Advanced Journal of Microbiology Research Vol. 2003
Available online at http://internationalscholarsjournals.org/journal/ajmr
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Full Length Research Paper

Efficacy of IgG, Fab, and F(ab')$_2$ fragments of horse antivenom in the treatment of local symptoms after *Cerastes cerastes* (Egyptian snake) bite

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Accepted 18 June 2003

The ability of horse antivenoms, consisting of immunoglobulin G (IgG) and its fragments F(ab')$_2$ and Fab were comparatively studied in mice to neutralize several effects of *Cerastes cerastes* venom. The three antivenoms were produced from the same batch of hyperimmune horse plasma. Neutralization was only partial when antivenins were administered intravenously at various time intervals after envenomation. No significant differences were observed among IgG, Fab, and F(ab')$_2$ antivenoms concerning neutralization of hemorrhagic effects. Fab fragments were slightly more effective in neutralizing edema while IgG and F(ab')$_2$ antivenoms were better in neutralizing myonecrosis in experiments involving independent injection of venom and antivenom.Thus these results disagree with the theory that "Fab " fragments are more effective than whole IgG and F(ab')$_2$ in the neutralization of local symptoms accompanying *C. Cerastes* venom.

Key words: *Cerastes cerastes*, Egyptian snake, IgG, F(ab')$_2$, Fab.

INTRODUCTION

Various local tissue alternations accompanying snakebite such as hemorrage, edema and myonecrosis, may result in tissue loss or organs dysfunction (Ownby, 1982; Gutierrez, 1995). These effects develop very rapidly after snake envenomation, making neutrlization by antivenoms very difficult, especially if serotherapy is delayed due to either late access to medical care or scarcity of antivenoms (Gutierrez et al., 1998).

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In the anti-sera production plant of VACSERA (Egyptian Organization for biological products and Vaccines) antivenoms have been produced by fractionation of hyperimmune horse plasma in order to obtain either immunoglobulin G (IgG) or F(ab')$_2$ fragments (Raw et al., 1991; Pope et al., 1939) which was modified by (Grechushkina-Sukhorukova et al., 1984) . It has been postulated that Fab fragments, obtained by papain digestion of immunoglobulins, may constitute a more convenient therapeutic tool (Dart and Horowitz, 1996). Various Fab antivenins have been produced (Laing et al., 1995; Landon and smith, 1996) and some have been evaluated in clinical trials (Karlson- Stiber et al., 1997; Meyer et al., 1997). Two theoretical advantages of Fab fragments are their wide volume of distribution, and their

ability to reach tissue compartment at a faster rate when compared to IgG and $F(ab')_2$ preparations (Covell et al., 1986; Scherrmann, 1994).

These two pharmacokinetic characteristics would be of value in the neutralization of locally-acting toxins, since Fab fragments might be able to reach and neutralize toxins present in the tissue more readily than whole IgG and $F(ab')_2$. The present study tested this hypothesis by comparing the ability of horse whole IgG, Fab and $F(ab')_2$ fragments antivenoms to neutralize local hemorrage, edema and myonecrosis induced by *C. Cerastes* venom in mice.

MATERIALS AND METHODS

Venom and hyperimmune plasma

The venom used in this work was obtained from *C. Cerastes* snakes collected in Helwan farm of the Egyptian Organization for biological products and Vaccines (VACSERA). Once obtained, the venom was lyophilized and stored at -20ºC until used. Plasma was collected from horses that had been immunized with the venom of *C. Cerastes* with increasing doses as discribed by Estrada et al. (1992).

Preparation of antivenoms

Fractionation of IgG: To obtain whole IgG, plasma was fractionated by caprylic acid precipitation according to (Rojas et al., 1994). Briefly caprylic acid is added drop wise directly to undiluted plasma whose pH has been adjusted to 5.8 by the addition of 1.76 N acetic acid. Caprylic acid was added up to a final concentration 5% (v/v) followed by vigorous stirring for one hour at room temperature before filtration. The mixture was filtered through Whattman filter K300 followed by EKS2 respectively. The filtrate was dialyzed for 48 hours against phosphate buffered saline (PBS) at pH 7.2 followed by ultrafilteration to remove caprylic acid. Afterwards, NaCl and tricresole were added to a final concentration of 0.15M and 0.35%, respectively. The preparation was sterilized by filteration through 0.22-µm membranes.

