Passive Hemagglutination Inhibition Test for Diagnosis of Brown Recluse Spider Bite Envenomation

Steven M. Barrett, Maxine Romine-Jenkins, and Kenneth E. Blick

Our goal was to recreate a passive hemagglutination inhibition (PHA1) test to diagnose brown recluse spider (BRS; Loxosceles reclusa) bite envenomation for treatment trials. Guinea pigs received intradermal injections of concentrated spider venom from the following species: Loxosceles reclusa, Argiope aurantia, Argiope trifasciata, Phidippus audax, and Lycosa frondicola. Skin lesion exudate was collected and tested with the BRS venom PHA1 assay. From 51 separate collections of exudate, test sensitivity was 90% as long as 3 days after venom injection. Specificity was 100% with venom from the other spider species listed above in vivo (7 test samples) and in vitro (5 test samples), as well as with random bacterial exudate and without added serial dilutions of BRS venom (10 test samples). The test was reproducible over repetitive assays to within one 10-fold dilution. A positive PHA1 test result could function as an entry criterion for BRS bite victims in human treatment trials.

Indexing Terms: toxicology · arachnidism

During the warmer months in Oklahoma, an average of one to two patients per week present to our university emergency department for evaluation and treatment of necrotic skin lesions. A common cause of necrotic skin lesions is envenomation from various spider species, including members of the following genera: Loxosceles (fiddleback, violin, or brown spider), Argiope (orb-weaver), Phidippus (jumping spider), Chiracanthium (running or sac spider), and Tegenaria (1, 2). Members of the wolf spider family Lycosidae have been reported to cause necrotic arachnidism (1, 2), but generally the lesions were also secondarily infected (3). There are no commonly available laboratory tests for diagnosis of spider bites (4). The diagnosis is instead based on circumstantial evidence: A spider captured nearby at the time of envenomation is identified as the cause of the skin lesion. Most of this circumstantial evidence in the Midwest seems to implicate Loxosceles reclusa (Figure 1), the brown recluse spider (BRS), as the venomous culprit.

To investigate the veracity of this implication for further studies, we formulated a passive hemagglutination inhibition (PHA1) assay specifically to diagnose necrotic arachnidism caused by the BRS (Figure 2). This test was previously described in guinea pig experiments with BRS envenomation (5), but test specificity was not studied and skin lesions were tested only up to 24 h after envenomation. The PHA1 assay is based on the property of certain BRS venom components to spontaneously adsorb to formalin-treated erythrocyte (RBC) membranes and on the ability of BRS venom to inhibit the anti- serum-induced agglutination of venom-coated RBCs (5).

Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee and the Institutional Review Board of the University of Oklahoma Health Sciences Center.

Preparation of spider venom. Food was withheld from adult BRS for 2 days before venom collection. Spiders were frozen and thawed before microdissection to acquire the white gelatinous venom sacs located directly under the carapace of the anterior cephalothorax. We extracted 10 to 20 paired venom glands and placed them in 1 mL of 0.1 mol/L sodium phosphate-buffered saline solution (PBSS). We prepared a purified venom fraction by the methods of Babcock et al. (6) and Rees et al. (7), as follows. We lightly crushed venom sacs for 5 min with a glass stirring rod, and removed the particulate matter by centrifugation (10 min, 8000 × g, 4 °C). We removed the supernate (S1) and resuspended the precipitate in 1 mL of PBSS. This suspension was crushed lightly for another 5 min and the centrifugation repeated. The second supernate (S2) was removed and the precipitate discarded. We pooled supernates S1 and S2 (S9) and separated the pool into 0.5-mL aliquots, which we froze with a mixture of methanol and solid CO2 and stored at −20 °C. We used the Bio-Rad (Hercules, CA) protein

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1 Section of Emergency Medicine, Department of Surgery, and 2 Radioimmune Assay/Computer Operation Department, Department of Pathology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73126.
3 Address for correspondence: 650 Clarenda Falls Drive, Sugar Land, TX 77479.
4 Nonstandard abbreviations: BRS, brown recluse spider; PHA1, passive hemagglutination inhibition; RBC, erythrocyte; PBSS, sodium phosphate-buffered saline solution; PHA, passive hemagglutination; and HU, hemagglutination unit.

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Fig. 1. Loxosceles reclusa, brown recluse spider
Note the dark violin-shaped marking on the dorsal cephalothorax. The scale shown is in centimeters

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assay to determine the protein concentration in thawed S^3.

