Phylogeny of Arthropod Immunity

An Inducible Humoral response in the Kalahari Millipede, Triaenostreptus triodus (Attems).

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Running title: Humoral Immune Response in a Millipede

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Humoral immunity has been demonstrated in a number of different invertebrates for example earthworms, crabs and snails (1). However amongst the arthropods work has focussed on more highly evolved short-lived species of insects such as the silk worm, wax moth, fruit fly and others (2-8). Unfortunately information on how arthropods other than insects respond to bacterial infection is limited. Long-lived terrestrial arthropods like scorpions and millipedes may have evolved different mechanisms for coping with pathogens.

This paper reports on an inducible antibacterial response to gram-negative bacteria (E. coli) in the Kalahari millipede, Triaenostreptus triodus (Diplopoda: Spirostreptidae). The potent humoral response to infection appears to be similar to that found in short-lived insects. Although T. triodus produces several proteins in response to bacterial challenge as illustrated by polyacrylamide gel electrophoresis, only one protein expressed bactericidal activity when subjected to the gel-overlay. This is the first report of a humoral response in a long-lived Diplopod. Inducible proteins responsible for antibacterial activity in arthropods may therefore have a long phylogeny.

The Kalahari millipede (Fig. 1) is one of the most successful arthropod species in the arid regions of South Africa, Botswana and Namibia. They are especially abundant in the Kalahari desert after rain. Here these long-lived `Songololos' feed on the newly sprouted vegetation, especially the leaves and yellow flowers of

Tribulus sp, so commonly covering the Kalahari sands. They also feed on detritus, algae, fungi and a myriad of small mammal scat found on the desert floor. After surface activity of about 4 months they return underground to await next years rains. Certainly their life style and feeding habits are conducive to exposure and attack from bacteria. In a North American millipede, Orthoporus ornatus, field observations of microbial mortality have been observed (9). T. triodus is an ideal animal in which to study immune responses in the laboratory, as aside from its life style possibly replete with pathogens, large quantities of hemolymph can be sampled from the same individual without harm.

Millipedes were collected at Nossob in the Kalahari Gemsbok Park, South Africa (20°30"N; 21°50"E). The millipedes were kept at room temperature (24°C) in glass aquaria on Kalahari sand and fed on a diet of rolled oats and lettuce. Because of the reported synthesis of wound healing proteins upon injection of any pathogen (10) and the necessity of using pyrogen free water and saline to make up all solutions, non-injected as well as saline injected millipedes were used as controls. Saline injected controls often produced proteins that were slightly positive on the gel-overlay method, a phenomenon also noted by other workers (11). The bacterium used was the gram negative Escherichia coli K12. This organism is commonly used in immune studies. The bacteria were cultured in 93 mm X 15 mm sterile petri dishes

containing either Nutrient Agar or McConkey Agar (Merck, Germany). Bacterial suspensions for injection were made up by scraping the bacteria off the culture plates with a heat sterilized spatula and suspending them in insect saline (Ø,7% w/v NaCl). The bacterial suspensions were counted under a microscope in an Improved Neubauer Counting Chamber (Spencer, Brightline, U.S.A.) under oil. Hemolymph was collected by inserting a 26G hypodermic needle between two adjacent segments in the mid-dorsal region (Fig. 1). Hemolymph was drained into an Eppendorf tube kept on ice. Samples were taken at various time intervals after injection (24, 48, 72, 96 hours). This was done to determine the maximum temporal synthesis of antibacterial proteins. To determine whether any antibacterial activity was induced by the injection of E. coli the plate assay technique was first employed (12). Specific antibacterial activity in the proteins separated by acidic electrophoresis was detected by means of a gel-overlay (6,13). Vaccinated and control hemolymph samples were separated towards the cathode in a 1Ø% non-denaturing acidic polyacrylamide gel (pH 4), in duplicate. After electrophoresis one gel was incubated in nutrient broth with Ø,2M potassium phosphate buffer pH 7,4 for one hour. The gel was then overlayed with nutrient agar which was seeded with $1Ø^5$ bacteria.ml^{-1} of E. coli K12. The gel-overlay was incubated at 35^oC for 24 hours to develop zones of inhibition (6). The duplicate gel run under the identical conditions was stained to detect the proteins.

