The detection and isolation of a paralysis toxin present in *Argas (Persicargas) walkerae*

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**ABSTRACT**


One-day-old leghorn chickens were used in a laboratory assay to determine the toxicity of crude extracts of the tick *Argas (Persicargas) walkerae* and of fractions obtained during the isolation procedure. Extracts of unfed and engorged larvae, nymphae and females were tested using this in vivo test procedure. Only extracts of replete *A. (P.) walkerae* larvae produced paralysis.

A toxic fraction was isolated from replete larval extracts by gel-permeation and ion-exchange chromatography. This fraction with a pI of 4.5, showed 2 major bands corresponding to a M of 32 kDa and 60 kDa after SDS-polyacrylamide gel electrophoresis.

**INTRODUCTION**

*Argas (Persicargas) walkerae* causes paralysis in poultry during infestation. This tick, also called Walker's fowl tampan, is the commonest fowl tick in the Republic of South Africa (Howell, Walker & Nevill, 1978). It has been recorded in the Transvaal (Gothe & Schrecke, 1972; Gothe & Koop, 1974), the Cape Province at Fort Beaufort and Queenstown, Lesocho and Namibia (Kaiser & Hoogstraal, 1969), in Zimbabwe (Novac, Short & Chrisholm, 1985) and in Zambia (Colbo, 1973).

Larvae of *A. (P.) arboresus* (Gothe & Verhalen, 1975) and of *A. (P.) radiatus* (Gothe & Englert, 1978) have a greater paralysis-inducing capacity than those of *A. (P.) walkerae*, whereas the larval toxicity of *A. (P.) persicus* (Gothe & Verhalen, 1975; Gothe & Englert, 1978; Gothe, Koop & Schrecke, 1981), *A. (P.) sanczezi* (Gothe & Englert, 1978) and *A. (A.) africolumbae* is less pronounced.

Gulyás (1952) attempted to explain the pathogenesis of the paralysis by an anaemic state caused by blood-loss during infestation by the ticks. He excluded the possibility that the symptoms were due to a toxic effect. Gothe, Kunze & Alt (1970), however, showed that no haematological changes occurred during the course of the paralysis, even in very extensive cases which terminated in death. The paralysis could rather be defined as a toxicosis (Gothe, 1971a) which showed the typical signs of a generalized affection of the peripheral nervous system (Gothe, Kunze & Hoogstraal, 1979). Partial immunity was also produced in some hosts after repeated infestations (Gothe, 1971b).

From the earlier literature, conflicting opinions existed as to which stage or stages of the tick are capable of causing paralysis during feeding. According to Lounsbury (1904), nymphae as well as adult ticks are capable of causing paralysis. Emmel (1945) and Neitz (1962) were of the opinion that all post-embryonatal stages may cause this toxicosis, whereas Coles (1959), quoted by Stampa, S. 1959, reported that paralysis is caused by the simultaneous feeding of both larvae and adults. The confusion was eventually unravelled through intensive studies by Gothe et al. (1970). They unambiguously determined that only larvae caused paralysis after at least 4 days of feeding. Of interest was the finding that the larval body mass increased abruptly from approximately the 4th feeding day. It was concluded that the onset of symptoms may be correlated with an increased rate of feeding. The interval between infestation and the onset of paralysis (at least 4 days) was found to be independent of the age of the host and of the number of ticks infesting it. The degree of paralysis, however, was dependent on these factors.

The inability of post larval ticks to cause paralysis during infestation is well established (Gothe et al., 1970). It is not known, however, if this is due to the absence of toxin production or to an inability of these ticks to transfer the toxin to the host during their short feeding time. Larval ticks of this species imbibe blood during several days, whereas all the other stages are fully engorged within 1-2 h (Howell et al., 1978).

To help solve this enigma, the paralysis-inducing capability of all post-larval stages of *A. (P.) walkerae* was investigated in the present study by inoculation of crude extracts into chickens. In addition, an attempt was made to isolate the implicated toxin from replete whole tick extracts.

**MATERIALS AND METHODS**

All glassware and equipment were sterilized with 70% (v/v) ethanol and buffers by filtration through 0.22 μm filters (Millipore).

**Origin and rearing of ticks**

Laboratory colonies of *A. (P.) walkerae* (Pretoria strain) were maintained and reared in an incubator at 27 °C and 80% relative humidity. Three weeks after the hatching of eggs, the batch of larvae from 1 female was allowed to attach freely under the wings of white Leghorn chicken (approximately 7-week-old) as described by Alt (1971). The replete larvae were collected on the morning of the 5th, 6th, 7th and 8th day after attachment. To obtain replete nymphae and the unfed nymphae and adults were fed overnight on 7-month-old white Leghorn hens, using approximately 100 ticks per animal. On the morning following the attachment, replete nymphae were collected. The same procedure was followed for feeding the subsequent stages.