**Preparation of spider antivenom.** New Zealand White rabbits were hyperimmunized with sublethal doses (20 μg) of S^3 fraction BRS venom suspended in adjuvant (Hunter's Titermax adjuvant; Cytrix, Norcross, GA) by both intramuscular and intradermal routes. Each rabbit received a booster injection of venom 10 to 14 days before blood was collected from an ear vessel. The blood was allowed to clot overnight before centrifugation and serum separation. The antisera were frozen at -20 °C in 0.5-mL aliquots. Before and after BRS venom injections, rabbit serum samples were compared for total protein content to verify antibody production by measuring the refractive index (Protometer; National Instrument Co., Baltimore, MD). The potency (working dilution) of the antisera is determined by performing the passive hemagglutination (PHA) assay.

**Hemagglutination and hemagglutination inhibition tests.** For PHA and PHAI testing we used the methods of Finke et al. (5). Briefly, we incubated at room temperature serial twofold dilutions of S^3 fraction venom for 1 h with formalin-treated group O human RBCs (10 g/L) (8) diluted with PBSS in a U-bottom microtiter plate (Fisher Scientific, Plano, TX). After washing twice with 10 g/L albumin in PBSS, the venom-coated RBCs were incubated with serial twofold dilutions of heat-inactivated (56 °C for 30 min) antivenom at 4 °C. After 6 to 24 h, we evaluated the microtiter plate wells for PHA. The greatest dilution of antivenom and venom that produces agglutination is defined as one hemagglutination unit (1 HU). Four hemagglutination units of antivenom (i.e., fourfold more concentrated than 1 HU) was used as the working venom and antivenom dilutions for the PHAI test.

For the PHAI procedure, we incubated the formalin-treated RBCs (10 g/L) with the working venom dilution for 1 h at room temperature in a microtiter plate, then centrifuged the microtiter plate (3 min, 450 × g; IEC Model PR6; International Equipment, Fisher Scientific) and decanted the supernate. We washed the venom-coated cells twice with 10 g/L albumin in PBSS. The working dilution of antivenom (4 HU) was incubated with 25 μL of test sample (wound exudate or known BRS spider venom control titer) for 20 min and then added to the washed venom-coated RBCs.

After incubation at 4 °C for 6 to 24 h, the test samples were considered positive for venom if the RBCs settled into a button at the bottom of the microtiter well. That is, antibody reacted to venom in the test sample, so that binding of antibody to the venom-coated cells was inhibited. The test samples were considered negative for venom if the RBCs were suspended (agglutinated) in the microtiter well; i.e., RBC agglutination signifies that the antigen–antibody reaction was not inhibited by the test sample. We used venom-coated RBCs incubated without antivenom, uncoated RBCs in PBSS, and uncoated RBCs with antivenom as positive controls. For the negative control, we incubated venom-coated RBCs with 4 HU of antivenom. All controls were reassayed for each lot number of reagents. The antivenin plus uncoated RBCs (positive control) and the venom-coated RBCs incubated with 4 HU of antivenom (negative control) were included with each test run (Figure 3).

**Assay optimization.** To optimize the interpretability of the assay, we manipulated the following PHAI test variables:

1. Confirmation of negative PHAI test results. Negative tests were repeated on a diluted test sample to rule out prozone effects (9) (interference with antigen–antibody binding because of excessive antigen concentrations).

2. Human RBC preparation and stabilization techniques. Group O human RBCs (10–20 g/L) treated with formalin proved to be a stable preparation and allowed accurate and reproducible test interpretability.

3. Viscosity and ionic properties of solutions to facilitate antigen–antibody binding (9, p. 15). Adding dextran to decrease viscosity did not seem to enhance test interpretability. Including albumin, 10 g/L, in PBSS to wash venom-coated RBCs reduced cellular surface charges, thereby facilitating antigen–antibody binding.

4. Choice of test tubes. Because polypropylene test
tubes provided more consistent test results than glass test tubes, we suspect that the nonspecific binding properties of BRS venom may extend to boro silicate glass.

Specificity and sensitivity studies. The above venom preparation method was also used for other captured spiders: A. aurantia, A. trifasciata, P. audax, and Lycosa frondicola. All dissected spiders were classified to the species level with a taxonomy system (10). Each guinea pig received two intradermal injections of venom from various spider species at weekly intervals. Subcutaneous injections only erratically produced necrotic skin lesions, whereas intradermal injections consistently caused characteristic lesions. Injections consisted of -24 μg of BBS venom, 20–40 μg of Argiope or Phidippus venom, or 14–28 μg of Lycosa venom. These injection amounts approximate the inoculum that occurs from actual spider bite envenomations (5–23 μg) (5).

