Use of an ELISA for detection of antibody responses in Argentine boa constrictors (*Boa constrictor occidentalis*)

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Objective—To develop mouse monoclonal and rabbit polyclonal antibodies against immunoglobulin of Argentine boa constrictors and to demonstrate the ability of these reagents to detect antibody responses in boa constrictors by use of an ELISA and western blot analysis.

Animals—Two 3-year-old Argentine boa constrictors.

Procedure—Boa constrictors were immunized with 2,4-dinitrophenylated bovine serum albumin (DNP-BSA). Each snake received biweekly inoculations of 250 µg of DNP-BSA (half SC, half IP) for a total of 6 inoculations followed by monthly inoculations for 3 months. Preimmune blood samples were collected. Subsequently, blood was collected immediately prior to each booster inoculation. Anti-DNP antibodies were isolated from immune plasma samples by affinity chromatography. Affinity-purified boa anti-DNP immunoglobulin was used for production of polyclonal and monoclonal antibodies. An ELISA and western blot analysis were used to monitor immune responses, for purification of boa anti-DNP immunoglobulin, and for assessment of polyclonal and monoclonal antibody specificity.

Results—A 6-fold increase in optical density (OD_{405}) of immune boa plasma, compared with preimmune plasma, was detected by the polyclonal antibody, and a 12- and 15-fold increase was detected by monoclonal antibodies HL1787 and HL1785, respectively, between weeks 4 and 8. Results of western blot analysis confirmed anti-DNP antibody activity in immunized boa plasma and in affinity column eluates. Polyclonal and monoclonal antibodies detected specific anti-DNP antibody responses in immunized boas.

Conclusions and Clinical Relevance—Polyclonal and monoclonal antibodies recognized boa constrictor immunoglobulin. These antibodies may be useful in serologic tests to determine exposure of snakes to pathogens. (*Am J Vet Res* 2003;64:388–395)

A number of species of snakes in the families Boidae (boas) and Pythonidae (pythons) are endangered, and all are considered threatened as a result of factors such as overcollecting for the pet and leather trade, loss of habitat, and pollution. Members of both families are generally referred to as boid snakes. The relationship between disease susceptibility and environmental and human impact factors is poorly understood, in part because of a lack of diagnostic reagents with which to monitor the health status of wild and captive boid snake populations. The urgent need to develop diagnostic tests is in part the result of the recognition of a variety of diseases in captive snakes, including inclusion body disease, a potentially devastating disease in collections of boid snakes, and chlamydiosis, which was recently described in a captive collection of emerald tree boas.[1,2] As causative agents of various infectious diseases are identified, standardized serodiagnostic tests will be needed to determine exposure of boid snakes to these pathogens.

Worldwide increases in the prevalence and severity of several infectious diseases of reptiles have driven the development of appropriate serodiagnostic tests during the past 10 years. Tests have been developed for herpesvirus-associated diseases, such as fibropapillomatosis and lung-eye-trachea disease, of green turtles (*Chelonia mydas*)[3,4] and herpesvirus in Mediterranean (*Testudo graeca*) and Hermann's (*Testudo hermanni*) tortoises.[5] In addition, serologic assays have been developed for mycoplasma-associated diseases such as upper respiratory tract disease in gopher (*Gopherus polyphemas*) and desert (*Gopherus agassizi*) tortoises caused by *Mycoplasma agassizi*[6] and *M alligatoris* in American alligators (*Alligator mississippiensis*).[7] These tests have played an important role in monitoring the health status of individual animals and in understanding the epizootiologic nature of these diseases in wild and captive reptiles.[3,4,5,6,7,8]

A number of polyclonal and monoclonal antibodies against different classes of reptile immunoglobulins have been produced.[3,4,5,6,7] These antibodies are an essential component of the ELISAs developed to determine exposure of reptiles to certain pathogens. However, studies on development of antibodies against immunoglobulins of snakes for use in assays have been extremely limited.[9,10] The purpose of the study reported here was to develop mouse monoclonal and rabbit polyclonal antibodies against immunoglobulin of

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Argentine boa constrictors (*Boa constrictor occidentalis*) and to demonstrate the ability of these reagents to detect antibody responses in boa constrictors by use of an ELISA and western blot analysis.

Materials and Methods

Animals—Two 3-year-old subadult (1 male, 1 female) captive-bred Argentine boa constrictors (*Boa constrictor occidentalis*) housed at a private breeding facility in north central Florida were used in this study. Snakes were maintained individually in 60-L newspaper-lined plastic containers with stainless steel water bowls. Rats, bred at the same facility, were fed to the snakes every 7 to 10 days, and water was changed on a daily basis. Cages were inspected daily and cleaned as needed by removing soiled newspaper substrate and replacing it with fresh newspaper. Cage temperatures ranged from 30 to 32°C during the immunization period. Detailed records of food offered (accepted or refused), defecation, shedding, weight, growth, and other observed behaviors were annotated on individual note cards throughout the study period. Health monitoring was limited to visual inspection of the snakes on a daily basis and notation of acceptance of offered food and positive growth (increase in length and weight).