Purification of $F(ab')_2$ fragments: $F(ab')_2$ fragments were prepared by pepsin digestion after adjusting the pH of plasma to 3.3 by 1.76N acetic acid followed by the addition of 3.5g pepsin/liter plasma. Digestion was performed at 22- 25ºC for 1 hour, and the pH was elevated to 3.6 using 1N NaOH for 30 min. Afterward plasma pH was readjusted to 5.8 and the mixture was incubated for 15 min. at 56ºC followed by centrifugation for 10 min. at 900 x g to remove fibrinogen. Caprylic acid was added dropwise to the undiluted plasma to attain a final concentration of 5% (v/v). The mixture was stirred vigorously for 24 h, followed by the same extraction and purification steps as previously stated for IgG preparation (Dos Santos et al., 1989)

Purification of Fab fragments: Fab antivenom was obtained by adjusting the pH of plasma to 7 followed by addition of 20 g/l papain and 1g/l cysteine. Digestion was performed at 22-25ºC for 2 hours, and caprylic acid was added to attain a final concentration of 5% (v/v). The mixture was vigorously stirred during 24 h, and then filtered. The filtrate was dialyzed against PBS and sterilized by filtration through 0.22 µm membrane, after the addition of 0.15 M NaCl and 0.35 % tricresol as described by Leon et al. (2000). The three-antivenin preparations were adjusted to have the same neutralizing potency (75 U/ml) against *C. cerastes* to ensure that if variations arise in experiments with independent injection of venom and antivenoms, they would be due to the pharmacokinetic profile of the products and not to differences in their ED50. It was demonstrated in a previous preliminary studies that ED50 (75 U/ml) could completely abolish these previously mentioned symptoms after snakebite in experiments in which venom and antivenoms were incubated prior to injection (VACSERA research).

Neutralization studies

Neutralization of hemorrhage: In experiments with independent injection of venom and antivenoms, groups of four swiss albino mice (20-22g) were initially injected intradermally with 0.1ml PBS containing 25 µg of venom/mouse in the abdominal region. At various time intervals (0, 10, 20 and 30 min), 0.2 ml of each antivenom was administered intramuscularly in the right gastrocnemius. Hemorrhage was assessed in the skin 2 h after venom injection. The mice were sacrificed, their skin removed and the hemorrhage area measured. Control mice were injected with the same amount of venom without antivenom as described by Gutierrez et al. (1985).

Neutralization of edema: Groups of four mice (20-22 g) were injected in the right foot pad with 10 µg venom dissolved in 50 µl of PBS, whereas the left foot pad received 50 µl of PBS alone. Then 0.2 ml of either native IgG, Fab or $F(ab')_2$ fragments were administered intravenously immediately or after 15, and 30 min). Control mice received 10 µg venom only. Edema was assessed one hour after envenomation. The mice were sacrified by cervical dislocation, and their feet were cut and weighed. Edema was expressed as percentage of increment in the weight of the right foot as compared to the left one (Gutierrez et al., 1986).

Neutralization of myotoxicity: The method of Gutierrez et al. (1981) was followed. Groups of four mice were injected intramuscularly with 25 µg venom in the right gastrocnemius at different time intervals (0, 20 and 40 min.), and 0.2 ml of either IgG or Fab and $F(ab')_2$ antivenoms were administered intravenously. Control mice were injected with venom alone. Plasma creatine kinase activity was assessed 3 h after venom injection. Blood samples were collected from the tail into heparinized tubes, and plasma creatine kinase activity was determined by the Sigma kit No. 520. Creatine kinase activity was expressed as units/ml, one unit resulting in the phosphorylation of one nanomole of creatine/min at 25ºC.

RESULTS

Neutralization of haemorrhagic activity

When antivenoms were administrated intramuscularly at different time intervals after venom injection, only partial neutralization was achieved. Figure 1 shows that Fab give the better neutralizing activity than $F(ab')_2$ and IgG. All the three antivenoms gives better results when administered immediately after envenomation.

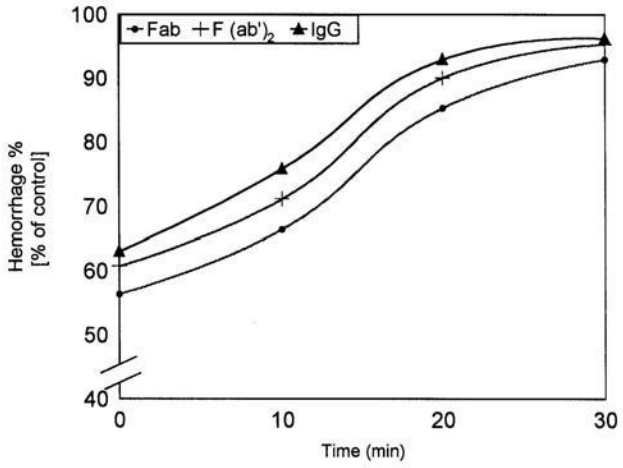

Figure 1. Neutralization of hemorrhagic effect induced by *C. cerates* venom after administration of Fab, F(ab')2 and IgG antivenoms in experiments involving independent injection of venom and antivenom. Control mice were injected with venom alone having as much as 100% hemorrhagic effect.

