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COMPARISON OF THE PURITY AND EFFICACY OF AFFINITY PURIFIED AVIAN ANTIVENOMS WITH COMMERCIAL EQUINE CROTALID ANTIVENOMS

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S. B. CARROLL, B. S. THALLEY, R. D. G. THEAKSTON and G. LAING. Comparison of the purity and efficacy of affinity purified avian antivenoms with commercial equine crotalid antivenoms. *Toxicon* **30,** 1017–1025, 1992.—Antivenoms were raised in laying hens by repeated immunizations with detoxified crotalid snake venoms and purified from egg yolks by affinity chromatography. While the affinity purified avian antivenoms were essentially pure IgG, commercial equine (Wyeth) and W.H.O. international reference antivenoms (*Trimeresurus flavoviridis*) contained several non-immunoglobulin contaminants. In standard mouse protection assays, the purified avian *Crotalus atrox* and *T. flavoviridis* antivenoms were 6.3 and 2.0 times as potent, respectively, as these equine antivenoms in neutralizing venom lethality. The purity, efficacy, and ease of manufacture of avian antivenoms, and their inability to fix mammalian complement, make them an attractive alternative to equine and other mammalian antivenoms.

INTRODUCTION

MOST antivenoms used to treat envenoming by snakes, scorpions, and other poisonous animals are produced in horses and are partially purified from sera by batch-type techniques such as ammonium sulfate fractionation and/or pepsin digestion (see reviews by CHRISTENSEN, 1979; RUSSELL, 1988). These antivenoms, while capable of neutralizing the toxic and potentially lethal effects of venoms, often cause significant clinical side effects which compromise their safety and efficacy.

The primary cause of side effects elicited by commercial antivenoms is their impurity (SUTHERLAND, 1977; W.H.O., 1981; SULLIVAN, 1987). Because only batch-type techniques are used to purify serum fractions, the resulting antivenom contains both non-immunoglobulin protein and immunoglobulin which is not reactive with venom components. The large amounts of non-therapeutic horse serum proteins can cause both early and delayed reactions including: (1) anticomplement reactions in which horse immunoglobulin fixes human complement and triggers the inflammatory pathways of the complement cascade and may result in circulatory collapse (SUTHERLAND, 1977); (2) anaphylactic shock, a potentially fatal reaction in a significant percentage of patients who are sensitive to horse

proteins (HABERMEHL, 1981); and (3) serum sickness, a very common delayed syndrome caused by the patient's own immune response to the large burden of foreign protein presented by the antivenom (W.H.O., 1981; ELLENHORN and BARCELOUX, 1988).

Clearly, commercial antivenoms containing only venom-reactive immunoglobulin are needed. However, there are two significant limitations to the commercial development of highly pure antivenoms. First, the antivenoms must preserve the broad spectrum of antibody reactivity necessary to neutralize the numerous toxins found in most venoms. And second, this must be achieved at a reasonable cost to users in developed as well as developing areas of the world. To date, most efforts at further purification of antivenoms have focused on products to treat bites of North American snakes. For example, RUSSELL *et al.* (1985) reported the purification of commercial (Wyeth) crotalid antivenin by affinity chromatography over polyacrylamide resins in which a number of different venoms had been entrapped. When IgG specific for individual venoms was challenged with the homologous and heterologous venoms, a significant increase in efficacy over the unpurified antivenom was usually observed.

We have also focused on the development of antivenoms for North American snakes (THALLEY and CARROLL, 1990) but have taken a different approach to conventional antivenom production. In order to circumvent the complement fixing properties of mammalian immunoglobulins, and in order to significantly reduce production costs, we have developed antivenoms by immunizing laying hens and collecting and purifying antivenom antibodies from their egg yolks. Previously, we have shown that egg-derived antivenoms could be harvested and purified in significant quantities from hyperimmunized hens and that these antivenoms were capable of neutralizing the venoms against which they were raised. Here, we describe the production of two additional snake antivenoms and a comparison of avian antivenom purity and efficacy with commercial and W.H.O. international reference antivenoms.