Immunization and blood collection—Boas were immunized with **2,4-dinitrophenylated bovine serum albumin (DNP-BSA)**[a] that was in a monophosphoryl lipid A plus synthetic trehalose dimycolate (ie, Ribi's) adjuvant system.[b] Each snake received biweekly inoculations of 250 µg (half SC and half IP) of DNP-BSA for a total of 6 inoculations, followed by monthly inoculations of the same DNP-BSA-adjuvant dose for another 3 months. Blood samples (6.0 mL) were collected into lithium heparin tubes from the boas under isoflurane anesthesia by cardiocentesis. A preimmune blood sample was collected from each snake immediately prior to the first immunization. Subsequently, blood was collected immediately prior to each booster inoculation with DNP-BSA following the described schedule. Blood samples were immediately placed into a portable cooler and transported to the laboratory. Plasma was obtained by low-speed centrifugation (300 $\times$ g for 10 minutes) and frozen at –70°C until analysis was performed. Plasma samples with the highest titer (subsequently described) were used for affinity purification of boa anti-DNP antibody. This antibody was then used for production of polyclonal and monoclonal antibodies.

Purification of boa anti-DNP immunoglobulin—Purification of boa anti-DNP immunoglobulin was performed by use of a commercial DNP-Sepharose column.[c] The DNP-Sepharose (2.5 mL) was poured into a plastic column (length, 6.0 cm; diameter, 0.75 cm; provided by the manufacturer), which was then washed with 3 column-bed volumes (9 to 10 mL) of **1$\times$ PBS solution and 0.02% sodium azide (PBS-AZ)**. Immune boa plasma (1.2 mL) diluted in PBS-AZ to a total volume of 5.0 mL was gently loaded onto the column and allowed to filter through the DNP-Sepharose matrix 3 times. An aliquot of each column flow through was saved and stored at 4°C for subsequent analysis. The column was washed by use of 10.0 mL of PBS-AZ, and this material was saved and stored at 4°C. An elution buffer was prepared by dissolving 0.24 mg of DNP-e-aminocaproic acid[b] in 275 µL of dimethyl sulfoxide and diluting 225 µL of this solution in 14.8 mL of PBS-AZ. Boa anti-DNP immunoglobulin was initially eluted from the column by use of 15.0 mL of this elution buffer (eluate-1). A second elution step was performed by use of a concentration of DNP-e-aminocaproic acid 3-fold higher than the first elution (eluate-2). Eluted fractions were concentrated to a 0.55-mL (eluate-1) and 175-µL (eluate-2) volume, and the DNP-e-aminocaproic acid was removed by use of a centrifugal filtration device with a 30,000 molecular weight cut-off.[d] Elution fractions 1 and 2 were stored separately at 4°C.

Polyclonal antibody production—The DNP affinity-purified boa immunoglobulin was delivered to a private biological company,[e] and polyclonal antiserum was produced in 2 rabbits by use of the company's standard 70-day protocol. Plasma samples were obtained from each rabbit before immunization (preimmune plasma). Two rabbits were immunized with 200 µg of affinity-purified boa immunoglobulin with complete Freund's adjuvant (first injection). Subsequent immunizations using incomplete Freund's adjuvant were given on days 21, 35, and 49. Plasma samples were obtained on days 44, 59, and 64. A final immunization (without adjuvant) was administered IP on day 75. Rabbits were anesthetized and exsanguinated on day 79. Plasma was separated and stored at –70°C.

Monoclonal antibody production—Mouse monoclonal antibodies against Argentine boa Ig were produced by standard protocols used by the Interdisciplinary Center for Biotechnology Research Hybridoma Core Laboratory at the University of Florida.[11,12,13] Briefly, two 6-week-old female Balb/cByj mice were immunized SC, with either 25 or 50 µg of boa immunoglobulin (DNP affinity purified) by use of Ribi's adjuvant system.[b] Spleen cells from the immunized mice were fused with myeloma cells at a ratio of 7:1 by use of 50% polyethylene glycol.[f] Supernatants from the resulting HAT (ie, hypoxantine + aminopterin + thymidine) resistant hybridoma cells were evaluated by an ELISA for the presence of antibody that bound to the immunogen (DNP-purified boa immunoglobulin), and the assay was repeated for those samples that initially had positive antibody results. Hybridomas from wells with positive antibody results (**optical density at 405 nm [OD_{405}]** 3 to 20$\times$ over background) were transferred to 24 well plates and screened by an ELISA a second time with antimouse whole molecule or heavy chain-specific (gamma or mu) secondary antibodies. Those supernatants that were **immunoglobulin G (IgG)** positive were again screened by an ELISA by use of wells coated with DNP-**keyhole limpet hemocyanin (KLH)**, followed by a 1:4,000 dilution of boa immune plasma. Sixty-six supernatants had positive antibody results from the first round, and 17 had positive antibody results from the second round of ELISA screening on DNP-purified boa immunoglobulin; of these, 7 had positive antibody results on the DNP-KLH ELISA. Hybridoma supernatants that had positive antibody results on all 3 ELISAs were evaluated by western blot analysis (subsequently described). Two hybridomas with positive antibody results were selected and cloned by use of a standard procedure.[12] Cloned hybridoma cell lines were designated HL1785 and HL1787.

