31. THE PREPARATION AND PURIFICATION OF ANTIVENOMS

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This short talk on the preparation and purification of antivenoms must of necessity be influenced by the attitude adopted at the South African Institute for Medical Research, but knowing that there is room for improvements, and having noted the titles of the many papers to be presented today, this attitude may well have changed before the end of this Symposium.

My experience with the preparation of scorpion and spider antivenoms is rather limited, our main concern being the production of snake antivenoms, but the basic problems are the same, namely, 1, which animal to choose as serum producer, 2, which venom to use as antigens, 3, how to use the venoms as antigens and 4, how to treat the resulting serum to make it suitable for use.

The use of sera from animals other than the horse may carry less risk of serum reactions in some persons, but the horse is the natural choice in climates where it thrives and can be obtained at a reasonable price. It is easy to handle, yields a large volume of serum, and methods of purification of horse antitoxins have been thoroughly studied and are technically more advanced than is the case with antitoxins derived from other animals.

With regard to which type of horse to choose, there can be no strict rule, and one's views are based on general impressions rather than controlled experimentation. It is natural to prefer big horses because they yield more serum, but young horses do not respond better than old, rather the opposite may be true, and leniently immunized, the life expectancy of antivenom producing horses is very long, though not always as long as that of a horse dying earlier this year at the age of 29 after 18 1/2 years’ continuous antivenom production.

Most laboratories making antivenoms prepare other antitoxins as well, and it is common practice to screen new horses for naturally induced antibodies to diphtheria and Clostridium perfringens toxin and to immunize them prophylactically against tetanus. Such horses can be allocated to the production of diphtheria, tetanus or gas gangrene antitoxin according to immediate requirement, and can be transferred from the production of one kind of antitoxin to that of another without much delay. The response of a horse to one antigen does not necessarily indicate its potential response to another, and horses previously immunized with bacterial toxins, but no longer required or discarded because of falling titre, are as good antivenom producers as any other horse. It can be argued that the presence of significant amounts of other antitoxins in the raw serum will reduce the ratio between antivenom potency and protein content of the final product because purification methods do not discriminate between antitoxins, but this is a minor objection.
Which venoms to use as antigens must obviously be determined by the frequency of bites by different snakes, the severity of the effects, and the availability of venom.

Availability of venom is essential for continued large scale serum production but goes usually hand in hand with the frequency of bites by different species and to some extent with the severity of the effects, because larger snakes delivering much venom tend to do most damage.

In the majority of snakebites the culprit is not seen or is not identified and this is one of the reasons why polyclonal sera are preferable to monovalent, except in areas where dangerous bites are almost exclusively due to a single species. The choice of venoms for the production of polyclonal serum must of course be influenced by the cross-neutralizing properties of monovalent sera prepared with the different venoms, but too much reliance should not be placed on paraspecific action. The immunological overlapping of protective antibodies, in which the earliest workers placed much faith, is very limited, as we know from the work of Dr. Vital Brazil and others who followed him. Not only is the paraspecific titre of a serum lower than the specific but a serum’s therapeutic value must depend not only on titre but also on the firmness of the union between antigen and antibody, and paraspecific venom-antivenom complexes tend to dissociate.

Some laboratories prepare polyclonal sera by blending several monovalent sera, others, including ourselves, prefer to immunize the horses with all the antigens. The obvious argument against blending monovalent sera is that each serum is being diluted by the addition of the others. This must happen in the case of antibodies with strict specificity but not with antibodies to common antigens, just as antibodies to related antigens could show some additive effect. The dilution of antibodies could be counterbalanced if the horses receiving a single venom reached much higher titres than those immunized with several venoms. Horses given only one venom could respond better because larger doses could be injected or because a ‘crowding’ effect would suppress the response to important toxins in the horses receiving several venoms. However, horses given only one venom soon reach a state of immunity which does not improve unless the dose of venom is increased quite out of proportion to the rise in antibody titre. Furthermore, such monovalent horses usually maintain their titre to this particular venom after they have been transferred to the production of polyclonal serum.

Once immunized, a horse will tolerate large single venom doses, but new horses are easily killed. A horse immunized many years ago for the preparation of monovalent *Naja naja* antivenom received single doses of up to 2 g of venom without symptoms, yet 15 mg of the same venom given in error killed a diphtheria antitoxin producing horse before the mistake was discovered and the outcome prevented with antivenom. To give new horses their first, basal, immunity with unmodified venom is a tedious process, and the initiation of basic immunity is therefore the first part of the next problem, how to use venoms as antigens.