MATERIALS AND METHODS

Venoms and equine antivenoms

Western diamondback rattlesnake (*Crotalus atrox*), South American rattlesnake (*Crotalus durissus terrificus*), eastern diamondback rattlesnake (*Crotalus adamanteus*), Mamushi (*Agkistrodon halys*), and Habu (*Trimeresurus flavoviridis*) venoms used for immunization were purchased from Sigma. International reference venoms for *C. atrox* and *T. flavoviridis* were obtained from the W.H.O. (Department of Biological Standardization, Statens Serum Institut, Copenhagen, Denmark). Wyeth crotalid polyvalent antivenom (CRILEY, 1956) (lot #4908220) was purchased commercially and *T. flavoviridis* monovalent antivenom (National Institute of Health, Japan), a W.H.O. international reference antivenom, was obtained from the W.H.O. (Copenhagen, lot #18).

Venom detoxification

To determine the best course for raising high titer egg antibodies against the different venoms, the effects of various methods of venom modification upon venom activity and immunogenicity were examined.

Crotalus atrox *venom modification.* Whole venom (Sigma) was modified by formaldehyde, glutaraldehyde or heat treatment. In order to monitor the inactivation of venom, the inhibition of total venom protease activity was measured according to a modified method of PHILPOT *et al.* (1978). The assay consisted of mixing 10 μl of venom or buffer control with 25 μl of PBS for 5–15 min. Protease activity is indicated when the supernatant turns red due to hydrolysis of the azo dye. Results were quantitated at A_{525} on a spectrophotometer (Gilford).

Complete inhibition of protease activity is not essential for inactivation. It is simply desirable to minimize the impact of immunization on the animal being immunized.

For formaldehyde treatment, a 10 mg/ml dilution of whole *C. atrox* venom was prepared in various concentrations of formaldehyde (0.25–8.0% w/v) and left for 1 hr at room temperature. It was observed that 8.0% formaldehyde completely inhibited venom protease activity, and this concentration was used to prepare the formaldehyde-treated immunogen.

For glutaraldehyde treatment, a 10 mg/ml dilution of whole *C. atrox* venom was prepared in glutaraldehyde and left for 1 hr at room temperature. It was observed that 1% glutaraldehyde totally inactivated venom protease activity, and this concentration was used to prepare the formaldehyde-treated immunogen.

For heat treatment, a 10 mg/ml dilution of whole *C. atrox* was heated to 95°C in a water bath for 5 min and then plunged into ice. This treatment eliminated all protease activity and was therefore the treatment used to prepare heat-treated immunogen.

Trimeresurus flavoviridis *and* Agkistrodon halys *venom detoxification.* Because heat treatment of *Crotalus atrox* venom proved to be the most effective means of inactivating the venom, yet preserved the greatest degree of antigenicity, this method was also used to inactivate *T. flavoviridis* and *A. halys* venoms.

Crotalus durissus terrificus *venom detoxification.* BREITHAUPT (1976*a*) has shown that crotoxin is very toxic to chickens, and our preliminary experiments confirmed this. Since brief heat treatment does not eliminate crotoxin toxicity (BREITHAUPT, 1976*b*), we sought to chemically inactivate whole *Crotalus durissus terrificus* venom. Toxoid was prepared by the formaldehyde method of RODRIGUEZ-ACOSTA and AGUILAR by adding a 37% solution of formalin to a 10–15 mg/ml solution of venom in phosphate buffer to an initial concentration of 0.2% on day 1, to 0.4% on day 3, to 0.6% on day 5, and to 0.8% on day 7 and incubating continuously at 37°C. The toxoid was dialyzed against 200–400 volumes of PBS in benzoylated dialysis tubing and stored at 4°C.