General ELISA procedure—Enzyme-linked immuno -sorbent assays were used for preliminary tracking of the immune response in Argentine boa constrictors, for evaluation and validation of the polyclonal and monoclonal antibodies developed against boa immunoglobulins, and to evaluate the ability of these reagents to cross-react with other snake plasma samples. Wells of a high protein binding microplate[g] were coated with antigen (subsequently described) overnight at 4°C. The microplates were washed 4 times with PBS solution containing 0.02% sodium azide and 0.05% Tween-20 by use of a microplate washer.[h] Subsequent washings were performed in a similar manner. After washing, individual wells were blocked with various reagents (subsequently described) for 1 hour at room temperature (approx 25°C). After washing, a specific primary antibody diluted to a specified concentration was added to each well. After incubation for 1 hour at room temperature with gentle agitation,[i]

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wells were washed and loaded with a specified alkaline phosphatase labeled secondary antibody. All subsequent incubations were performed in a similar manner. After incubation and washing, each well received 0.1 mL of alkaline phosphatase substrate (1.0 mg/mL p-nitrophenyl phosphate).[b] Color development was monitored visually, and the absorbance at 405 nm was recorded after 30 and 60 minutes by use of a microplate reader.[j]

Detection of the immune response—Initially, a partially cross-reactive mouse monoclonal antibody, HL673, specific for the light chain of the desert tortoise[6] was used as a surrogate antibody to indicate successful immunization of Argentine boas. Subsequently, in all experiments, the polyclonal antibody (1:10,000 dilution) and both monoclonal antibodies (hybridoma supernatant, 1:2 dilution) were used as primary antibody reagents in ELISAs to monitor the immune response (ie, an increasing anti-DNP Ig titer) in Argentine boas. Wells were coated with 50 µL of DNP-KLH (1.0 µg/mL). Fifty microliters of select preimmune and immune boa plasma samples was used as the primary antibodies in all wells, whereas goat antirabbit and rabbit antimouse IgG were used as secondary antibodies for those wells in which an immune response was detected with polyclonal or monoclonal antibodies, respectively. Boa plasma samples were diluted 2-fold from 1:500 to 1:64,000.

Cross-reactivity of rabbit polyclonal and mouse monoclonal antiboa Ig antibodies—The ability of the polyclonal antibody and both monoclonal antibodies to cross-react with plasma from 14 snake species **(Table 1)** was evaluated by an ELISA as already described. Wells were coated with 50 µL of a 1:100 dilution of plasma samples from each of the 14 snake species. Fifty microliters of a 1:10,000 dilution of rabbit polyclonal antiboa immunoglobulin or a 1:2 dilution of mouse monoclonal antiboa Ig hybridoma supernatant was then evaluated for reactivity on these plasma samples. Appropriate secondary antibodies were used as described in the previous section.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis—Ability of the DNP-Sepharose affinity column to isolate anti-DNP boa immunoglobulin from immune boa plasma was demonstrated by **sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)** analysis. Immune boa plasma and affinity-purified boa anti-DNP Ig (elute 2, 1.28 mg/mL) were separated (45 minutes at 200 V) by PAGE, under denaturing conditions, with a precast 10% (wt/vol) polyacrylamide gel and morpholinepropanesulfonic acid running buffer.[k] The separated proteins were stained by incubating in stain[l] for 3 hours and then destained overnight in water.

Immunoblot analysis of polyclonal and monoclonal antibodies—For further evaluation of the polyclonal antibody and specificity of the monoclonal antibodies, immune boa plasma,

Table 1—Results of a cross-reactivity ELISA* on various snake plasma samples by use of the rabbit polyclonal (PAb) and mouse monoclonal (MAb) antiboa antibodies

Snake species	PAb	MAb	
		HL1785	HL1787
Argentine boa constrictor (*Boa constrictor occidentalis*)	+++	+++	++
Emerald tree boa (*Corallus caninus*)	+++	+++	–
Anaconda (*Eunectus murinus*)	+++	+++	–
Green tree python (*Morelia viridus*)	+	–	–
Blood python (*Python curtus*)	++	–	–
Indigo snake (*Drymarchon coraeus coperii)*	–	–	–
Black pine snake (*Pituophis melanoleucus lodingii*)	++	–	++
Red ratsnake (*Elaphe guttata*)	+	–	–
Mountain viper (*Vipera ursinii*)	+	–	–
Rhinoceros viper (*Bitis nasicornis*)	+	–	–
Bushmaster (*Lachesis muta*)	++	–	–
Lancehead rattlesnake (*Crotalus polystictus*)	+	–	–
Timber rattlesnake (*C horridus*)	+	–	–
Copperhead (*Akistrodon contortrix*)	++	–	–

*Readings of optical density at 405 nm taken at 60 minutes. Results expressed as follows: +++ = > 3.0; ++ = > 2.0 < 3.0; + = > 1.0 < 2.0; and - = < 1.0.

