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DEVELOPMENT OF ANTIBODY RESPONSE AND CLINICAL AND HEMATOLOGICAL ALTERATIONS IN HORSES IMMUNIZED WITH SNAKE VENOMS FOR THE PRODUCTION OF ANTIVENOM IN COSTA RICA

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ABSTRACT: The development of antibody response, as well as the clinical and hematological alterations occurring in horses immunized with snake venoms for the production of polyvalent antivenom in Costa Rica were studied. One horse receiving its first immunization gradually increased antibody response, and underwent an inversion in their albumin/globulin ratio. Neutralizing antibody response in horses that had been previously immunized and received a booster injection of venom showed marked individual variability. Horses injected with booster doses of venom showed increments in total serum preteins and a slight drop in hematocrit and hemoglobin. Regardin clinical alterations after venom injection, there were no signs of systemic alterations (hypotension, hemorrhage, shock), but all horses developed small local lesions at the site of venom injection, characterized by edema, fibrosis and abscesses. These local lesions were treated and healed successfully. At the end of extensive production bleedings, there was a slight drop in hematocrit, whereas in the following weeks there was a conspicuous increase in hematocrit and hemoglobin.

KEYWORDS: Snake venom, antivenom, horses, hematological changes.

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INTRODUCTION

A polyvalent antivenom is produced in Costa Rica since 1967 for the treatment of pit viper envenomations[1,2]. Horses are immunized with a mixture of equal parts of venoms from *Bothrops asper, Crotalus durissus durissus* and *Lachesis muta*[2]. There is little information in the scientific literature regarding the development of immune response in horses being immunized for the production of antivenoms[4]. In addition, we are not aware of studies on clinical and hematological alterations occurring in horses during immunization with snake venoms.

At the Instituto Clodomiro Picado, Costa Rica, an individual record is kept on the antibody response as well as in clinical and hematological changes in horses being injected with venoms for the production of the polyvalent (botropic, crotalic and lachetic) antivenom. In this work we present data on a group of horses that had been previously immunized with venom and received booster doses, as well as in one horse being immunized for the first time.

MATERIALS AND METHODS

Horses: Eight healthy adult horses (2-5 years old), from both sexes, weighing between 450 and 500 kg were used in this study. One horse (Nº 21) had not been injected previously with venom and was submitted to a first immunization. The other horses (Nºs 3, 12, 23, 29, 37, 42 and 44) had been previously immunized and bled, and received booster doses of either 20 or 50 mg of venom. Throughout the study, horses were fed with a combination of king grass, hay and a mixture of powder food reinforced with vitamins and minerals in specific amounts for horses.

Venoms and immunization schedule: Venoms of *Bothrops asper, Crotalus durissus durissus* and *Lachesis muta* were obtained from adult specimens kept at the serpentarium of Instituto Clodomiro Picado. Once collected, venoms were centrifuged at 3000 rpm, frozen and lyophilized. The immunization mixture was prepared by combining equal weights of lyophilized venom from the three species. The venom was dissolved in phosphate-buffered saline solution, pH 7.2, and equal volumes of venom solution and adjuvant (complete Freund, incomplete Freund or sodium alginate) were mixed. Injections were done subcutaneously in the costal arc. Horse Nº 21 was immunized according to the protocol described in Table 1. Seven horses (Nºs 3, 12, 23, 29, 37, 42 and 44) that had been previously immunized and bled, were injected with a booster dose of venom of either 20 or 50 mg using sodium alginate as adjuvant. This booster injection was administered 40-50 days after the last production bleeding. In all cases, before the addition of adjuvant, venom solutions were sterilized by filtration in nitrocellulose membrane of 0.22 (μ)m.