Immunizations

Two, 1-year-old white leghorn hens were each immunized with 1 mg of *Crotalus atrox* venom after it had been inactivated by one of the methods described above (total of six immunized hens). The hens were injected with the inactivated venom in complete Freund's adjuvant (CFA) (Gibco) on day 0 subcutaneously in multiple sites (both sides of the abdomen, both breasts, and in both wings to involve as much of the lymphatic system as possible). The hens were subsequently injected with 1 mg of the same inactivated venom in incomplete Freund's adjuvant (IFA) (Gibco) on days 11 and 19. Once sufficient antibody titers were obtained, booster immunizations were performed as described by THALLEY and CARROLL (1990).

Trimeresurus flavoviridis and *A. halys* immunizations were carried out using 10 mg of heat treated venoms on day 0 in CFA and 10 mg of the same in IFA on days 14, 28 and 61. Hens were boosted with 2 mg, 2.5 mg, 5 mg, 10 mg, 12 mg and 15 mg of each native venom on days 82, 103, 129, 143, 158, 172, 186, 204, 222, and 238, respectively. *Crotalus durissus terrificus* immunizations were with 5 mg of toxoid in CFA on day 0 and 2.5–5 mg of toxoid in IFA on days 14, 20, 63, 103, 128, and 147.

Collection and purification of antivenom

Whole chicken egg yolk immunoglobulin (IgY) was extracted with polyethylene glycol (PEG) according to a modification of the methods of POLSON *et al.* (1980) as described by THALLEY and CARROLL (1990). The whole IgY fraction obtained from the 12% PEG precipitation step was then dissolved in the original yolk volume of egg extraction buffer and stored at 4°C.

Specific antibodies to whole venoms were purified from 3.5% PEG or 12% PEG IgY fractions of egg yolks by passage over individual *C. atrox*, *T. flavoviridis*, or *C. durissus terrificus* venom antigen matrices prepared by coupling 5–10 mg of venom/ml Actigel A (Sterogene Biochemicals, San Rafael, CA, U.S.A.) affinity resin (THALLEY and CARROLL, 1990). In order to minimize damage to the antivenom antibodies during their elution from the affinity matrix, a gentle, non-denaturing neutral pH elution medium (ACTISEP; Sterogene Biochemicals) was applied to the matrix, incubated for 90–120 min, and active antibody recovered by dialysis of the eluate against many volumes of 50 mM Tris, pH 7.2, 150 mM NaCl.

Assessment of the effect of modification treatments upon C. atrox *venom antigenicity*

Eggs were collected between days 25 and 28 after the start of immunization to assess whether the treated venoms were sufficiently antigenic to have elicited an immune response. Eggs from the two hens in each modification group were pooled and antibody was collected as described above. Antigenicity was assessed on Western blots according to the method of TOWBIN *et al.* (1979). One-hundred microgram samples of three distinct venom types (*C. adamanteus*, *C. atrox*, *Agkistrodon piscivorus*) were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (WEBER and OSBORN, 1975) and transferred to nitrocellulose using the ABN polyblot electro-blotting system according to the manufacturer's instructions (Fisher Biotech, NJ, U.S.A.). After blocking remaining protein binding sites in phosphate buffered saline (PBS) containing 3% bovine serum albumin (BSA) overnight, the blot was cut into strips and each strip was incubated with IgY preparations (12% PEG fractions) of eggs from immunized and control hens diluted 1:250 in PBS containing 1 mg/ml BSA for 2 hr at room temperature. The blots were washed with PBS, borate buffered saline containing 0.1% (v/v) Tween-20 and PBS successively. Goat anti-chicken IgG alkaline phosphatase conjugated secondary antibody (Fisher Biotech) was diluted 1:400 in PBS containing 1 mg/ml BSA and incubated with the blot for 2 hr at room

temperature. The blots were washed with PBS and BBS-Tween, followed by one change of PBS and 0.1 M Tris–HCl, pH 9.5, and developed in freshly prepared alkaline phosphatase substrate buffer.