To use venom-antivenom mixtures for this purpose is also tedious because such mixtures are dangerous when they are under-neutralized, and fully neutralized they are poor antigens. Many ways of rendering venoms atoxic without destroying their antigenicity have been suggested over the years, but only detoxification with formalin seems to have been used on any scale. But the loss in venom toxicity due to formalin is accompanied by a large loss in antigenicity, and the use of formol-toxoided venoms, or anavenoms, is wasteful of venom and can be the cause of much suffering of the horses. Actually no detoxification is neces-
sary if the venom is adsorbed on some inert carrier; the harmlessness of adsorbed venoms is presumably due to a decreased rate of absorption from the site of injection and the good response is probably due to prolonged stimulation by antigens held in subcutis, apart from any possible adjuvant effect of the adsorbant.

In principle this method originated with Calmette who in 1894 recorded that a small piece of chalk impregnated with venom and coated with collodion and inserted under the skin of a rabbit would serve as a continued stimulation from an artificial gland, a suggestion he credited to Dr. Roux, in whose laboratory he was working.

Criley (1956) had good results with venoms adsorbed on aluminium hydroxide, a method we found unsuccessful many years ago, possibly because our aluminium hydroxide gels were unsatisfactory, and I turned to the use of venoms adsorbed on bentonite, with good results during the last fifteen years.

The first basic immunity of the horses is achieved with a few injections of from 25 to 100 mg of venom adsorbed on a 2% suspension of bentonite in distilled water. The venom solutions are sterilized by filtration, the bentonite suspension by steam under pressure. This method saves time, venom and labour, but it would appear that not all preparations of bentonite are suitable. As soon as traces of circulating antibody are detectable by mouse protection tests, the immunization is continued with venom solutions sterilized by filtration and preserved with 0.25% cresol, but without the addition of bentonite. The injection of a suspension of bentonite does in some cases cause the formation of a small sterile abscess which requires incision, but the lesion heals in few days and does not upset the horses, and although some antigenic material must be evacuated through the incision, enough remains to stimulate antibody formation.

The greatest advantage of the use of plain solutions of unmodified venoms for the continued immunization is the lack of untoward reactions in basically immune horses. A healthy horse will not only live longer but will presumably in the long run respond better to antigenic stimulation than a horse in poor condition due to repeated injury, such as that caused by some adjuvants. The use of Freund’s complete adjuvant gives amazing results in horses immunized with diphtheria or tetanus toxin (Mason, 1963), but tends to cause rather severe reactions. Unpublished experimental work by J. H. Mason has shown that the severity of the reactions is considerably reduced if antigen and Freund’s adjuvant are incorporated in a multiple (water-in-oil-in-water) emulsion of the type described by Herbert (1965). As far as venoms are concerned, we have failed to observe any difference in the response of comparable groups of horses immunized with or without the addition of Freund’s adjuvant in the form of simple or multiple emulsions.

South Africa’s needs for scorpion and spider antivenoms are met by one or two immunized horses, too few to allow one to form any opinion on the value of different methods of immunization, but the methods in current use are briefly as follows.

Scorpions of the genus Parabuthus, regardless of species, are kept alive and the collected venom is dried under vacuum in a desiccator and dissolved in saline as required. Filtration through Seitz pads removes about 75% of the toxin, and the solution is therefore sterilized by the addition of phosphate buffer and beta-propiolactone to a concentration of 0.2%. Having stood overnight at
about 3%, the solution is mixed with procaine immediately before injection in order to make it painless. Sterilization by means of beta-propiolactone is very convenient even if it does reduce the toxicity by about 30%.

_Latrodectus indistinctus_ antivenom is prepared with extracts of dried cephalothoraces. The tedious task of isolating the chelicerae with the venom glands for extraction and use as antigen was discontinued because extracts of the remaining cephalothoraces were toxic and sera prepared with either chelicera extract or cephalothorax extract gave complete cross-neutralization. This was more than likely due to incomplete removal of the venom glands but the practical implication was to save the effort of collecting chelicerae. One might add that a serum prepared against the extremely toxic extract of spider abdomens is ineffective against the true venom, just as the ordinary antivenom fails to neutralize the toxic material obtained from the abdomens. The extracts have hitherto been sterilized by filtration through Seitz pads or with beta-propiolactone but we intend to resort to membrane-filtration in order to minimize the loss of toxin which is quite considerable.