Production bleeding: Each production bleeding was performed in four days, as follows: Day 1: Eight liters of blood are collected. Day 2: Initially, four liters of blood are collected. Then, sedimented erythrocytes from the blood collected on day 1 are resuspended in 0.15 M NaCl to a volume of four liters; resuspended erythrocytes are then transfused to the same animal. Then, a second bleeding of four liters is performed. Day 3: Initially, four liters of blood are collected. Then, sedimented erytrocytes from the blood collected on the second day are resuspended

in 0.15 M NaCl to a volume of four liters; this volume is then transfused to the horse. Day 4: Sedimented erythrocytes from the blood collected on the third day are resuspended in 0.15 M NaCl to a volume of four liters and returned to the horse. Then, 8-12 liters of Ringer's lactate solution are administered. This solution has the following composition: 130 mEq/L sodium, 4 mEq/L potassium, 4mEq/L calcium, 111 mEq/L chloride, and 27 mEq/L lactate.

Antibody response and serum protein concentration: On the days of venom injection, horses were bled from the jugular vein just before venom injection. Blood was allowed to clot at 37°C and serum was collected and used immediately or stored at 4°C. Antibody response against indirect hemolytic activity of *B. asper* venom was assayed by the method of Gutiérrez *et al.*[7] Neutralizing ability of sera was expressed as Effective Dose 50% (ED_{50}), defined as the ratio of (μ)l of serum/mg of venom that reduced hemolytic activity 50%. In order to facilitate data interpretation, neutralizing ability of sera was expressed as $1/ED_{50} \times 10^5$. Besides neutralization tests, total serum protein concentration was determined by a modification of the Biuret method[12], using bovine serum albumin as standard. In addition, albumin concentration was determined by the Bromocresol Green method[12] and the globulin concentration was calculated by subtracting albumin concentration from total protein concentration.

Hematologial changes: On the days of venom injection, horses were bled just before injection, as described above. To prevent clotting, 4% sodium citrate was used as anticoagulant (1 part of anticoagulant per 9 parts of blood). Hematocrit was determined using heparinized capillary tubes[11] and hemoglobin by the cyanomethemoglobin assay[11]. Hematocrit and hemoglobin were also determined during extensive bleeding, in order do detect the effect of acute blood loss. In this case, the Student's *t* test was performed to determine the significance of the differences observed.

Clinical alterations: Clinical examination of horses involved in this study was carried out twice a week and local and systemic alterations were recorded. Local alterations, i.e. pathological changes at the site of venom injection, were observed, especially edema, abscesses, fibrosis and fistules. In addition, sings of systemic poisoning were also followed, in order to detect evidences of hypotension, hemorrhage and cardiovascular shock.

RESULTS

Development of antibody response and changes in serum proteins: Response in horse Nº 21: Fig. 1 shows the development of antibody response, together with changes in serum albumin and globulins, in this horse that received venom injections for the first time. Antibody response peaked around the 100th day, in agreement with previous results[4]. There was and inversion of the albumin: globulin ratio in the course of immunization, correlating with an increase in antibody response (Fig. 1).

Response in horses that had been previously immunized and received a booster injection of venom: Fig. 2 shows a great individual variability in the antibody response after a booster injection of either 20 or 50 mg of venom. In general terms,

venom injection elicited an increase in antibody titers. However, the magnitude of this increment varied drastically and, in some cases, there was not an adequate response after venom injection. Some horses (N.ºs 12, 29, 42 and 44) maintained a high antibody titer even 40-50 days after the last venom injection.

Regarding changes in serum protein concentration in these horses, there was an increment in total serum proteins and globulins after venom injection (Fig. 3). This increment was associated with the elevation of antibody titers against indirect hemolytic activity. However, there is not a strict quantitative correlation between these two parameters.

Effect of venom inoculations on hematocrit and hemoglobin: In the majority of studied horses, injection of booster doses of venom (20 or 50 mg) induced a slight reduction in hematocrit and hemoglobin (Fig. 4). Hematocrit reduction after each injection ranged from 3 to 6%.

Effect of extensive production bleeding on hematocrit: Table 2 presents the data on hematocrit variation at different times during these four days of bleeding. At the end of the fourth day, after returning the resuspended erythrocytes but before administering the Ringer's lactate solution, there was a slight decrease in hematocrit, although this drop was not statistically significant ($P > 0.1$). Then, after Ringer's lactate infusion, hematocrit dropped significantly ($P < 0.05$). On the other hand, in the weeks that followed these extensive production bleedings, there was a conspicuous increase in hematocrit and hemoglobin (Fig. 4).