Protein assays

Numerous methods for determining protein concentration were investigated. The most consistent method was A_{260}/A_{280} method of WARBURG and CHRISTIAN (1941), which yielded values for purified IgG that were in accord with these values obtained using known extinction coefficients (e.g. A_{280} of 1.3 = 1 mg/ml chicken IgG).

In vivo *neutralization of venoms with antivenoms*

Lethal toxicity of venoms (LD_{50}, median lethal dose) was assessed by i.v. injection of different venom doses in 0.2 ml physiological saline into 18–20 g male TFW strain mice (Tuck and Sons, Ltd, Kent, U.K.). Six animals were used at each dose level and survival after 24 hr was recorded. The LD_{50} was calculated by probit analysis as recommended by W.H.O. (W.H.O., 1981) and THEAKSTON and REID (1983).

Antivenom efficacy (ED_{50}, median effective dose) was assessed by mixing $2 \times LD_{50}$ with different amounts of antivenom, incubating for 30 min at 37°C and injecting into mice. The ED_{50} was calculated according to the recommended W.H.O. guidelines (W.H.O., 1981).

RESULTS

In order to determine which mode of venom detoxification—formaldehyde, glutaraldehyde or heat treatment—yielded the most immunogenic form of venom, the antivenom titers of eggs from hens that had been immunized with equivalent amounts of each venom preparation were compared. After three immunizations, the eggs from hens immunized with heat-treated *C. atrox* venom showed far greater reactivity with three representative crotalid venoms (Fig. 1D) than did those from hens immunized with formaldehyde-treated venom (Fig. 1B) or glutaraldehyde-treated venom (Fig. 1C). The latter exhibited reactivity that was comparable to the background reactivity of pre-immune eggs (Fig. 1E). Based upon these observations, antivenoms to a cocktail of North American crotalid venoms and a pair of Asian crotalids (*T. flavoviridis* and *A. halys*) were prepared using heat-treated venoms initially and, once a high titer was achieved ($> 100\ \mu g$ venom antibodies per ml of egg yolk), by following with booster immunizations with native venoms. An antivenom to *C. durissus terrificus* venom was raised using toxoided venom. In all cases, immunization was well tolerated for extended periods with no local or systemic problems.

Antivenoms to *C. atrox* venom and *T. flavoviridis* venom were purified by affinity chromatography on affinity matrices of each respective venom. By SDS-polyacrylamide gel electrophoresis, these antivenoms appear to be pure IgG (Fig. 2, lanes 1 and 3) whereas commercially available equine antivenoms are not pure IgG (Fig. 2, lanes 2 and 4). In fact, it appears that the majority of proteins present in the equine Wyeth antivenin are not immunoglobulins (Fig. 2, asterisks). Quantitative affinity chromatography of the Wyeth equine antivenin indicates that only about 10% of the immunoglobulin present in the antivenom is venom-reactive (CARROLL, THALLEY and WAINWRIGHT, unpublished observations). Overall, these results indicate that removing non-immunoglobulin proteins and non-venom-reactive immunoglobulin should yield a more effective product.

Preliminary neutralization experiments in mice indicated that each purified antivenom was capable of neutralizing venom lethality. Nearly complete protection against $\geq 1 LD_{50}$ of venom was observed with a 2:1 ratio of anti-*C. atrox* antivenom: *C. atrox* venom, with a 3.5:1 ratio of anti-*T. flavoviridis* antivenom: *T. flavoviridis* venom, and a 10:1 ratio of anti-*C. durissus terrificus* venom: *C. durissus terrificus* venom. In order to quantify more accurately the neutralizing potency of the avian antivenoms, and to compare their