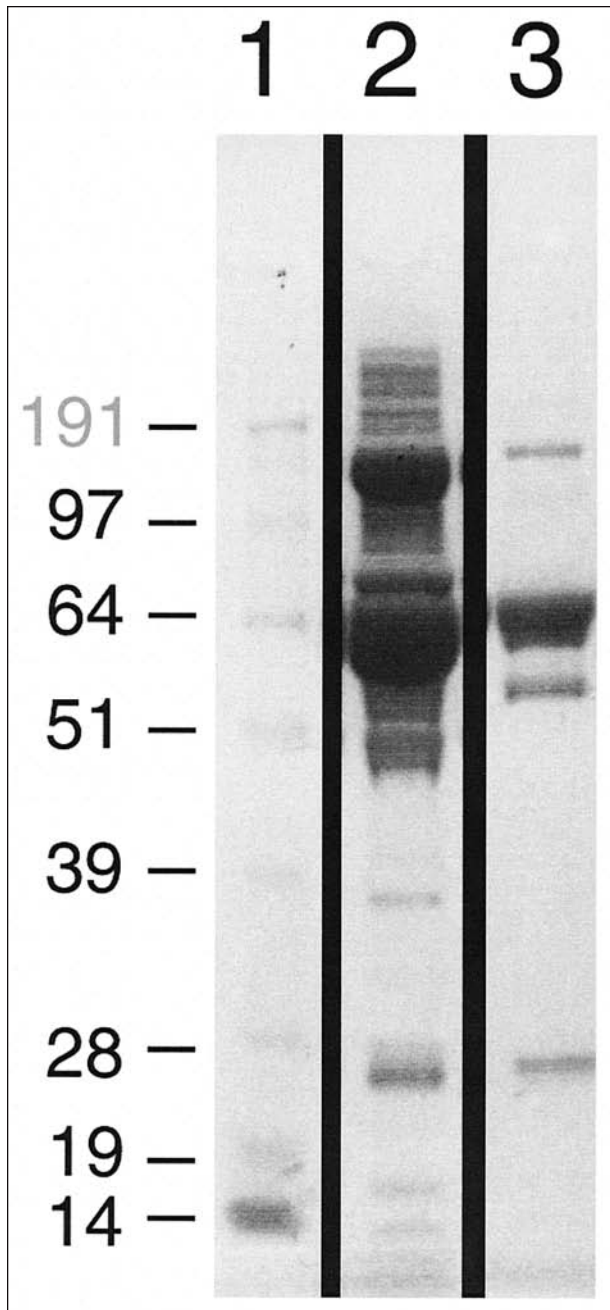

Figure 1—Results of sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of immune boa plasma and DNP-affinity column eluate (elute 2). Lanes (left to right): prestained molecular mass standards (lane 1), immune boa plasma (lane 2; diluted 1:50 in PBS solution), and affinity column eluate (lane 3; elute 2, 1.28 mg/mL). Numbers along the left-hand side represent molecular markers in kd.

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diluted 1:10 in PBS solution, was separated by gel electrophoresis as described and then electrophoretically transferred from the gel to a nitrocellulose sheet[k] by use of a western blot transfer apparatus. A Tris-glycine buffer[k] in 20% methanol was used as the transfer buffer. The blotting time was 60 minutes at 30 V. After transfer, the nitrocellulose was immediately blocked overnight with PBS-AZ containing 5% nonfat dry milk at room temperature (approx 25°C). The nitrocellulose blot was washed with PBS solution containing 0.02% sodium azide and 0.05% Tween-20 and placed into a developer.[m] A total of 900 µL of primary antibody (polyclonal or monoclonal antibodies) was loaded into each channel and incubated on the nitrocellulose for 60 minutes at room temperature. After washing, the nitrocellulose was removed from the manifold and incubated with the appropriate secondary antibody: alkaline phosphatase labeled goat antirabbit IgG (diluted 1:2,000 in **bovine serum albumin [BSA]**) or goat antimouse IgG (diluted 1:4,000 in BSA) for the polyclonal antibody and monoclonal antibodies, respectively, for 60 minutes. After washing, the blot was developed with nitroblue tetrazolium 5-bromo-4-chloro-3 inolyphosphate *p*-toluidine substrate, per manufacturer's instructions.[b]

Cross species immunoblot analysis—Cross-reactivity of polyclonal and monoclonal antibodies was evaluated by western blot analysis by use of anaconda (*Eunectes murinus*) plasma as antigen. This blot was performed in an identical manner to that already described except for the substitution of anaconda plasma for immune boa plasma.

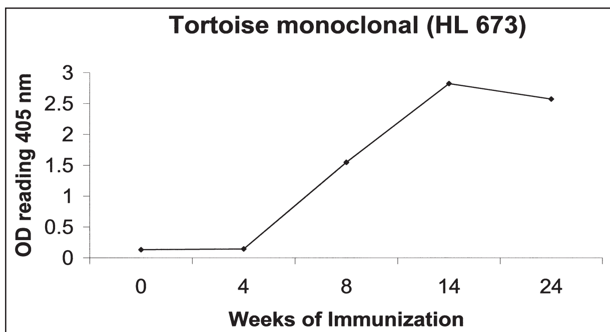

Figure 2—Results of ELISA evaluation of immune and preimmune (0 weeks) boa plasma (1:16,000 dilution) by use of the surrogate antibody HL673 (mouse monoclonal specific for light chain of desert tortoise) at a 1:300 dilution. A 12-fold increase in optical density at 405 nm (OD_{405}) was detected between 4 and 8 weeks of immunization, which persisted throughout the duration of the study.

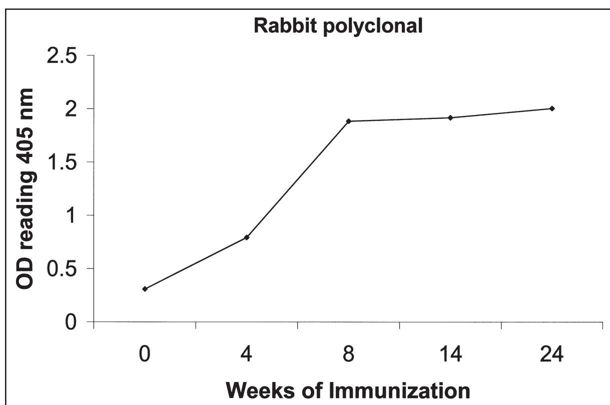

Figure 3—Results of ELISA evaluation of immune and preimmune (0 weeks) boa plasma (1:16,000 dilution) by use of the rabbit polyclonal antiboa Ig (1:10,000 dilution) as a secondary reagent. A 6-fold increase in OD_{405} was detected by 8 weeks of immunization, which persisted throughout the duration of the study.