Clinical alterations: No evidences of systemic alterations, i.e. hypotension, hemorrhage, and cardiovascular shock, were observed in the horses used in this study. In contrast, all horses developed local tissue damage at the site of venom inoculation, characterized by edema and fibrosis. In addition, abscesses were observed at the site of injection. Five horses (Nos. 3, 12, 37, 42 and 44) developed fistules at this site. In these cases, topical treatment was performed with antiseptics of external use and healing was successful in all cases.

DISCUSSION

Our results confirmed earlier observations that there is a conspicuous individual variability in the antibody response of horses immunized with snake venoms for the production of antivenoms[4]. The present study extends these findings to horses that had been previously immunized and received booster doses of venom. In the case of *B. asper* venom and antivenom, the study of neutralization of indirect hemolytic activity is a simple and useful parameter to follow the development of antibody response, since there is a good correlation between neutralization of lethality and neutralization of indirect hemolysis[7]. On the basis of the correlation between hemolysis and lethality neutralization, it has been established in our laboratory that hemolysis neutralization titers of 70 correspond to a lethality neutralization titer of 3 mg of venom neutralized per ml of serum.

Drastic variability in antibody response against venom was observed not only between horses but also in the same horse at different times, even when the amount of venom injected was the same. Our results suggest that, in order to increase efficacy in antivenom production centers, it is necessary to evaluate the immune response development individually. *In vitro* assays such as the one used

in this work, or others like ELISA[13], might be useful for this individual monitoring.

There was a clear relationship between development of antibody response and some changes in serum protein concentration. After immunization, an increment in total serum proteins took place, as well as in globulins, concomitantly with a reduction in the albumin/globulin ratio. This finding agrees with observations carried out in horses immunized with *Bothrops* venoms in Brazil[3]. These changes are likely to depend on the increment in antibody synthesis in hyperimmunized animals.

Attention was paid to the study of the hematological and clinical alterations in horses as a consequence of venom injection. Only slight changes in hematocrit and hemoglobin were observed even after injections of 20-50 mg venom. In some horses there was a slight drop in these parameters, but no drastic changes were found at any time. These observations correlate with the absence of signs of systemic alterations, probably because these horses had been previously immunized with venoms.

Horses showed signs of local tissue damage at the site of venom injection. The most common findings were edema, abscesses, and fibrosis. Local tissue damage is due to the combined effect of hemorrhagic toxins, edema-forming toxins and myotoxins.[9,10].

Venoms of the three species used for immunization, i.e. *Bothrops asper, Crotalus durissus durissus* and *Lachesis muta*, induce myonecrosis, hemorrhage and edema[5,6]. The formation and extent of local abscesses has been drastically reduced at the Instituto Clodomiro Picado since the introduction of routine filtration of venom solutions prior to immunization. However, small abscesses were observed in these horses, probably due to the local tissue damage induced by venom. Fistules developed in five out of eight horses, and they were successfully treated with topical application of antiseptics.

Another element in the reduction of local lesion intensity in these horses has been the decrease in the amount of venom injected. In the past, booster doses were of 100 mg, whereas current injections are of 20 mg and, exceptionally, of 50 mg. The clinical consequences of this reduction have been evident. Laboratory and clinical observations taken together suggest that horses previously immunized, and injected with booster doses of 20 or 50 mg of venom, undergo only mild pathophysiological alterations. Similar observations were made by Estrada *et al.*[4] in horses being immunized for the first time to produce this antivenom.

Extensive production bleedings have effects on hematocrit and hemoglobin. In order to prevent anemia, sedimented erythrocytes from the previous bleeding are resuspended in saline solution and transfused to horses before the next bleeding. This procedure decreases the extent of hemodynamic alterations in extensive bleeding. In addition, the infusion of Ringer's lactate at the end of bleeding compensates the fluid and eletrolyte deficit caused by plasma depletion and prevents hemoconcentration. No signs of dehydration nor cardiovacular shock are observed in horses used for the production of polyvalent antivenom.