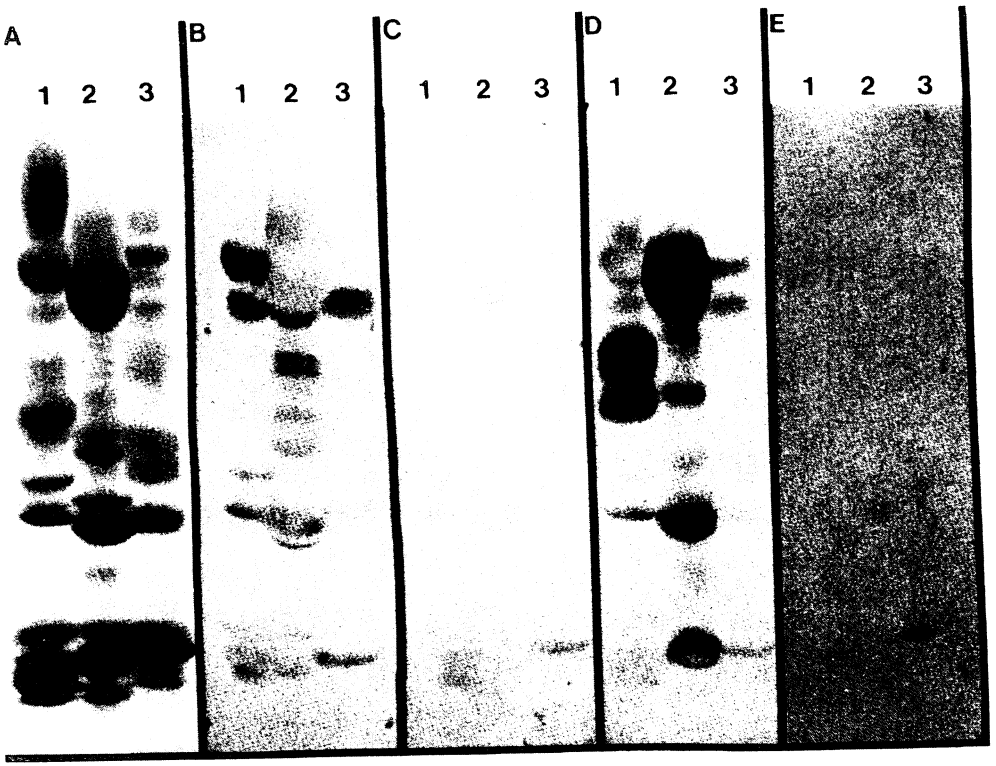

FIG. 1. THE EFFECT OF DIFFERENT MODES OF VENOM DETOXIFICATION ON VENOM IMMUNOGENICITY IN LAYING HENS.

Venom proteins were separated by SDS-PAGE and either visualized by Coomassie blue staining (A) or transferred to nitrocellulose and probed with egg antibodies obtained from hens immunized with different forms of detoxified *C. atrox* venom (B–D) or control hens (E). The venoms used in each panel were *C. adamanteus* (lane 1), *C. atrox* (lane 2) and *A. piscivorus* (lane 3). A 1:250 dilution of whole egg IgG was tested in each case. Eggs from hens immunized with heat-treated venom (D) exhibited greater reactivity than those from hens immunized with formaldehyde-treated venom (B) or glutaraldehyde-treated venom (C). Pre-immune eggs exhibited a small degree of background reactivity (E).

performance with that of the equine antivenoms currently used, a larger scale mouse protection study was undertaken using W.H.O. reference venoms and antivenoms under standard assay procedures (THEAKSTON, 1986; see Materials and Methods). Since no reference venom or antivenom currently exists for *C. durissus terrificus*, it was not included in the larger study.