When subjected initially to the plate assay method, growth of E.

coli was unaffected by control hemolymph (Fig. 2a). However, hemolymph taken from a millipede 48 hours after infection inhibited E. coli growth (Fig. 2b). This is seen as a clear zone of inhibition whose magnitude appears similar to that found for various vaccinated insect species subjected to this method (11,12). Hemolymph proteins of vaccinated and control millipedes separated on duplicate gels is seen in Figure 3a and 3b. One gel was stained with both silver and PAGE BLUE (Fig. 3b) (14), the other gel was used for the gel-overlay assay (Fig. 3a). The gel-overlay (Fig.3a) indicates the presence of an inducible antibacterial component in the hemolymph of millipedes 48 hours after bacterial infection. A protein band(s) in a similar position is seen in Figure 3b. No such protein band appears in the control (Fig. 3b, well 3). The antibacterial protein(s) induced in the vaccinated millipede is therefore likely to be a basic protein(s). Maximal levels of this antibacterial protein(s) were found between 48 and 96 hours after infection. Also, using the normal non-denaturing PAGE gel-overlay (15) no acidic antibacterial proteins could be detected. In insects, basic proteins that have been shown to be active against gram-negative bacteria, belong to two families of antibacterial proteins, namely attacins and ceropins, both of small molecular mass and with a non-specific mode of action against many gram-negative bacterial species (16). At this stage it is not clear whether the induced protein(s) are similar to either of the two classes mentioned. Further characterization of the millipede

proteins is underway. It is interesting to note that several other basic proteins were separated in vaccinated millipede hemolymph (Fig. 3b). However no immunological functions could be ascertained using the methods employed.

Fig. 1 (millipedes)

The Kalahari millipede Triaenostreptus triodus. Showing both sampling and injection site (arrowed).

Fig. 2

Plate Assay. Nutrient Agar which has been seeded with a 10^5 bacterial.ml^{-1} E. coli.

A. saline injected hemolymph sample. (28 ul of hemolymph).

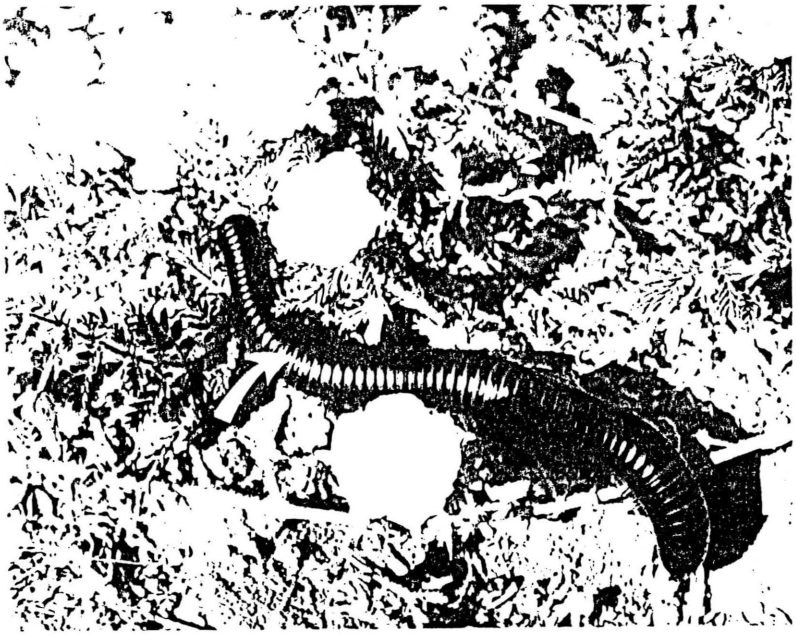
B. Hemolymph sample (30 ul hemolymph) from millipede 48 hours after injection of 10^7 bacteria.g^{-1} E. coli. Loaded 1,5 mg of total protein in each well.