In the weeks that follow a production bleeding, there is a trend to increase hematocrit and hemoglobin, probably as a response to the acute blood loss. Usually, when horses are injected again with the next booster dose of venom, i.e. 40-50 days after bleeding, they have normal hematocrit and hemoglobin and are, therefore, prepared for the next production bleeding. Routine determinations of her-

TABLE 1
Immunization schedule used for the production of polyvalent antivenom in horses immunized for the first time.

Day nº	Venom injected (mg)*	Adjuvant
0	0.5	Complete Freund
10	1	Sodium alginate
20	1.5	Sodium alginate
30	3	Sodium alginate
40	5	Sodium alginate
50	10	Sodium alginate
60	15	Sodium alginate
70	30	Incomplete Freund
90	30	Sodium alginate
100	30	Sodium alginate

* Injections are made subcutaneously (see Materials and Methods).

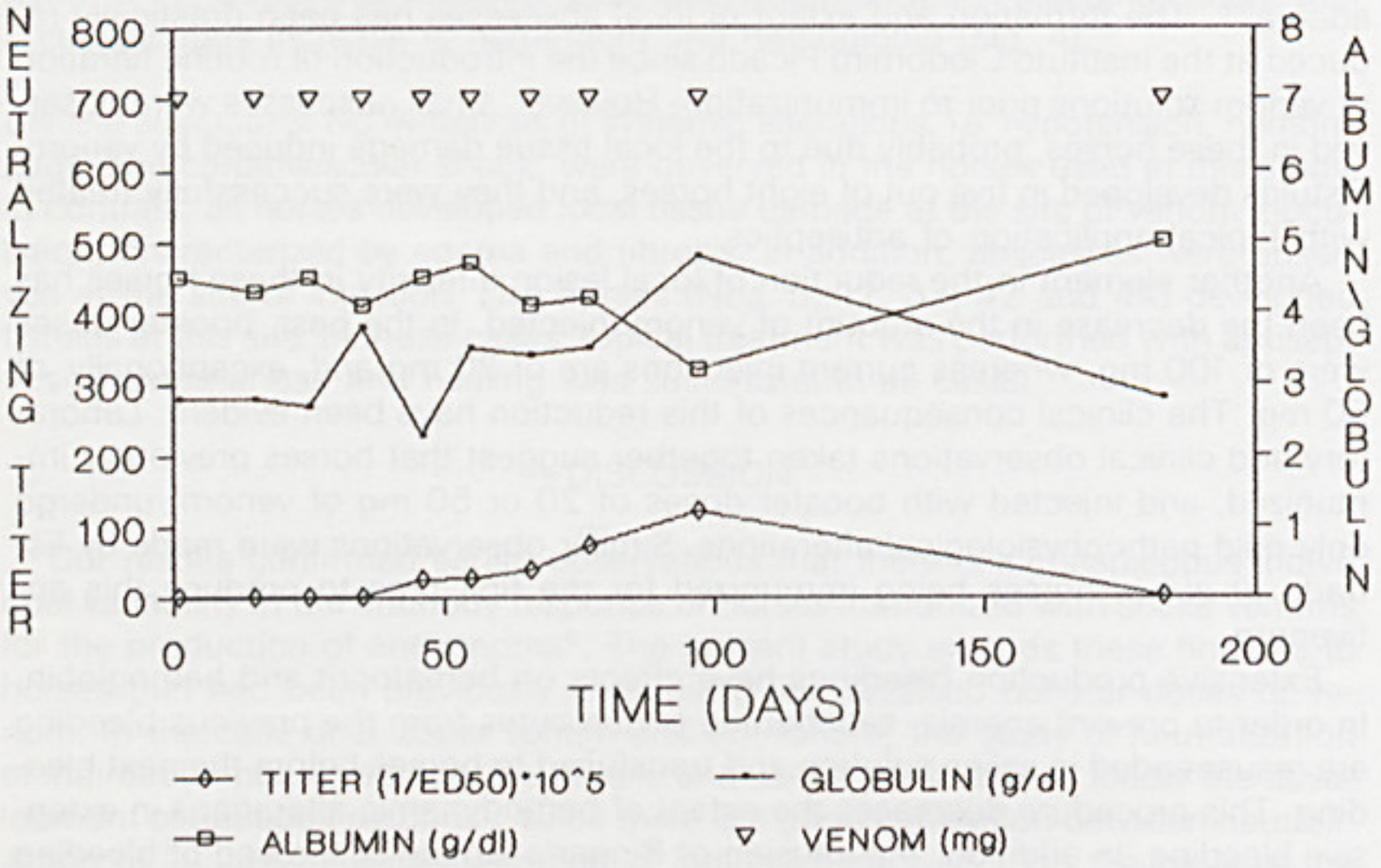

FIG. 1. Development of antibody response against indirect hemolytic activity of *B. asper* venom in horse nº 21, immunized for the first time. Changes in serum albumin and globulins are also shown. Neutralizing titer is expressed as $1/ED_{50} \times 10^5$ (see Materials and Methods). Triangles (▽) represent venom injections, according to the immunization protocol shown in Table 1.