Initially, LD_{50} determinations were performed on international reference venoms for *C. atrox* and *T. flavoviridis*. The i.v. LD_{50} was 96.15 μg/18–20 g mouse for *C. atrox* venom (95% confidence limits 93.17–101.62 μg/mouse) and 193.12 μg/18–20 g mouse for *T. flavoviridis* venom (95% confidence limits 183.68–201.72 μg/mouse). The venom-neutralizing capability of each antivenom was then tested and the ED_{50} calculated. As shown in Table 1, 1.9 mg of Wyeth antivenom was required to achieve the same degree of protection against *C. atrox* venom obtained with 0.3 mg of purified avian *C. atrox* antivenom while 4.1 mg of the W.H.O. reference *T. flavoviridis* antivenom was required to

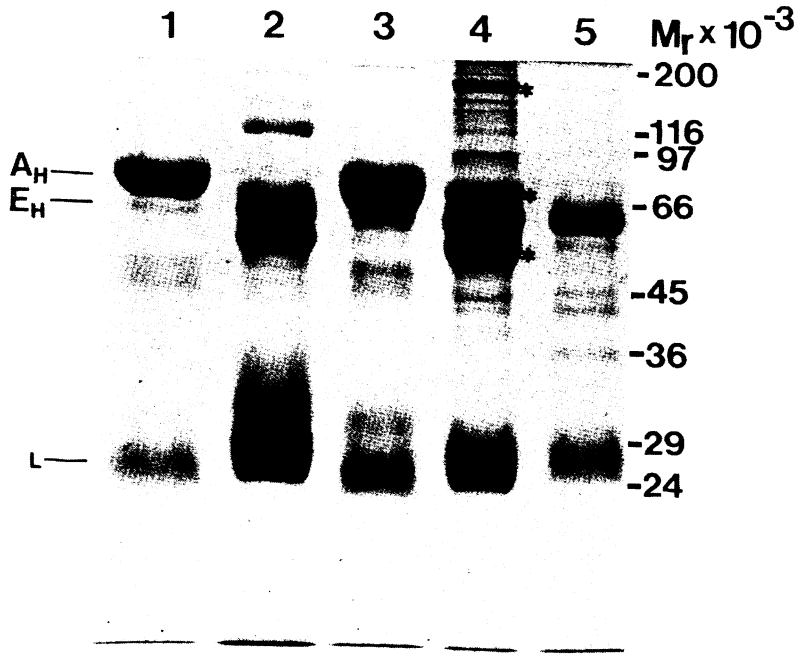

FIG. 2. THE RELATIVE PURITY OF AFFINITY PURIFIED AVIAN ANTIVENOMS AND COMMERCIAL EQUINE ANTIVENOMS.

Thirty micrograms of protein from avian anti-*T. flavoviridis* (lane 1), equine anti-*T. flavoviridis* (lane 2), avian anti-*C. atrox* (lane 3), and equine crotalid polyvalent (lane 4) antivenoms and a purified equine IgG standard (lane 5) were separated on a 12.5% SDS-polyacrylamide gel and stained with Coomassie blue. The avian antivenoms consist of only heavy (A_H) and light (L) chains of IgG, while the equine antivenoms contain several higher molecular weight proteins in addition to the heavy (E_H) and light (L) chains of IgG. Note, for example, the prominent bands (asterisks) in the crotalid polyvalent antivenom (lane 4) which are not immunoglobulin proteins.

achieve the same degree of protection as that obtained with 2.1 mg of avian antivenom. Thus, on a protein weight basis, the avian antivenoms were 6.3 and 2.0 times more effective than the Wyeth and W.H.O. reference antivenoms, respectively.

DISCUSSION

All three avian snake antivenoms raised here are venom-neutralizing and we have shown that two of these antivenoms, the anti-*C. atrox* and anti-*T. flavoviridis* preparations, are 6.3 and 2.0 times more potent on a weight basis than their currently available equine counterparts in standard mouse protection assays using international reference venoms. Similar analysis of the anti-*C. durissus terrificus* antivenom, using validated lots of *C. durissus terrificus* venom, indicates that this preparation is at least ten times more potent than commercial (Wyeth) polyvalent crotalid antivenom (CARROLL, THALLEY and WAINWRIGHT, unpublished observations). These results demonstrate that laying hens respond well immunologically to the wide range of different toxins present in these crotalid venoms and that, when purified, the avian antivenoms are significantly more potent than existing antivenoms.