Results

Purification of boa anti-DNP immunoglobulin—Results of SDS-PAGE analysis of immune boa plasma and affinity-purified eluates indicated that the DNP affinity chromatography column selected certain proteins (**Fig 1**). Immune boa plasma (lane 2) had a protein pattern with multiple bands ranging in molecular mass from > 191 kd to 14 kd. The affinity column eluate (lane 3) had a more selective protein pattern relative to lane 2, with 4 major bands present at 190, 63, 55, and 25 kd.

Detection of anti-DNP antibody titer—Results of an ELISA with HL673 (a partially cross-reactive mouse monoclonal antibody specific for the light chain of the desert tortoise)[6] indicated that the immunized boas had developed an anti-DNP titer, compared with results obtained with preimmune boa plasma. The use of HL673, diluted 1:300 (**Fig 2**), resulted in the detection of a 12-fold increase in antibody titer between immune and preimmune boa plasma samples on DNP-KLH-coated wells. Detection of the antibody titer by use of the antiboa polyclonal antibody (1:10,000 dilution; **Fig 3**) was 6-fold higher in immune boa plasma samples, com-

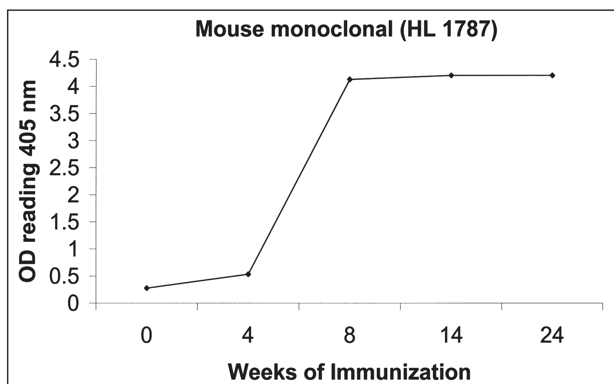

Figure 4—Results of ELISA evaluation of immune and preimmune (0 weeks) boa plasma (1:16,000 dilution) by use of HL1787 (presumed heavy chain specific) hybridoma supernatant at a 1:2 dilution. A 12-fold increase in OD_{405} was detected between 4 and 8 weeks of immunization, which persisted throughout the duration of the study.

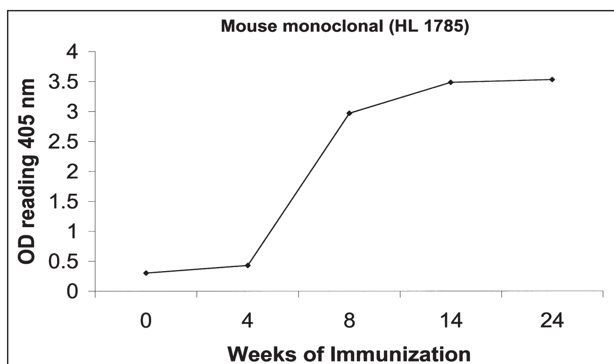

Figure 5—Results of ELISA evaluation of immune and preimmune (0 weeks) boa plasma (1:16,000 dilution) by use of HL1785 (presumed light chain specific) hybridoma supernatant at a 1:2 dilution. A 15-fold increase in OD_{405} was detected between 4 and 8 weeks of immunization, which persisted throughout the duration of the study.

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pared with preimmune samples, on DNP-KLH-coated wells. By use of antiboa monoclonal antibodies on DNP-KLH-coated wells, a 12-fold increase in antibody titer between immune and preimmune boa plasma samples was detected by HL1787 hybridoma culture supernatant diluted 1:2 (presumed boa Ig light chain specific; **Fig 4**). The use of the monoclonal antibody HL1785 (presumed boa Ig heavy chain specific; **Fig 5**) at a 1:2 dilution resulted in a 15-fold increase in antibody titer. The increase in antibody titer was detected between 4 and 8 weeks following immunization and persisted throughout the duration of the study. No detectable increase in the OD_{405} was seen in BSA- and KLH-coated wells.

Immunoblot analysis—Western blot reactivity on immune boa and nonimmune anaconda plasma of the polyclonal antibody, monoclonal antibody HL1785 (presumed light chain specific), and monoclonal antibody HL1787 (presumed heavy chain specific) was determined (**Fig 6** and **7**). For immune boa plasma, the polyclonal antibody (Lane 2) reacted with multiple bands ranging from > 191 kd to 15 kd. Major bands were detected at 64 and 55 kd for both immune boa and nonimmune anaconda plasma, consistent with reptile immunoglobulin heavy chain.[6,7,10,14,15] Findings on the blot (lane 4) indicated that monoclonal antibody HL1785 reacted with a single band at 25 kd (immune boa plasma) and at 28 kd (nonimmune anaconda plasma), consistent with reptile immunoglobulin light chain.[6,7,14,15] Monoclonal antibody HL1787 (lane 5) reacted with a predominant band at 55 kd (immune boa plasma), consistent with reptile immunoglobulin heavy chain.[6,7,14,15] Lane 3 served as a negative control, with preimmune rabbit plasma being used as the primary antibody.