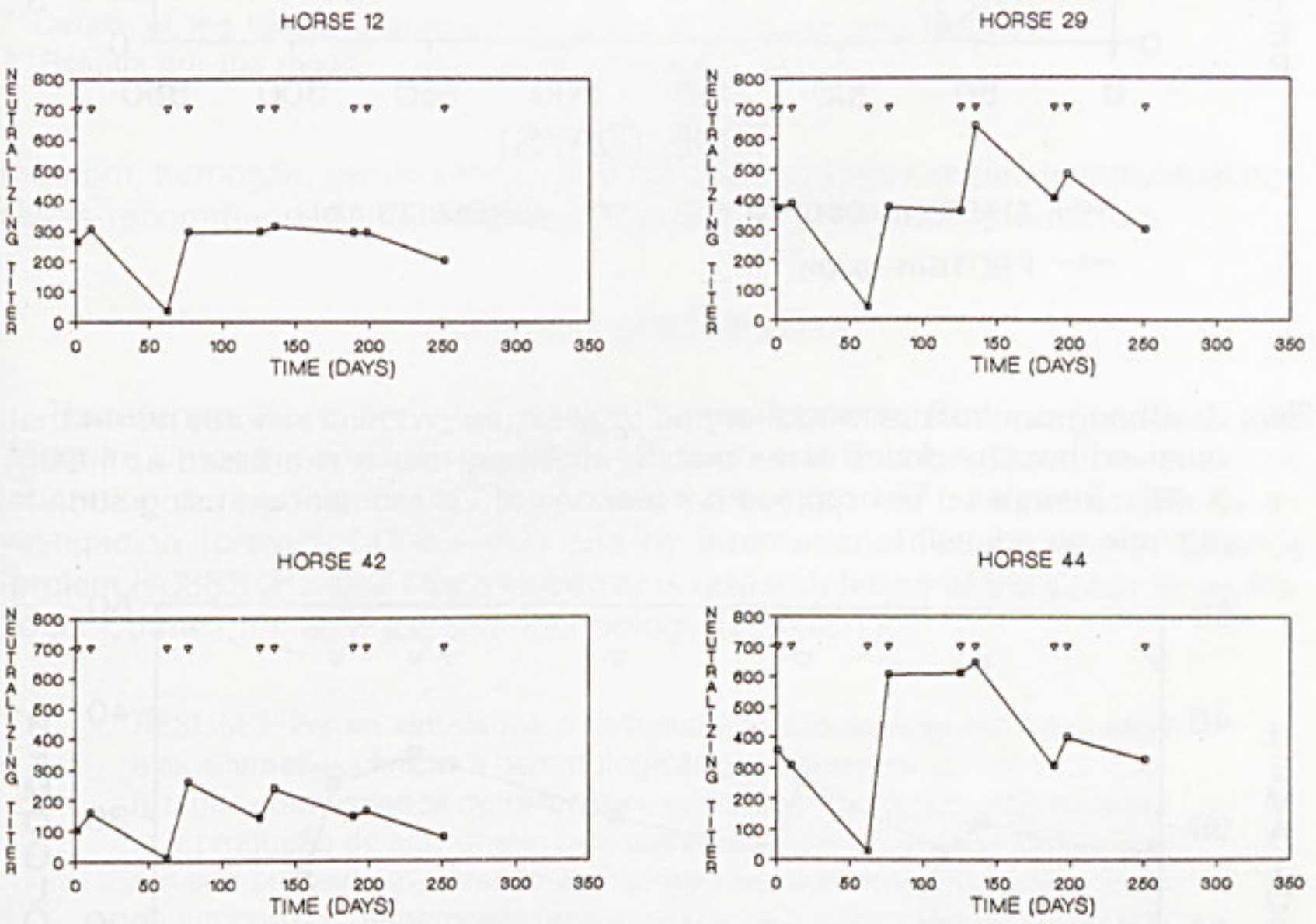

FIG. 2. Changes in neutralizing titer in four horses that had been previously immunized with snake venoms and received booster doses of venom. Neutralizing titer is expressed as $1/ED_{50} \times 10^5$. Triangles ($\bigtriangledown$) represent injections of 20 mg venom, using sodium alginate as adjuvant.