TABLE 1. RELATIVE EFFICACY OF EQUINE AND AVIAN ANTIVENOMS

Venom	Antivenom	Antivenom protein concentration (mg/ml)	Antivenom ED_{50} (mg/mouse)	Relative ED_{50}* (equine/avian)
C. atrox	Wyeth equine Crotalid polyvalent	124.3	1.9	6.3
	Avian anti-*C. atrox*	5.1	0.3	
T. flavoviridis	W.H.O. equine *T. flavoviridis*	62.8	4.1	2.0
	Avian anti-*T. flavoviridis*	24.0	2.1	

$2 \times LD_{50}$ of venom was incubated with different amounts of each antivenom at 37°C for 30 min and the mixture injected intravenously into groups of six mice each. The ED_{50} of each antivenom was determined by Probit analysis of deaths occurring within 24 hr.

*The relative ED_{50} was calculated by dividing the equine antivenom ED_{50} by the ED_{50} for the corresponding avian antivenom and indicates the relative weight of each equine antivenom required to achieve the same degree of neutralization obtained with each respective avian antivenom.

The degree to which individual purified avian antivenoms are more potent than comparable equine antivenoms varies and depends upon several factors. First, because equine antivenoms are batch purified from whole hyperimmune sera, their purity and potency (i.e. the titer of venom-specific antibodies) vary considerably. In the case of two equine antivenoms, we have found that the W.H.O. reference antivenom against *T. flavoviridis* contains a higher percentage of *T. flavoviridis*-reactive antibody than the Wyeth antivenom does of *C. atrox*-reactive antibody. Therefore, the purified avian *C. atrox* antivenom exhibited greater relative potency than the purified avian *T. flavoviridis* antivenom. Second, the methods used to purify the antivenom can affect their relative potency. Antivenom antibody elution during affinity chromatography requires disruption of antigen–antibody interactions; the eluents employed may have deleterious effects on antibody activity and the antigenicity of the venom matrix. We know that the neutral pH elution buffers employed here, while significantly less denaturing than conventional eluents, still cause some reduction in the specific activity of the antibody as measured by quantitative enzyme immunoassays (THALLEY and CARROLL, unpublished observations). Third, it should be noted that the venoms used to raise and purify the avian antivenoms were obtained from a different source than those employed in the mouse protection assays. It is possible that antigenic differences exist between different lots of venom and may influence the relative potency measurements.

Clearly, a several-fold increase in antivenom potency is clinically desirable due to the expected reduction in both the incidence of early anaphylactic and complement reactions and late serum sickness reactions. Of course, this could be achieved by purifying existing mammalian antivenoms. The added clinical advantages of the avian antivenom are that first, the avian antibodies do not fix mammalian complement (BENSON *et al.*, 1961; ROSE and ORLANS, 1962) and therefore circumvent the risk of anticomplement reactions. The removal of the complement-reactive F_c portion of mammalian immunoglobulins, as currently performed by pepsin digestion, reduces the yield and potency of the resulting

antivenom product, adds to the cost of manufacturing, and still does not eliminate the risk of early reactions (SUTHERLAND, 1977). Second, the antibodies are not equine and therefore should be safe to use in patients that are hypersensitive to equine or other mammalian serum products. In addition to these clinical considerations, there are several manufacturing advantages to antivenom production in laying hens which make them a practical source of commercial quantities of antibodies. The volume of egg yolk produced per animal, the smaller quantities of venom antigens required for hyperimmunization (due to the low body weight of hens), and the ease and simplicity of collecting eggs and obtaining antivenom from the egg yolk each contribute to the cost effectiveness of the avian system.

A purified polyvalent crotalid antivenom for North and South American crotalids has been developed and proven efficacious against several of the most significant crotalids in these regions (CARROLL, THALLEY and WAINWRIGHT, unpublished results). This polyvalent antivenom will be the first antivenom tested for its clinical performance, which of course will be the final measure of the safety and efficacy of these new antivenoms.

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