ELISA evaluation of cross-reactivity with plasma of other snake species—Polyclonal and monoclonal antibodies cross-reacted with various snake plasma samples in different patterns (Table 1). Of 14 different snake plasma samples tested, 13 reacted with the polyclonal antibody, 3 with HL1785 (presumed light chain specific), and 2 with HL1787 (presumed heavy chain specific). Only plasma from a single species (Argentine boa) reacted with all 3 reagents. Of the 4 species that reacted with the monoclonal antibodies, only 1 (black pine snake) was a nonboid snake. No plasma samples from a nonboid snake cross-reacted at the highest (> 3.0) OD_{405} reading.

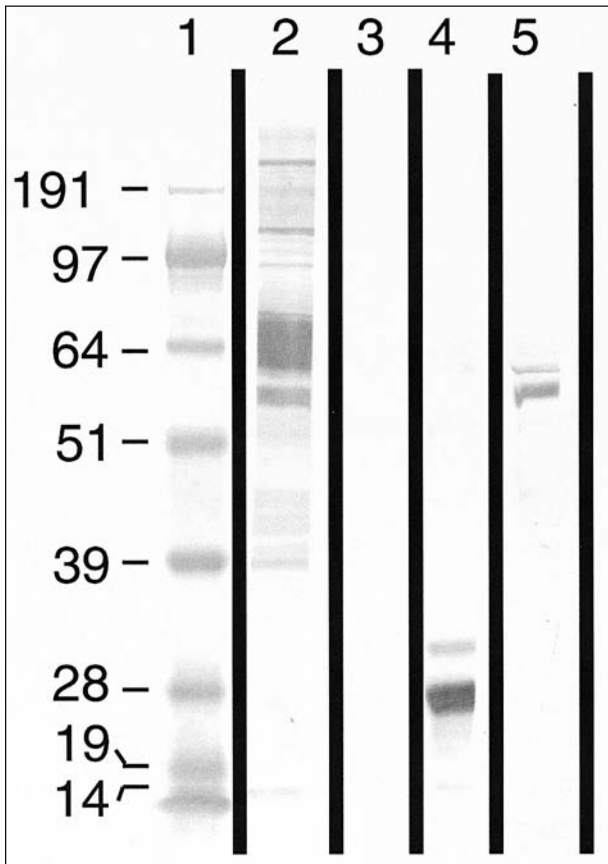

Figure 6—Results of western immunoblot analysis of polyclonal and monoclonal antibodies binding to immune boa plasma. Lanes (left to right): prestained molecular mass standards (lane 1), polyclonal antibody (lane 2), preimmune rabbit plasma (lane 3; negative control), HL1785 (lane 4), and HL1787 (lane 5). The predominant bands detected are consistent with reptile 28 kd Ig light chain and 63 and 55 kd heavy chains. Numbers along the left-hand side represent molecular markers in kd.

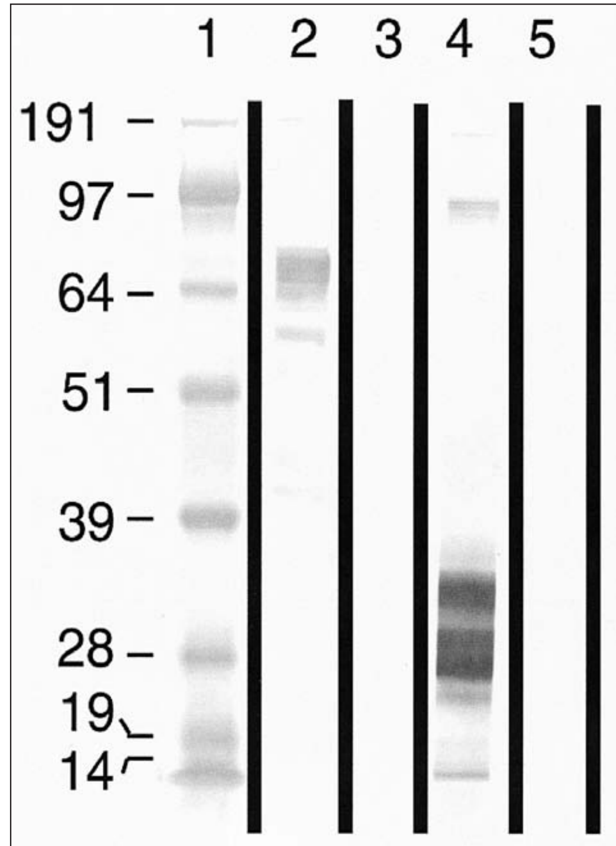

Figure 7—Results of western immunoblot analysis of polyclonal and monoclonal antibodies binding to anaconda (*Eunectus murinus*) plasma. Lanes (left to right): prestained molecular mass standards (lane 1), polyclonal antibody (lane 2), preimmune rabbit plasma (lane 3), HL1785 (lane 4), and HL1787 (lane 5). The predominant bands detected in lane 2 are consistent with reptile 63 kd heavy chain and 55 kd heavy chain, whereas those in lane 4 are consistent with reptile 28 kd Ig light chain. Numbers along the left-hand side represent molecular markers in kd.