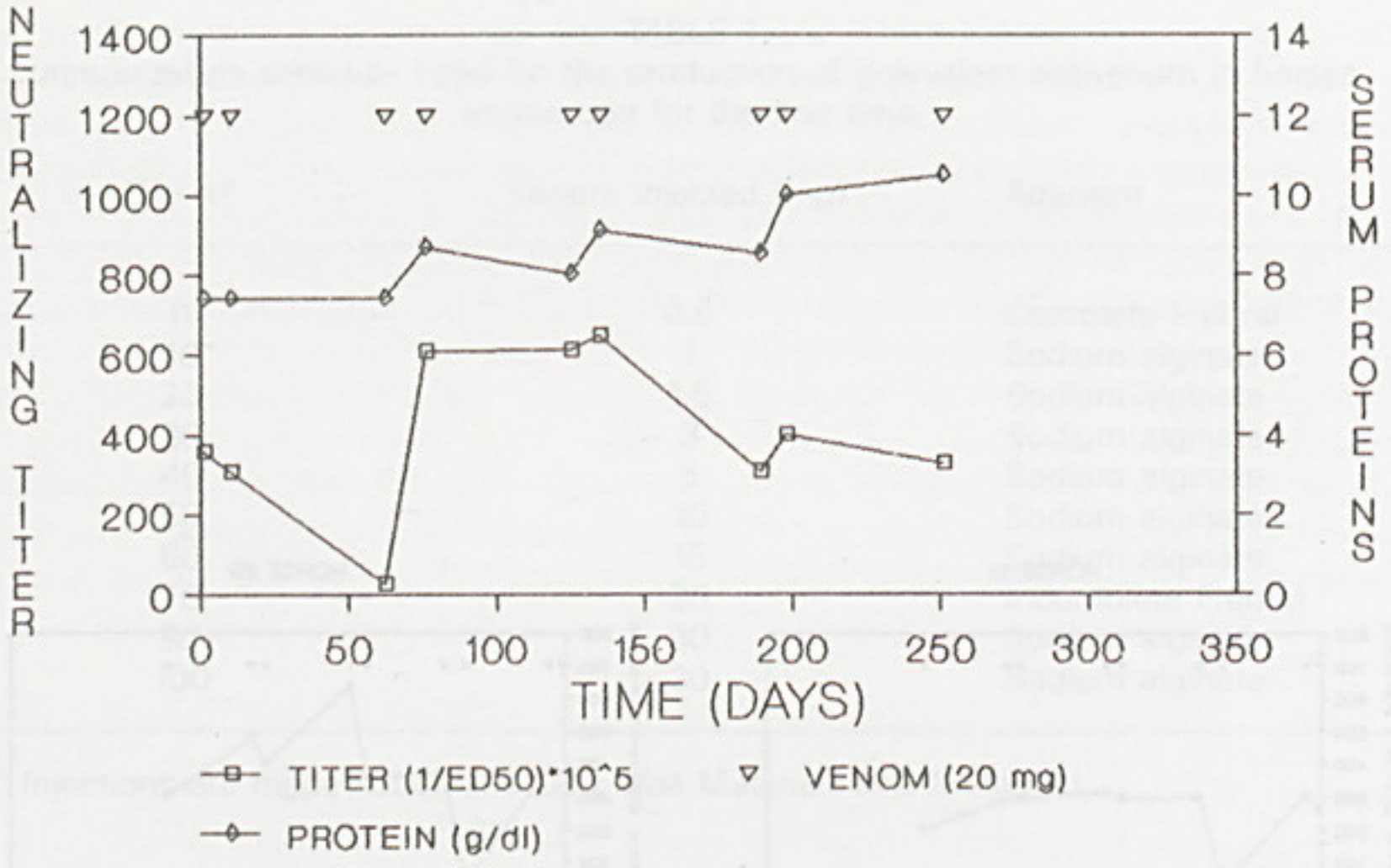

FIG. 3. Changes in neutralizing titer and total serum proteins in horse nº 44 that received booster doses of venom. Neutralizing titer is expressed as $1/ED_{50}$ X 10^5. Triangles (▽) represent injections of 20 mg venom, using sodium alginate as adjuvant.