Irrespective of which kind of serum they produce, snake, scorpion or spider antivenom, the horses rest for about five weeks between courses of immunization lasting about two weeks. The crude serum, or rather plasma, obtained at the end of each course of immunization is improved for therapeutic use by purification, which removes inactive material, and by concentration, which reduces the volume to be injected.

Purification by means of salt fractionation came into general use after Dr. Vital Brazil had shown that the distribution of antibodies in antivenoms was similar to that in bacterial antitoxins, but, although they are still used, such earlier methods have been superseded by others involving treatment with proteolytic enzymes, pepsin in particular.

The interest in the use of pepsin for this purpose began in 1902 and was smouldering until Parfentjev (1936), Pope (1939a, 1939b) and Hansen (1941) published methods suitable for large-scale purification of bacterial antitoxins. In all the three methods, the serum is treated with pepsin at a controlled pH, but the methods of Parfentjev and Hansen rely on adsorption for the removal of inert material, whereas the unwanted protein is removed by heat-coagulation in Pope's method which was further developed by Harms (1943). _Latrodectus mactans_ antivenom was successfully purified already in 1942 by Pirosky and co-workers in Argentina using Pope's technique, and this is the method used at Instituto Butantan (Höxter and Decoussau, 1949) and at the South African Institute for Medical Research (Grasset and Christensen, 1947).

The advantages of pepsin-treated sera are too well known to warrant lengthy discussion. Such sera are stable when stored at reasonable temperatures and, of more importance, the incidence of serum reactions is reduced to a low level. It may be of interest to note that the incidence of serum sickness in children treated with pepsin-refined diphtheria antitoxin has been found significantly lower in Bantu patients than in Whites (Mason & Christensen, unpublished), which is gratifying because the Bantu is more likely to need treatment with antivenom, which will behave as other equine antitoxins with regard to serum reactions.

The drawback to the production of pepsin-refined antivenom is the cost. A contributing factor is a considerable loss of active material during the process. The well-known difficulties in assessing antivenom potency makes it difficult to
estimate the amount lost, but it is probably about 55%, which is not surprising as the antibodies are distributed in both the so-called pseudo- and eu-globulins, and even the water insoluble globulins of viper antivenom may contain some antibody (Christensen, 1955).

In retrospect, there have been no great forward strides in antivenom production apart from the introduction of better purification methods, and further developments in this direction will probably have to await improvements of bacterial antitoxins, which are easier to evaluate. The scope for improvement lies in the broadening of the specificity of polyvalent antivenoms and the use of purer antigens.

For reasons already stated, it is inadvisable to rely on paraspécific protection if specific sera can be produced, but taking the African region as an example, it might be necessary to immunize with ten or more venoms in order to cover the more important snakes. Each venom is likely to contain a dozen or more different antigens of which the most toxic is often the poorest antigen, and the solution to the problem may lie in the isolation of the important toxins from these venoms in quantities large enough for use as antigens, possibly after binding to a suitable carrier in order to enhance their antigenicity. Steps in this direction have already been taken by workers in Israel and France (Kochwa et al., 1959; Moroz et al., 1963), a lead we hope to follow in South Africa.

References


5. Hansen, A. — Studier over Isolering af det antitoxinbærende Protein fra andre Seruminbestanddele. Ejnar Munksgaard, Copenhagen, 1941.


**DISCUSSION**

A. Shulov: “Whether you tried in your vast experience direct bites of snakes and direct stings of scorpions in order to increase the title of the antisera. In our laboratory we received good results in donkey and camels, but not in sheep, goats and rabbits.”

P. A. Christensen: “No.”

P. Cohen: “Have you observed any differences in the antibody titers of horses immunized with bentonite-absorbed venom compared to horses which have received unmodified venom.”

P. A. Christensen: “Unmodified venom is not used for new horses. The basal immunity is achieved with venom adsorbed on bentonite, but continued with unmodified venom. No comparison has therefore been possible.”

P. J. Deoras: “Does the speaker have any observation to make to the fact that venom when used with debris gives reactions?”

P. A. Christensen: “No.”

C. Puranamanda: “When the speaker mentioned young horse, I want to know at what age?”

P. A. Christensen: “Dificil precisar a idade. Penso que é velho um que morreu aos 29 anos e moço um cavalho de 6 anos.”