

Effect of adjuvants on the antibody response of mice to *Bothrops asper* (Terciopelo) snake venom

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Abstract

The adjuvant properties of several immunostimulant molecules on the murine antibody response to *Bothrops asper* snake venom were evaluated. Mice receiving venom together with either sodium alginate, calcium alginate, aluminum hydroxide, muramyl dipeptide, killed *Brucella abortus* or *B. abortus* smooth lipopolysaccharide developed a similar antibody response. Despite the fact that in some cases animals injected with venom and *Salmonella montevideo* lipopolysaccharide developed a significantly higher antibody titer when compared to other experimental groups, no statistically significant differences were observed in most of the comparisons.

Key words

- Adjuvant
- Snake venom
- Antibody response

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Research supported by
Vicerrectoría de Investigación,
Universidad de Costa Rica
(No. 741-89-057), and by the
International Foundation for
Science (No. F/0883-4).

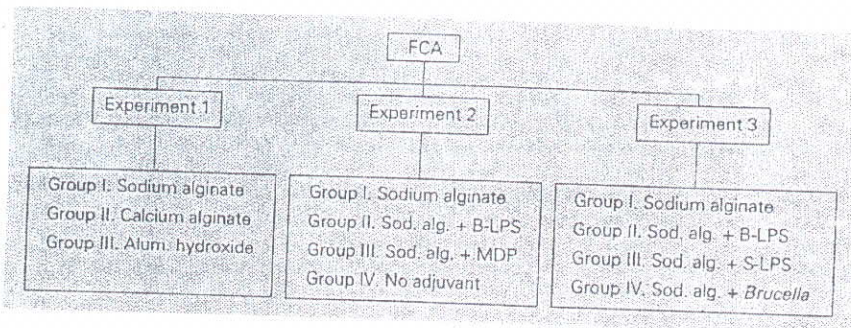
Received March 8, 1996
Accepted June 18, 1996

A polyvalent (Crotalinae) antivenom is produced at Instituto Clodomiro Picado for the treatment of pit viper envenomations in Central America (1). The immunization protocol involves the injection of increasing amounts of a mixture of *Bothrops asper*, *Crotalus durissus durissus* and *Lachesis muta* venoms in horses. The first two venom doses are administered in Freund's complete and incomplete adjuvants, respectively, whereas sodium alginate is used as adjuvant during the rest of the immunization. Other laboratories use aluminum hydroxide (2) or bentonite (3) instead of alginate. Claims on strong adjuvant properties of several bacterial molecules that do not induce hypersensitivity reactions have been consistently reported in the literature (4,5). In order to identify more effective adjuvants for antivenom produc-

tion, a study was carried out in mice immunized with mixtures of *B. asper* venom and various known adjuvants.

The following antigen and adjuvants were used: venom from adult *B. asper* specimens, Freund's complete adjuvant (FCA; Sigma Chemical Co., St. Louis, MO), sodium alginate (Sigma), calcium alginate prepared according to Chase (6), aluminum hydroxide prepared according to Herbert (7), muramyl dipeptide (MDP; Sigma), *Brucella abortus* smooth lipopolysaccharide (B-LPS) purified according to Moreno et al. (8), acetone-killed *B. abortus* S 19, and *Salmonella montevideo* lipopolysaccharide (S-LPS) extracted and purified according to Lindberg and Holme (9). An immunization protocol was designed to simulate the scheme followed in horses at Instituto Clodomiro Picado (10). Venom was

Figure 1 - Adjuvants used for the immunization of different groups of mice with *B. asper* venom. All mice received the first injection of venom emulsified in Freund's complete adjuvant (FCA) and the remaining injections were performed with venom and the adjuvants indicated. Group IV in Experiment 2 received the first venom dose in FCA and the remaining injections were of venom without adjuvant. B-LPS, *Brucella abortus* lipopolysaccharide; MDP, muramyl dipeptide; S-LPS, *Salmonella montevideo* smooth lipopolysaccharide.



dissolved in sterile 0.12 M NaCl, 40 mM phosphate buffer, pH 7.2 (PBS), and mixed with an equal volume of the adjuvant; a total volume of 100 μ l was used per injection. Groups of ten female Swiss-Webster mice (18-20 g) were used in three independent experiments. In all cases, mice were initially injected subcutaneously (sc) with 20 μ g of venom emulsified in FCA. Then, at two-week intervals, each group of mice was injected sc with increasing amounts of venom (20, 40 and 60 μ g), using different adjuvants as shown in Figure 1. An additional group was included in Experiment 2 in which the first venom dose was emulsified in FCA, and in the rest of injections venom was administered in PBS without adjuvant. Control animals were injected with PBS without venom at the same times. After the last injection, mice were bled from the tail every two weeks, and additional injections of 40 μ g venom, with the corresponding adjuvant, were performed one week after each bleeding.

Antibody levels were estimated by immunoenzymatic assay (ELISA). Plastic plates (Dynatech Immulon II) were coated with 1 μ g venom per well and free sites blocked with 2% bovine serum albumin (Sigma). Mouse serum at 1:2000 dilution, corresponding to a linear region of the enzyme immunoassay, was added. Bound antibodies were detected with a rabbit anti-mouse IgG (whole molecule)-peroxidase conjugate (Sigma), using o-phenylenediamine (Sigma) and H₂O₂ as substrate. Absorbances were recorded at 492 nm. The statistical significance of the

differences in mean absorbance between experimental groups at each sampling time was determined by one-way analysis of variance. When significant differences were detected ($P < 0.05$), pairs of experimental groups were compared by the Tukey-Kramer multiple comparison test. Since this study was performed in three independent experiments, only the results of groups within the same experiment were compared.