Neutralization of edema

Fab salso gave the better results in neutralizing edema in experiment where antivenoms were administered intravenously after envenomation. While F(ab')$_2$ and IgG showed similar neutralizing activity (Figure 2).

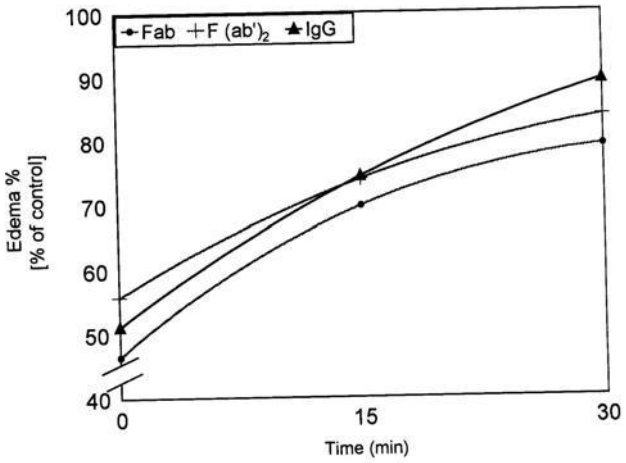

Figure 2. Neutralization of edema induced by *C. cerates* venom after administration of Fab, F(ab')2 and IgG antivenoms in experiments with independent injection of venom and antivenom. Edema is expressed in percentage, with as much as 100% effect induced by venom alone. Edema was assessed 1 h after venom injection.

Neutralization of myonecrosis

Mice injected with venom alone had plasma creatine kinase activity of 920±37.7 units/ml, whereas activity of plasma from mice receiving PBS alone was 64.4±4.3 units/ml. When antivenoms were injected after venom, neutralization of myotoxicity was only partial even in conditions where immunotherapy was performed immediately after envenomation (Figure 3). IgG and F(ab') $_2$ antivenoms showed better neutralizing ability at all times tested than Fab.

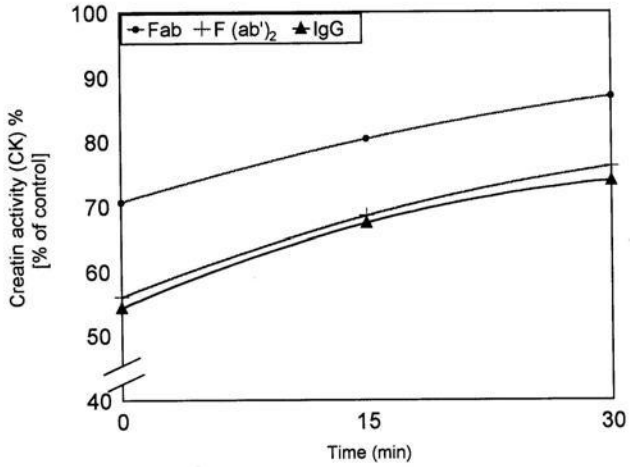

Figure 3. Neutralization of myonecrosis induced by *C. cerates* venom after administration of Fab, F(ab')2 and IgG antivenoms in experiments with independent injection of venom and antivenom. Myonecrosis was assessed 3 h after venom injection by quantitating plasma creatine kinase (CK) activity. Myonecrosis is expressed in percentage, with as much as 100% effect induced by venom alone.

DISCUSSION

Despite the recent marked improvement in the field of immunoglobulins structure and function, the resulting knowledge has not been fully applied to antitoxin or antivenin production (Morais et al., 1994). However antivenoms are considered to be the mainstay in the treatment of snakebite envenomation (Dart and Horowitz, 1996) because of their efficacy in neutralizing systemically acting toxins. Nevertheless, clinical evidence indicates that they are only partially effective in neutralizing venom induced local tissue damage (Gutierrez et al., 1998). This was the motivation for carrying out the present study.

IgG and its fragments F(ab')$_2$ and Fab were produced and purified using caprylic acid method, which eliminate irrelevant plasma proteins such as albumin and fibrinogen. Such purified preparation is devoid of undesirable effects, like anaphylactic, attacks induced by the eliminated irrelevant proteins (Morais et al., 1994).