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Discussion

Historically, the most common assays used for the measurement of pathogen-specific antibodies have been serum neutralization, immunoprecipitation, and agglutination.[5,14] In reptiles, serum neutralization assays have been used to detect antibodies against herpesvirus in Mediterranean and Hermann's tortoises.[5] This test was sensitive (97%) and specific (98%).[5] However, there are a number of limitations to serum neutralization as a serodiagnostic or seroepidemiologic tool. Serum neutralization assays are labor intensive and require a prolonged period (10 to 14 days) to obtain results.[5] They use virus and cell culture, which are slow, cannot be performed in the field, and represent a greater risk to personnel. Hemagglutination assays have been used to determine the prevalence of antibodies against paramyxovirus in snakes.[16] Although hemagglutination assays are less expensive to run and less labor intensive than serum neutralization, they are also less sensitive.

Testing by use of an ELISA has only recently been used to determine exposure of reptiles to specific pathogens.[4-7] Indirect ELISAs are generally considered the serodiagnostic method of choice.[17,18] They are rapid to perform, adaptable to field situations, inexpensive, safe, and easily applied to large numbers of samples.[17,18] However, indirect ELISAs require the availability of specific antispecies monoclonal or polyclonal antibodies that, up until recently, have been unavailable for use in serologic testing of reptiles. Because none are commercially available, those investigators wishing to work with these types of reagents will have to produce their own.

A wide array of antigens has been used to induce an immune response in reptiles including virus, foreign erythrocytes, serum albumins (BSA, horse serum albumin), KLH, and a variety of bacteria and their toxins.[3-10,14,15,19] Some of these immunogens are potential pathogens that could cause illness or death in study animals. Foreign serum albumins are soluble antigens that, either alone or conjugated to DNP, have been shown to induce an antibody response and to be benign immunogens, producing no reported adverse effects in reptiles.[3,9,19,20] The addition of DNP groups to antigens is a common method of making these materials more immunogenic.[21] In our study, DNP-BSA was chosen as the immunogen because of its proven ability to induce an antibody response in other reptiles,[3] its safety, and its commercial availability.

In our study, Argentine boas were immunized with DNP-BSA. This not only provided information on the immune response to this antigen but also allowed purification of specific antibody by use of commercially available DNP-Sepharose chromatography columns. We were previously unsuccessful in purifying snake immunoglobulin by use of more conventional approaches, such as ion exchange and size exclusion chromatography. The approach used in our study allowed purification and validation of the monoclonal and polyclonal antibodies by demonstrating seroconversion following immunization with DNP-BSA. This is a major benefit of the use of this approach in snakes and other animals.

The injection site, dose, antigen type, use of an adjuvant, and schedule of booster inoculations used in our study tried to take into account many of the immune response variables known to affect reptiles[14] and were similar to a previously reported[3] successful protocol used in green sea turtles. When BSA is used as an antigen in lizards, lower antibody titers result when adjuvants are not used for immunization or if injection site of immunogen is IM rather than IP; maximum antibody titers were independent of concentration of antigen used over a range of 1 to 200 mg, and booster injections of antigen resulted in high antibody titers that persisted over a longer period.[14] Intraperitoneal injections were used to take advantage of an enhanced immune response, when antigen was inoculated by this route, whereas IM injections were given in the cranial half of the snake's body. A number of authors state that drugs injected into the caudal portion of the body may be carried by the renal portal system to the kidneys, leading to rapid excretion and possible nephrotoxicity.[22,23] However, these assertions are not based on experimental data. Two studies[24,25] in turtles suggest that the renal portal system and site of injection have no effect on drug kinetics. Because of unknown and possible effects of the renal portal system on DNP-BSA kinetics, all immunizations were given in the cranial half of the body.

Adjuvants are commonly used during immunization as nonspecific stimulators of the immune system and are essential to induce a strong antibody response to soluble antigens such as BSA.[21] Although the exact mechanism of action of adjuvants is not completely understood, complete Freund's adjuvant or incomplete Freund's adjuvant are frequently used in reptile immunization studies[14] and are successful in stimulating strong and prolonged reponses. Complete Freund's adjuvant contains the bacteria *Mycobacteria tuberculosis* and often invokes aggressive and persistent granulomas in mammalian subjects and is not used in humans for this reason.[21] Newer synthetic adjuvants, such as Ribi's, are potent inducers of the immune system with few adverse effects.[21] Ribi's adjuvant has been used to immunize green sea turtles[3] with success and was chosen for our study because of its safety and efficacy.

The physiologic characteristics of reptiles, including immune function, are influenced by ambient temperature.[14,25-28] Reptiles have a body temperature range that is ideal for each species. As the body temperature increases, there is generally an increase in activity of these processes.[14,25-28] Because of this, ambient temperature needs to be controlled when performing physiologic studies. As a result of a unique collaboration, the snakes in our study were maintained under ideal environmental and husbandry conditions for this species.[29] Both snakes reacted to the immunogen, producing a 6- (polyclonal antibody) to 15-fold (monoclonal antibody) increase in anti-DNP antibodies between 4 and 8 weeks following initial immunization. This time course is similar to that reported[3-7,9,10,19,20]for other species of immunized reptiles. Possibly a greater response could be achieved at higher ambient temperatures.