**Preparation of crude extracts from eggs, larvae, nymphae and adults for the detection of the paralysis toxin**

*A. (P.) walkerae* eggs and fed as well as unfed larvae, nymphae and adult ticks were homogenized

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in distilled water with an Ultra Turrax at low speed. Eggs and larvae were homogenized for 2 min and the other stages for 5 min at 4°C. The homogenates were then sonified for 15 s with a Branson Model B-30 sonifier with the output set at 5 (continuous cycles). The sonified homogenates were centrifuged in a Beckman Model B microfuge for 10 min at 8,000 × g and 4°C. The resulting supernatants were freeze-dried and suspended in 0.154 M NaCl before use. Aliquots were injected in the neck of one-day-old white Leghorn chickens (Table 1). At least 3 animals were used per assay and were kept under observation for at least 4 days. The degree of paralysis was evaluated according to the description provided by Goethe & Englert (1978) (See Table 2).

Preparation of crude larval extracts

Preliminary studies showed no differences in toxicity when extracts were prepared in the presence of either distilled water or 0.154 M NaCl.

For the preparation of a crude extract, 100 replete larvae were homogenized in 1 ml of solvent. The supernatants were made up to 12 ml with solvent and centrifuged for 5 h at 80,000 g at 5°C. The supernatants were freeze-dried. Salt containing samples were made up to 2.5 ml with distilled water, desalted on a Sephadex PD-10 column, and freeze-dried.

Gel-permeation chromatography

Sephadex G-100 gel was packed into a K26/40 column. A 2 ml crude larval extract, of 200 larvae in 3 ml of 0.154 M NaCl, was applied to the column and separated, using 0.154 M NaCl (pH 7.0) or H2O (pH 7.0) as eluant by upward-flow elution at a flowrate of 0.1 ml/min at 30°C. Fractions showing clinical symptoms of paralysis in one-day-old chickens (Tables 1 and 2); extracts prepared from 200 unfed larvae failed to show paralysis symptoms.

Further investigations of the toxicity of crude larval extracts prepared from 3 larvae caused a F1 paresis, whereas extracts from 30 caused a total paralysis (Table 3). All the paralyzed animals recovered within 48 h.

No difference in toxicity between the crude larval extracts in either distilled water or 0.154 M NaCl was observed. The addition of detergent at room temperature, whether Tween 20, SDS or Chaps, had no effect on the toxicity. Heating in the presence or absence of detergent, however, resulted in total loss in toxicity.

Gel-permeation chromatography

Elution of the crude A. (P.) walkerae larval extract on the Sephadex-G-100 column did not separate the toxic component from the haemoglobin fraction when using H2O (pH 7.0) (fraction SN1 in Fig. 1 or 0.154 M NaCl pH 7.00), with either 0.05 % Tween 20, 0.1 % SDS or 0.1 % Chaps, as eluting buffer (fraction SND, in Fig. 2).
TABLE 1 Determination of the presence of paralysis toxin in crude extracts from various stages of Argas (Persicargas) walkerae

<table>
<thead>
<tr>
<th>Developmental stage</th>
<th>Number used</th>
<th>Mean ± SD quantity of protein injected (mg/chicken)</th>
<th>Clinical symptoms$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly laid eggs</td>
<td>2000</td>
<td>100.3 ± 12.8</td>
<td>N</td>
</tr>
<tr>
<td>Unfed larvae$^1$</td>
<td>2000</td>
<td>87.7 ± 3.2</td>
<td>N</td>
</tr>
<tr>
<td>Unfed larvae$^2$</td>
<td>2000</td>
<td>82.4 ± 8.1</td>
<td>N</td>
</tr>
<tr>
<td>Replete larvae$^3$</td>
<td>300</td>
<td>32.3 ± 3.1</td>
<td>VP, N</td>
</tr>
<tr>
<td>Unfed nymphae stage 1$^4$</td>
<td>2000</td>
<td>217.4 ± 17.4</td>
<td>N</td>
</tr>
<tr>
<td>Replete nymphae stage 1$^4$</td>
<td>400</td>
<td>123.1 ± 9.7</td>
<td>N</td>
</tr>
<tr>
<td>Replete nymphae stage 2$^4$</td>
<td>400</td>
<td>90.7 ± 10.5</td>
<td>N</td>
</tr>
<tr>
<td>Unfed nymphae stage 2$^5$</td>
<td>200</td>
<td>97.3 ± 14.9</td>
<td>N</td>
</tr>
<tr>
<td>Replete nymphae stage 3$^5$</td>
<td>200</td>
<td>82.6 ± 11.3</td>
<td>N</td>
</tr>
<tr>
<td>Replete nymphae stage 3$^5$</td>
<td>300</td>
<td>101.3 ± 18.4</td>
<td>N</td>
</tr>
<tr>
<td>Replete adult male ticks$^6$</td>
<td>200</td>
<td>89.9 ± 17.6</td>
<td>N</td>
</tr>
<tr>
<td>Replete adult female ticks$^7$</td>
<td>200</td>
<td>203.1 ± 23.7</td>
<td>N</td>
</tr>
</tbody>
</table>