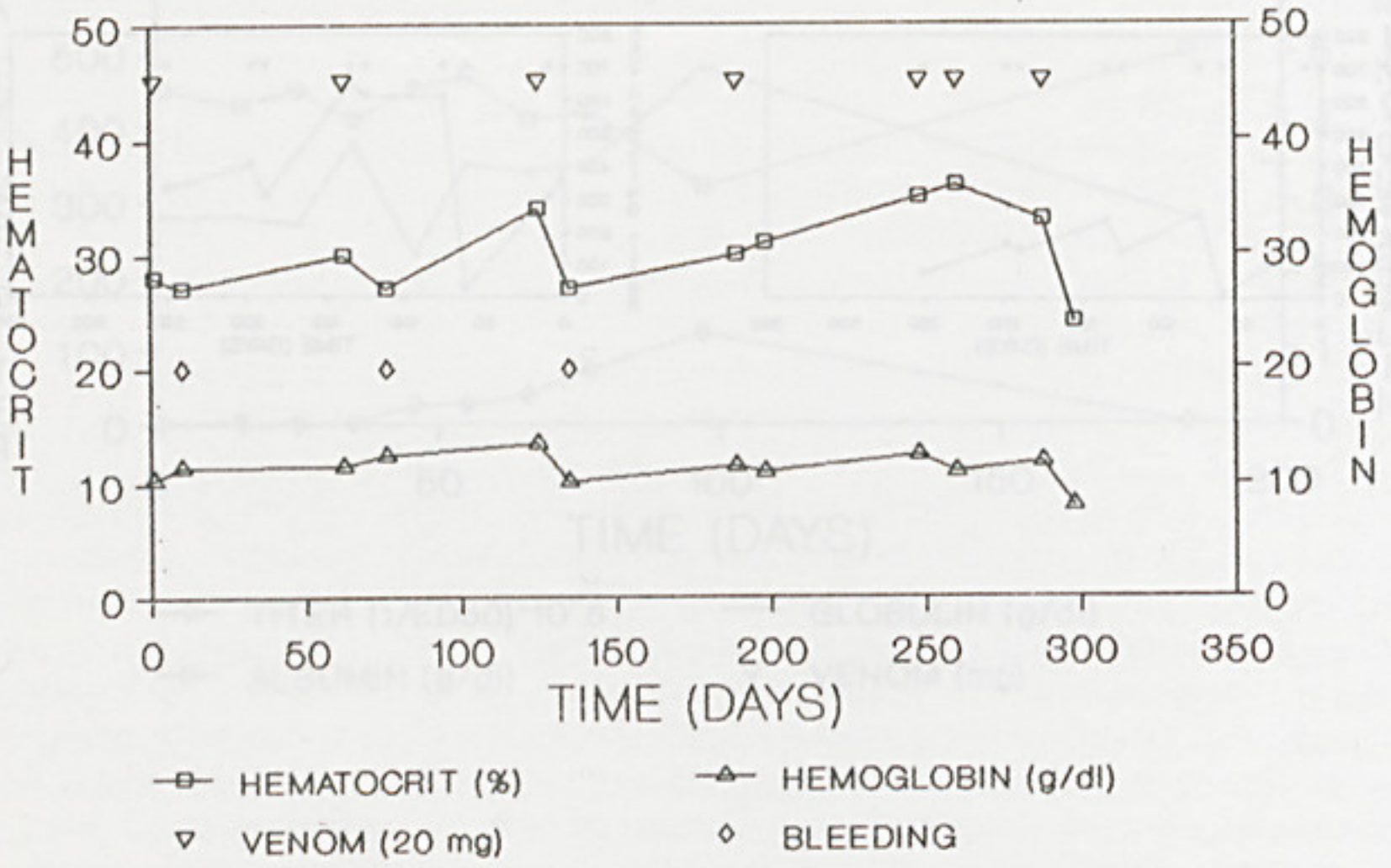

FIG. 4. Changes in hematocrit and hemoglobin in horse nº 37 that received booster doses of venom. Triangles (▽) represent injections of 20 mg of venom, using sodium alginate as adjuvant. Diamonds (◇) represent extensive production bleedings.

TABLE 2
Changes in hematocrit in horses during extensive production bleeding*

Day	Hematocrit (%)					
	Before bleeding	After bleeding of 4 liters	Before return of 4 liters	After return of 4 liters	After second bleeding of 4 liter	After Ringers lactate infusion
1	32±3.3**	31±3.0	—	—	31±3.4	—
2	29±3.4	26±1.7	—	30±1.4	31±2.9	—
3	28±3.1	27±2.1	—	30±1.7	—	—
4	—	—	33±2.9	30.2.9	—	25±3.4

* Details of the bleeding protocol are given in Materials and Methods.
**Results are the mean S.D. of four horses.

matocrit, hemoglin, serum protein and neutralizing ability of sera in individual horses is recommended in laboratories devoted to antivenom production.

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RESUMO: Foram estudados o desenvolvimento da resposta imunitária e as alterações clínicas e hematológicas que ocorreram em cavalos imunizados com venenos de serpentes, cujos plasmas foram utilizados para a produção de antiveneno polivalente, na Costa Rica. Um cavalo que em sua primeira imunização apresentou um aumento gradual do título de anticorpos, demonstrou uma inversão da proporcionalidade albumina/globulina.

As respostas em anticorpos neutralizantes observadas em cavalos que recebaram dose de reforço de veneno demonstraram marcante variabilidade individual. Em cavalos inoculados com doses de reforço de veneno foi verificado um aumento das proteinas séricas totais e ligeira redução no hemotócrito e da hemoglobina. No que diz respeito às alterações clínicas após a inoculação do veneno, não foram notados sinais de alterações sistêmicas (hipotensão, hemorragia, choque), mas todos os cavalos desenvolveram pequenas lesões locais no ponto de injeção do veneno, caracterizadas por edema, fibrose e abcessos. Estas lesões foram tratadas e cicatrizaram totalmente. Após extensas sangrias para produção houve uma queda ligeira no hematócrito, no entanto, nas semanas seguintes, ocorreram aumentos distintos tanto no hematócrito como da hemoglobina.

UNITERMOS: Veneno de serpentes, antiveneno, cavalos, alteração hematológica

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