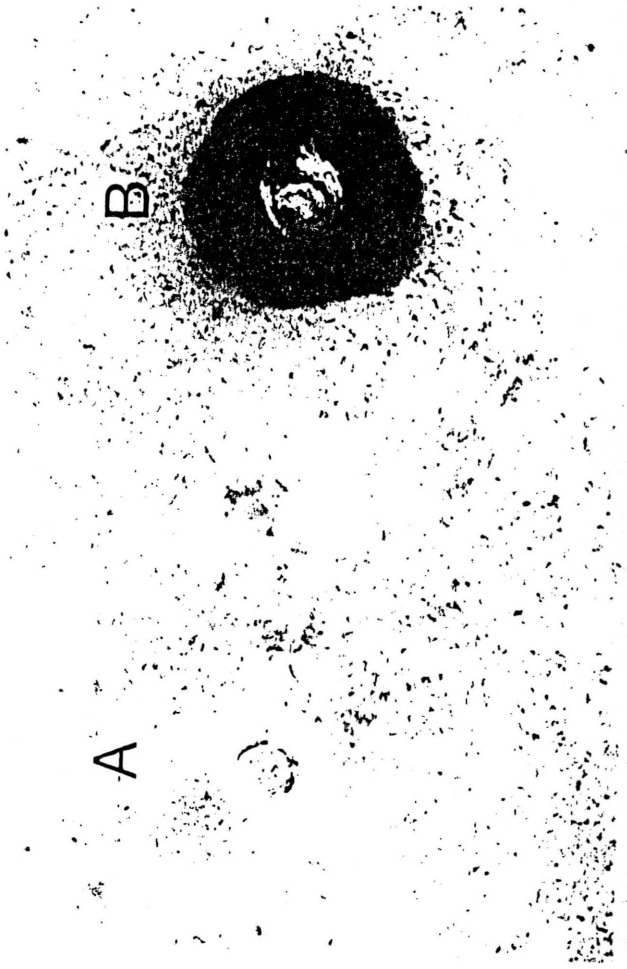
Fig. 3A

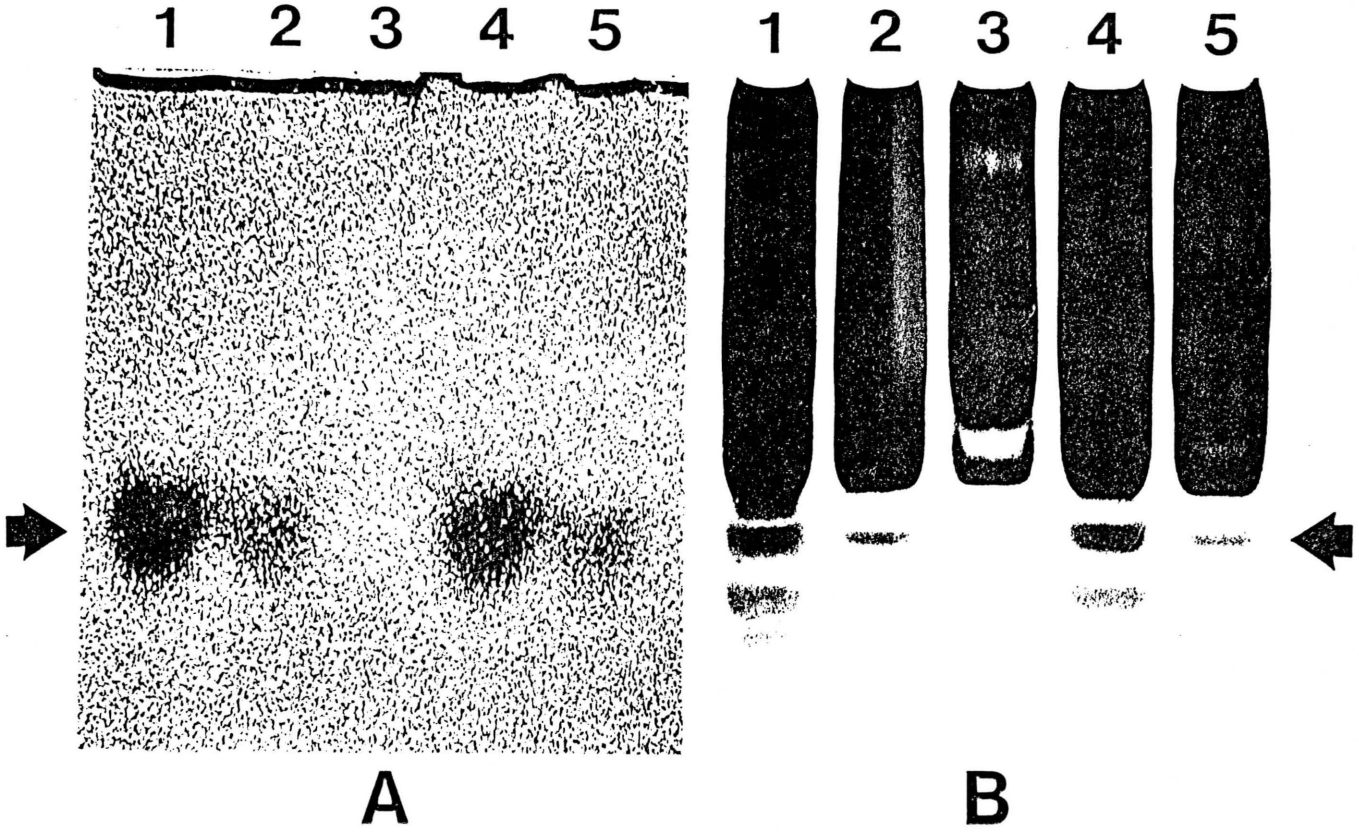
E. coli gel overlay. Zones of antibacterial activity against gram-negative E. coli are evident in wells 1, 2, 4 and 5 (arrowed). Antibacterial activity was absent in the control hemolymph sample (well 3, Fig. 3a). Excess protein (250 ug per well) was loaded to obtain detectable amounts of antibacterial activity. Apparent difference in protein and activity profile is a result of viscosity of solution which impaired accurate loading.

Fig. 3B

Duplicate gel of Fig. 3A, PAGE Blue and Silver stained. Wells: 1,2,4 and 5 -Hemolymph samples from two millipedes 48 hours after injection of 10^7 bacteria.g^{-1} *E. coli* K12. Well 3: control hemolymph from non-injected millipede. The proteins under these acidic conditions are separated towards the cathode (6,12). The gel was stained first with PAGE Blue and then with silver (14). Direction of protein migration is from top to bottom of figure 3.




B
A


1 2 3 4 5
1 2 3 4 5
A
B

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