$^1$ Extracts prepared 3 days after hatching
$^2$ Extracts prepared 2 weeks after hatching
$^3$ Extracts prepared immediately after collection
$^4$ Extracts prepared 2 weeks after moulting
$^5$ Further investigations reported in Table 3
$^6$ See Table 2
$^7$ Result for at least 3 animals

TABLE 2 Description of the clinical symptoms in chickens from inoculation of crude extracts of Argas (Persicargas) walkerae (Gothe & Englert, 1978)

<table>
<thead>
<tr>
<th>Paralysis</th>
<th>Clinical symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>No symptoms</td>
</tr>
<tr>
<td>Wings</td>
<td>Slight degree of paresis</td>
</tr>
<tr>
<td>F I</td>
<td>Medium degree of paresis</td>
</tr>
<tr>
<td>F II</td>
<td>High degree of paresis</td>
</tr>
<tr>
<td>Legs</td>
<td>Slight degree of paresis</td>
</tr>
<tr>
<td>B I</td>
<td>Medium degree of paresis</td>
</tr>
<tr>
<td>B II</td>
<td>High degree of paresis</td>
</tr>
<tr>
<td>B III</td>
<td>Total paralysis</td>
</tr>
<tr>
<td>VP</td>
<td>Total paralysis ending in death</td>
</tr>
</tbody>
</table>

TABLE 3 Toxicity determinations of crude extracts prepared from replete Argas (Persicargas) walkerae larvae

<table>
<thead>
<tr>
<th>Number inoculated per chicken$^1$</th>
<th>Mean ± SD inoculated (mg/chicken)</th>
<th>Clinical symptoms$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 larvae</td>
<td>0.32 ± 0.05</td>
<td>F I</td>
</tr>
<tr>
<td>5 larvae</td>
<td>0.51 ± 0.07</td>
<td>F II</td>
</tr>
<tr>
<td>10 larvae</td>
<td>0.94 ± 0.06</td>
<td>F III/B I</td>
</tr>
<tr>
<td>15 larvae</td>
<td>1.39 ± 0.11</td>
<td>B I/B II</td>
</tr>
<tr>
<td>20 larvae</td>
<td>1.97 ± 0.15</td>
<td>B II</td>
</tr>
<tr>
<td>25 larvae</td>
<td>2.53 ± 0.13</td>
<td>B III</td>
</tr>
<tr>
<td>50 larvae</td>
<td>5.04 ± 0.17</td>
<td>VP</td>
</tr>
</tbody>
</table>

$^1$ At least 3 animals tested
$^2$ See Table 2

In all the cases, virtually 100 % of the protein as well as the toxicity was recovered.

The Sephacryl S-200 column gave reasonable resolution when the column was eluted with distilled water (pH 4.00) (Fig. 3). A colourless peak (SH₂) was obtained which represented 30 % of the original protein and 40 % of the original toxicity. This peak eluted at a volume corresponding to a molecular mass of 80 to 100 kDa. A red fraction (SH₄) adsorbed onto the column which could be eluted with 0.154 M NaCl. This fraction represented approximately 65 % of the sample protein and 70 % of the sample toxicity.
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By means of the batch Sephacryl S-200 method, most of the haemoglobin could be separated from the sample. This toxic fraction (equivalent to SH$_2$) represented approximately 50% of the original toxicity.

Chromatofocusing

This method gave good recoveries with respect to protein and toxicity (above 85%). Two toxic fractions (C$_1$ and C$_3$), with pI of approximately 7.6 and 8.3, each associated with haemoglobin, were observed (Fig. 4).

Ion-exchange chromatography

DEAE-Sephacryl chromatography of the toxic filtrate obtained from the Sephacryl S-200 batch method resulted in 2 major peaks (Fig. 5), the 2nd of which showed high toxicity.

SDS-PAGE of this fraction revealed 2 bands with $M_r$ of 32 kDa and 60 kDa. A faint band with $M_r$ of 18 kDa was present when a high sample concentration was used. One band with a pI of 4.5 was visible after IEF. At high sample concentrations, additional faint bands with higher pI were visible.