Absorbances from sera of immunized mice corresponding to Experiment 1 are shown in Figure 2. At the first bleeding, animals receiving calcium alginate had a significantly higher antibody response than mice injected with sodium alginate, whereas neither of these groups showed significant differences compared to the group receiving aluminum hydroxide. No significant differences between any pair of groups were observed at the second and third bleedings. Mice receiving venom with either sodium alginate or aluminum hydroxide did not develop local lesions at the site of injection, whereas animals injected with venom and calcium alginate developed granulomas and local ulceration.

In Experiment 2, no significant differences were observed between the groups corresponding to sodium alginate, MDP and B-LPS at any of the bleeding times tested (Figure 2). In contrast, mice injected initially with venom emulsified in FCA and later with venom in PBS, with no adjuvant, gave a very weak response. In Experiment 3, mice injected with venom and S-LPS developed

an antibody response slightly higher than those receiving B-LPS (first bleeding) and sodium alginate (second bleeding) (Figure 2). However, with the exception of these two cases, no statistically significant differences were observed between any of the groups in the other comparisons (Figure 2).

No consistent differences were observed between mice immunized with venom in aluminum hydroxide, sodium alginate or calcium alginate, the latter inducing conspicuous local ulceration. The use of bacterial products as adjuvants for antibody response to *B. asper* venom was of no significant benefit. Our results with muramyl dipeptide agree with those of Laing and Theakston (11). When *S. montevideo* LPS was used as adjuvant, mice developed a significantly higher antibody response in two cases. However, in most instances mice receiving *S. montevideo* LPS did not differ in their antibody response when compared to the other groups. Thus, despite the fact that bacterial LPS are well-known immunomodulators (5), and their usefulness as immunostimulants for venom immunization has been described by Laing and Theakston (11), our results did not demonstrate an improvement in the immune response of mice who received mixtures of venom and LPS when compared to other adjuvants. It is likely that the immunization protocol followed, based on repeated venom injections, may explain the lack of consistent differences between the adjuvants compared in this study.

Most lipopolysaccharides are known to induce severe toxic side effects, precluding their use as adjuvants. Therefore, it was of interest to study the adjuvant activity of *Brucella abortus* LPS, which lacks most of the endotoxic activities associated with enterobacterial LPS, while being effective as adjuvant (12). However, our results do not support the use of killed *Brucella* cells or LPS as adjuvants for antivenom production. In conclusion, our results indicate that no marked increase in the antibody response to *B. asper*

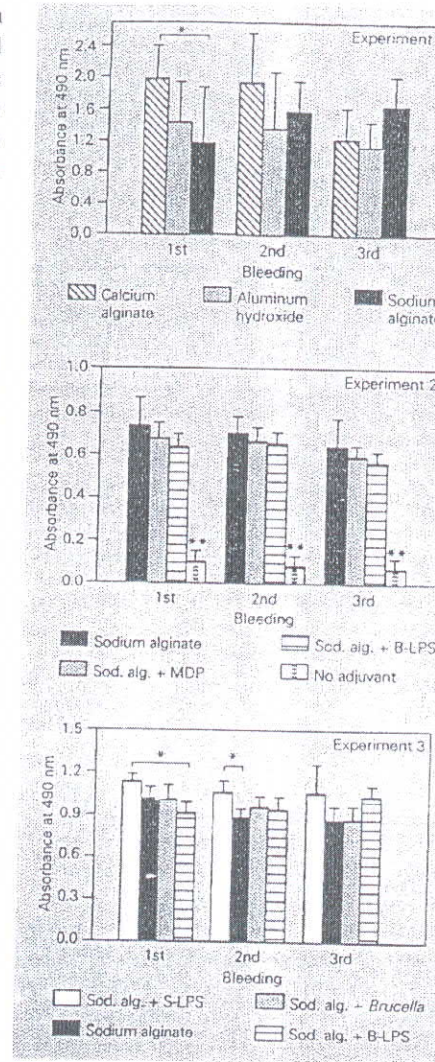


Figure 2 - Effect of different adjuvants on the antibody response of various groups of mice to *B. asper* venom. Mice from all experiments received a first venom dose emulsified in FCA and the remaining venom injections were administered to groups of mice (N = 10) with the adjuvants indicated. Mice were bled three times and antibody levels were estimated at 1:2000 dilution by ELISA (see text). Bars represent the mean (\pm SD) absorbance at 492 nm of triplicate determinations. Samples from mice injected with PBS alone (controls) gave absorbances lower than 0.1. Horizontal lines (*) link the experimental groups having a significantly different antibody response between them ($P < 0.05$). In Experiment 2, the group corresponding to mice receiving venom with no adjuvant had a significantly lower response than the remaining groups (**) (one-way ANOVA followed by the Tukey-Kramer test). Abbreviations are identified in the legend to Figure 1.

venom was obtained by using bacterial products when compared to adjuvants traditionally used in antivenom-producing laboratories.

Acknowledgments

The authors thank Drs. Bruno Lomonte and Alberto Alape for helpful discussions and Jorge Sanabria, Rodrigo Chaves and Javier Núñez for skillful technical collaboration.

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