For proper comparison among the antivenoms, their doses were standardized to have the same neutralizing potency against venom when incubated together before injection. It can therefore be assumed that any variations in neutralization capability would then be due to differences in the pharmacokinetics profiles of the preparations. In the present study, the time factor (time of injecting the antivenom) was very critical. Although the neutralizing capacity at all time points was only partial, the sooner the treatment was applied, the more pronounced was the neutralizing effect for all parameters tested. With longer delays in treating the victim with antivenin, the values of the tested parameters quickly approached the untreated control values. The cause of the partial ineffectiveness of antivenoms is probably related to rapid development of local effect after envenomation, therby precluding neutralization (Gutierrez et al., 1985) or probably due to relatively inadequate antivenom dose, but not the absence of neutralizing antibodies in the antivenom.

Although Fab did not show good activity to counteract myonecrosis produced by *C. cerastes* venom, as measured by release of creatine kinase into the blood, it ranked first among the three tested preparations in the other two parameters; namely hemorrhage and edema formation. This is probably because Fab is less immunogenic compared to IgG and $F(ab')_2$. Moreover, Fab has a relatively more favorable pharmacokinetic profile with its lower molecular weight and larger volume of distribution, together with its ability to reach the tissue compartment at a faster rate. This, unfortunately, is coupled with rapid renal clearance, which may decrease the tissue concentration of Fab. Such unfavorable property may be offset by the presumption that Fab molecules may have greater association with cellular material (Scherrmann, 1994; Riviere, 1997). On the other hand, IgG and $F(ab')_2$ with their higher molecular weights and shorter diffusion rates may be more localized in the tissues where toxins are present . The differences in diffusion rates may be more localized in the tissues where toxins are present. Therefore, the differences in diffusion into blood and tissue compartments may explain the difference in action on hemorrhage and myonecrosis. It should not be expected, however, that Fab or any of the other antivenom fractions, can completely counter act the hemorrhagic activity of the venom. Perez et al. (1984) claimed that hemorrhagic factors have several antigenic determinants and that it is unlikely that a single antibody could neutralize the hemorrhagic completely.

The rapid decrease in the ability of the antivenom fractions to neutralize toxins with time should be seriously considered. This may be due to the extremely rapid sequence of toxic events and the development of local tissue damage following envenomation (Gutrierrez et al., 1980; Moreira et al., 1992; Lomonte et al., 1994; Chaves et al., 1995). An important factor is the time lapse between envenomation and serotherapy. The speed with which the antivenom binding in the circulation and redistribution of the toxins from the tissues into circulation are important factors (Choumet et al., 1996; Riviere et al., 1997). A proper study of toxin and antivenom kinetics in the victim is therefore mandatory.

It has been reported (Morais et al., 1994) that when IgG or $F(ab')_2$ is used for the treatment of envenomation, they can equally induce anaphylactic shock through complement activation by classical and alternative pathways. Fab, however, does not induce anaphylaxis because it has only one binding site and does not cross-link to form immune complexes (Morais et al., 1994). This gives an advantage to Fab preparations, which may be administered in consecutive multiple doses to compensate for its rapid renal clearance because of its relatively low molecular weight. The relatively low toxicity accompanied with the use of Fab points to its possible successful use at higher dose levels without deleterious effects. The search should also be directed toward identifying an antivenin preparation that can better neutralize locally acting toxins as well.

Finally, we assert that neutralization of toxin in tissue is negligible in the studies involving separate injection of venom and antivenom at different intervals of time. This is may be due to the extremely rapid development of local tissue damage, edema, myonecrosis and hemorrhage within minutes after injection of venom (Gutierrez et al., 1980, 1984; Lomonte et al., 1994; Chaves et al., 1995). Furthermore, there is always a time lapse between envenomation and serotherapy. Thus, the most important mechanism is the antibody toxins binding in the circulation, with consequent redistribution of the toxins from the tissue to the circulation (Choumet et al., 1996; Riviere et al., 1997).

In conclusion when IgG or $F(ab')_2$ is used in the treatment of envenomation there was no differences in neutralization. Both can equally induce anaphylactic shock through complement activation by classical or alternative pathway (Morias et al., 1994). Fab preparation can be administered with multiple doses to compensate for its rapid clearance through the kidney due to its low molecular weight. And since Fab has only one binding site and do not cross link to form immune complexes, its low toxicity makes it possible for its use at higher concentrations than the other products tested, without deleterious effects. Thus the production of antivenom that includes IgG and its fragments, $F(ab')_2$ and Fab, is necessary to treat the local symptoms. Trials are underway to determine the addition of natural substances to the immune-sera to treat snakebite including neutralization of the local-acting toxins.

ACKNOWLEDGMENT

The authors are grateful to the chairman of VACSERA. Prof.Dr.Mohamed El -Abbadi for his generosity and kindness in supporting this work.

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