DISCUSSION

One-day-old leghorn chickens proved to be suitable for determining the toxicity of crude A. (P.) walkerae tick extracts and fractions obtained during the isolation procedures. With the use of this in vivo test system, it was found that only replete A. (P.) walkerae larval crude extracts produced paralysis. Unfed larvae as well as all the other stages, either fed or unfed, showed no overt effect when inoculated at high dosage rates into one-day-old chickens.

These observations are in agreement with those of Gothe (1971a) and Gothe & Verhalen (1975) regarding the paralysis-inducing capabilities and the infestation rate of larval, nymphal and adult ticks during feeding. They showed that only larvae, after at least 4 days of infestation, caused paralysis, the intensity of which was directly proportional to the rate of infestation.

The present results also substantiate the finding of Gothe (1971a) that the paralysis is due to a toxin and does not develop as a result of blood loss in the host caused by feeding ticks, as suggested by Gulyas (1952). Furthermore, it has been reported that the timely removal of ticks from paralyzed animals leads to a subsidence of symptoms and complete recovery (Gothe et al., 1979). Likewise, with the toxicity tests of crude larval extracts, it was observed on many occasions that animals, paralysed by a single inoculation of a sub-optimal paralysis-inducing dosage, recovered within 1–2 days. It is evident that the noxious substance responsible for the paralysis is not cell-bound, but circulates humorally. The toxin possesses membranophilic properties, but its somatic linkage is labile (Kunze & Gothe, 1978).

The combined results of the present investigations and those of Gothe et al. (1970) thus show that toxin...
production and transfer occur only after several days of feeding by larval ticks only. Post-larval stages do not produce toxin in amounts detectable by the present toxicity assays. The correlation between feeding behaviour and the development of pathophysiological syndromes has also been observed in other tick species that induce paralysis (Gothe, 1984).

It is clear that the major obstacle encountered during the isolation of the toxin is the presence of extremely high haemoglobin concentrations in the extracts prepared from fed larvae. Excision of salivary glands from hundreds of larvae is impracticable because of their small size. Thus the use of salivary glands to circumvent this problem was not possible.

Gel-permeation chromatography of crude larval extracts showed that elution in the presence of saline resulted in a toxic fraction which eluted at the void volume of Sephadex G-100 and Sephacryl S-200 columns. This fraction was reddish to brown in colour. Recovery of protein and toxicity were in excess of 90%. The inclusion of 0.05% Tween 20, 0.1% SDS or 0.1% Chaps to the sample and eluent had no effect on the elution pattern or on protein and toxicity recoveries. These results indicate the presence of large complexes between toxin and haemoglobin.

Elution of the Sephacryl S-200 column with distilled water (pH 4.00) resulted in the recovery of a colourless toxic fraction representing 40% of the original toxicity at a volume corresponding to a Mr of 80-100 kDa. Under these conditions, the haemoglobin is adsorbed onto the column.

Elution of this fraction was achieved with 0.154 M NaCl. It contained approximately 70% of the original toxicity. These results indicate the possibility that 2 toxins were present. More likely, however, is that the toxin is associated with haemoglobin through hydrophobic interaction. At low pH and low ionic strength, the toxin is partially liberated from a haemoglobin-toxin complex, with the result that some free toxin is present. The haemoglobin-toxin is adsorbed on the column, while the free toxin is eluted at a volume according to its molecular size and conformation.

Chromatofocusing was also tested as a means of toxin isolation starting with the crude larval extracts. Good resolution of peaks was obtained as well as good correlation between feeding by larval ticks only. Post-larval stages do not original toxicity at a volume corresponding to a Mr of 80-100 kDa. Under these conditions, the haemoglobin is adsorbed onto the column.

F.M. 20, 0,1% SDS or 0.1% Chaps to the sample and eluent had no effect on the elution pattern or on protein and toxicity recoveries. These results indicate the presence of large complexes between toxin and haemoglobin.

SDS-PAGE showed that the toxic fraction obtained after ion-exchange chromatography consists of 2 bands with Mr of 32 and 60 kDa. This result indicates that the toxin may exist as an oligomer, since gel-permeation chromatography revealed a Mr of 80-100 kDa. The Mr of 60 kDa is similar to that of the paralysis inducing toxins isolated from *Ixodes holocyclus* (Stone, Double, Binnington & Goolder, 1979) and from *Rhipicephalus evertsi evertsi* (Vlijoen, Beuzuidenbou, Oberem, Vermeulen, Visser, GoTHE & Neitz, 1986). The toxin from all 3 tick species have a pI in the acid range.

Although a homogeneous toxin was not obtained by these isolation attempts, useful information regarding the source, molecular mass, iso-electric point and the behaviour of the toxin with respect to ionic strength and pH has been obtained. This information is at present being exploited to further purify the toxin.

**REFERENCES**


DETECTION AND ISOLATION OF A PARALYSIS TOXIN PRESENT IN ARGAS (PERSICARGAS) WALKERAE


