms. One of the main aims of these studies was to discuss and develop standard assay techniques for the assessment and neutralization of these important venom activities. To the list of appropriate tests already mentioned, GUTIÉRREZ *et al.* also mention, quite rightly, the need to develop tests for assessing the neutralization of the hypotensive, nephrotoxic and oedema-forming activities of medically-important venoms.

2. With regard to methods for estimating the efficacy of an antivenom against lethality the standard method, as stated by GUTIÉRREZ *et al.*, is the premixing and incubation of a fixed amount of venom with different doses of antivenom before i.v. injection into the experimental mouse. WHO (1981) recommend this well-tested method, but more realistically it would perhaps be more satisfactory to inject the standard dose of venom s.c. followed at set time intervals by i.v. injection of antivenom. However, as GUTIÉRREZ *et al.* rightly state, the former method is simpler and estimates more reliably and repeatably the actual neutralizing activity of antibodies present in the antivenom. The efficacy of an antivenom is, as stated, best expressed as an ED_{50}.

3. It should be pointed out that, currently, a large scale project involving 13 collaborating laboratories throughout the world is concerned with this problem. This project is currently being coordinated by the WHO Collaborative Centre for the Control of Antivenoms at the Liverpool School of Tropical Medicine, U.K. Eight venoms of major medical importance throughout the world have been selected and have been characterized biologically for many of the activities mentioned above. Proposed standard monospecific antivenoms have been raised against each of these venoms. The protocol for the neutralization of the biological activities of these venoms by the standard antivenoms is

currently in preparation. It is hoped that these antivenoms, prepared under the auspices of WHO Biologicals, will eventually be designated by WHO as international reference reagents for use in research and for comparing both new and currently available commercial antivenoms.

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