Results of our study indicate that snakes repeated-

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ly immunized with a dose of 250 μg of DNP-BSA produced antibodies against the DNP hapten alone. Immune boa plasma reacted in the ELISA to DNP but not BSA or KLH. Further, SDS-PAGE analysis of fractions obtained from the DNP-Sepharose column revealed a selective protein pattern (Fig 1) with major bands at 63, 55, and 25 kd, which are in the size range of other reported[9,10,15] immunoglobulin heavy and light chains of snakes. These results are similar to those reported in green seas turtles immunized with similar doses of DNP-BSA[3] and can be explained by the antigenic dose that was used. In previous studies with anti-BSA titer development, pure BSA was used at much higher doses in turtles (18 to 80 mg/dose)[30] and snakes (2.5 to 5 mg/dose)[9] than were used in our study.

Cross-reactivity of the antidesert tortoise monoclonal antibody, HL673, with boa immunoglobulin was an unexpected finding. Chelonians (turtles and tortoises) are distantly related to snakes.[31] This reagent allowed analysis of preimmune and immune boa plasma samples, thus providing preliminary evidence for successful immunization (increasing titer) of the 2 boas in our study. Compared with the boa reagents, HL673 detected similar increases in antibody titer (12-fold) to HL1787 (12-fold) and HL1785 (15-fold) and a higher titer than the polyclonal antibody (6-fold). Although the exact nature of the cross-reactivity is unknown, results of studies in the evolution of the immune response in turtles indicated that there are similarities in the structure of antibodies between chelonians and mammals, and thus it is not unreasonable that snakes and turtles may also share similar conserved epitopes.[32]

Validation of polyclonal and monoclonal antibodies was accomplished by ELISA detection of an increasing DNP-antibody titer (Fig 3–5) and SDS-PAGE analysis (Fig 6) of immune boa plasma. Western blot analysis of immune boa plasma confirmed that the proteins were consistent in size range with other reported reptile immunoglobulins. The polyclonal and monoclonal antibodies were able to measure a 6-fold (polyclonal antibody) and a 12- (HL1787) and 15-fold (HL1785) increase in titer between 4 and 8 weeks of immunization. This is temporally similar to other reports[3-7,9,10,20,21] on reptiles and similar to that seen in our study with HL673 (Fig 2). This slow rate of seroconversion in reptiles could present challenges to diagnosis of infection by use of an ELISA. In 1 study[7] involving 3 species of crocodilians inoculated with pathogenic *M alligatoris*, false-negative results were obtained because half of the alligators and a third of the caimans died before serconversion. Testing of paired samples obtained at least 8 weeks apart would help minimize the risk of false-negative ELISA results.

The primary objective for developing these boa-specific reagents was to design practical serodiagnostic tests to determine exposure to pathogens, such as the causative agent of inclusion body disease, in captive and wild populations of boid snakes. However, because such reagents are not available for other snake species, assessment of the ability of the monoclonal and polyclonal antibodies to detect immunoglobulins in other boid and nonboid snake species seemed warranted.

Preliminary screening indicated that the polyclonal antibody cross-reacted with 13 of 14 snake species (Table 1) representing 12 different genera in 3 families. This may indicate conserved immunoglobulin epitopes across most snake species that can be detected by some of the multiple antiboa IgG antibodies in this polyclonal antiboa reagent. In contrast, but as expected, the reactions observed with the highly epitope-specific monoclonal antibodies were more restricted. Only the plasma from the Argentine boa reacted with both monoclonal antibody reagents. The emerald tree boa and anaconda reacted strongly with HL1785 (presumed light chain), whereas the black pine snake reacted with HL1787 (presumed heavy chain) specific antibody. As in mammals, the heavy chain (5 types) may be less diverse than the immunoglobulin light chains (2 types).[33] It is unknown why the only nonboid snake to react with the monoclonal antibodies was the black pine snake, but its reaction with the potentially less diverse heavy chain antibody may suggest a conserved epitope in this species. Western blot analysis of nonimmune anaconda (*E murinus*) plasma (Fig 7) by use of polyclonal and monoclonal antibodies revealed good correlation with ELISA data, indicating no reaction with HL1787. An immunoglobulin-banding pattern similar to Argentine boa provided evidence for similar classes of immunoglobulins in these closely related species.[34]

The availability of these specific reagents for serologic testing will be of value in clinical cases, retrospective seroepidemiologic surveys, and prospective monitoring of disease agents of concern. In addition, use of these reagents has the potential to be beneficial in the veterinary management of captive boid snakes, studying the epidemiologic characteristics of emerging diseases in the wild, and the conservation of boid snakes.

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[a]Molecular Probes, Eugene, Ore.
[b]Sigma Chemical Co, St Louis, Mo.
[c]Biosearch Technologies Inc, Novato, Calif.
[d]Millipore, Bedford, Mass.
[e]Strategic Biosolutions, Newark, Del.
[f]Roche, Indianapolis, Ind.
[g]Nunc Maxisorp, Fisher Scientific, Pittsburgh, Pa.
[h]Biotek Instruments, Winooski, Vt.
[i]Nutator, Adams, Fisher Scientific, Pittsburgh, Pa.
[j]Spectramax 250, Molecular Devices, Sunnyvale, Calif.
[k]Invitrogen, Carlsbad, Calif.
[l]Simply Blue Safestain, Invitrogen, Carlsbad, Calif.
[m]Fast Blot-Developer, Pierce, Rockford, Ill.

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