Collection of exudate. Exudate was collected as follows: The lesion edge was scraped with the point of a needle, and the resulting serous or serosanguineous exudate was blotted with filter paper. Some early lesions contained blisters; the tops of these blebs were removed and the blister fluid was blotted onto filter paper. The filter paper exudate blot (5 mm diameter) was trimmed, placed in the bottom of a 1-mL polypropylene micro tube (Fisher Scientific), and rinsed repeatedly with 50 μL of PBSS. The PHAI test was performed with the eluted sample.

Other studies. The PHAI assay was also performed with in vitro samples of concentrated venoms from various spiders. To assess reproducibility, we made 25 different PHAI test runs with serial dilutions of BRS venom. We also assayed 10 random human bacterial wound samples to which serial titers of BRS venom had been added, as well as unsupplemented wound exudates.

Results

The PHAI venom working dilution varied among lot numbers (separate pooled venom preparations) by ±1 microtiter well (i.e., 1 dilution factor). PHAI antisera working dilution varied among lot numbers (separate pooled rabbit antisera preparations) by ±2 microtiter wells. Therefore, venom and antisera working dilutions should be determined for each separate lot number of preparation.

Serial 10-fold dilutions (range 10⁻³ to 10⁻¹₂) of venom (740 mg/L) were assayed during 25 separate PHAI procedures. The PHAI assay was positive from 10⁻⁶ through 10⁻¹⁰ (±10⁻º) dilution of BRS venom.

We made 26 separate intradermal injections of concentrated BRS venom into guinea pigs. From these skin lesions, we collected 51 exudates, 1 to 3 days after venom injection. Forty-six of these 51 samples were PHAI-positive. Therefore, the in vivo sensitivity of the PHAI test for detection of BRS venom from induced skin lesions was 90% as long as 3 days after envenomation. Four of the five false-negative PHAI results occurred on the third day (72 h) after injection, and one on the first day. Although many of the PHAI tests were positive despite serosanguinous exudate, all five false-negative results were from exudate that was primarily bloody.

Three separate intradermal injections of concentrated venom from A. aurantia or trifasciata resulted in three skin lesions and four collections of exudate. Three injections of venom from P. audax resulted in two skin lesions and three collections of exudate. Three injections of concentrated venom (14–28 μg) from Lycosa frondicola did not cause skin lesions. In addition, one in vitro PHAI test was performed with Argiope venom, three with Lycosa venom, and one with Phidippus venom. PHAI test specificity was 100%: All 7 in vivo and 5 in vitro tests of venom from other spider species were negative, and 10 in vitro tests of random human bacterial exudate with none or added serially diluted BRS venom gave no false-positive results.

Discussion

The PHAI test for detection of BRS venom in skin lesions is sensitive, specific, reproducible, and easy to interpret. However, it is cumbersome to prepare and the results are available only after several (6–24) hours. Lesion exudate that is primarily bloody may yield a false-negative PHAI test. Sensitivity of the test with bloody samples might be improved if the samples were frozen before PHAI testing. Freezing would lyse the contaminating RBCs that might confuse the interpretation of the PHAI test.

Twenty of 24 guinea pig skin lesions caused by BRS venom were PHAI-positive at 72 h after envenomation. However, we evaluated a patient with a typical BRS bite necrotic skin wound, whose exudate was PHAI-positive 2 weeks after envenomation (Figure 3). With our particular injection technique, the guinea pig BRS skin lesions were essentially healed after 4 or 5 days.

The necrotic potential of cutaneous envenomation from Argiope and Phidippus spiders was confirmed in our study. However, injection of venom from Lycosa frondicola caused only mild erythema in guinea pig skin. The role of the Lycosidae family of spiders as a cause of necrotic arachnidism in the absence of secondary infection has recently been questioned (3).

If 80% of the acute necrotic skin lesions in our area patients are due to BRS bites (80% prevalence), then the predictive value of a positive PHAI test would be 100% and the predictive value of a negative test would be 71.4%. We are currently using a positive PHAI test as an entry criterion for a controlled clinical treatment trial in humans. Thus far, the majority (59 of 62) of patients with necrotic skin lesions clinically compatible with BRS bites have tested PHAI positive.

The BRS bite is only one of many potential causes of the necrotic skin lesion. Treatment options for presumed BRS-induced necrotic arachnidism over the years have included surgical excision, various medications, hyperbaric oxygen, and electric shocks. When planning controlled prospective evaluations of these various therapies, the specific cause of the necrotic skin lesion should be identified if possible.

In view of the very high specificity of the PHAI test...
with in vitro venoms and in vivo animal wound exudates, a positive test with human wound exudate would serve as solid evidence of the presence of BRS venom in the skin wound. Therefore, a positive test result could function as an entry criterion for BRS bite victims in human treatment trials.

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