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The use of aerolysin toxin as an aid for visualization of low numbers of African trypanosomes in whole blood[1]

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Summary

A method was developed for detection of low numbers of African trypanosomes in whole blood. The method is based on selective lysis of erythrocytes and leukocytes by aerolysin, a toxin produced by the bacterium *Aeromonas hydrophila*. African trypanosomes do not bind the toxin and their viability and motility are therefore unaffected by this treatment.

Key words: *Trypanosoma brucei gambiense; Trypanosoma brucei rhodesiense; Aeromonas hydrophila;* aerolysin; parasitemias.

Introduction

Detection of low numbers of African trypanosomes in wet mounts of whole blood is often necessary for determination of parasitemias in research and in clinical diagnosis of disease (WHO, 1979; Paris et al., 1980). Often, in light microscopy, the parasites are obscured by high numbers of host erythrocytes. Clearly, this is not an ideal situation for accurate detection and identification of trypanosomes, especially if the parasites are in extremely low numbers or are of low motility. In addition, it is often necessary to identify the different species of trypanosomes particularly in the field where infections are often mixed. This is an especially difficult task when the parasites are surrounded by host blood cells. In this paper we describe a method which allows specific lysis of host

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erythrocytes under isotonic conditions which do not affect either the viability or motility of the trypanosomes, thus enabling their visualization even in extremely dense wet blood mounts. Selective lysis of rat and mouse erythrocytes was achieved in whole infected blood by addition of extremely small quantities of aerolysin, a cytolytic toxin secreted by the Gram negative bacterium *Aeromonas hydrophila* (Bernheimer et al., 1975).

Materials and Methods

Animals. Female BALB/c mice were obtained from our own breeding colony and used at approximately 8 weeks of age. Female Long-Evans rats were obtained from Canadian Breeding Labs (St. Constant, Quebec) and maintained in our animal facility. They were used at approximately 10 weeks of age.

Trypanosomes. Uncloned *Trypanosoma brucei rhodesiense* EATRO 1895 (Hill et al., 1978) and cloned *Trypanosoma brucei gambiense* TTrT-1 (Seed and Negus, 1970) were obtained from Drs. George Hill and Dick Seed, respectively, and were maintained by serial passage in normal BALB/c mice. Usually 5×10^3 motile trypanosomes were injected intraperitoneally into either mice or rats and blood was taken from the tail vein 1–2 days later (*T. b. gambiense*) or 8–10 days later (*T. b. rhodesiense*) for determination of parasitemias.

Purification of trypanosomes. Trypanosomes were purified from infected mouse blood by passage over DE-52 cellulose according to the method of Lanham and Godfrey (1970) except that whole blood rather than the buffy coat layer was added to the columns. Purified trypanosomes were washed once by centrifugation of 10^8 trypanosomes in 10 ml of ice-cold PBS containing 1% glucose and were resuspended to 1×10^8/ml in the same buffer. Washed trypanosomes were kept on ice prior to use in experiments.

Aerolysin purification. The toxin was isolated in large amounts from cell-free culture supernatants of the Gram negative bacterium *Aeromonas hydrophila* by the method described by Buckley et al. (1981). The purified protein was stored at –20° C at 500 μg/ml in 0.1 M phosphate buffer, pH 7.0, and immediately before use was thawed quickly and diluted to 10 μg/ml in phosphate-buffered saline (PBS).

Iodination of aerolysin. Pure aerolysin (300 μg) was iodinated with ^{125}I using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (Iodogen, Pierce Chemical Co., Rockford, Illinois) according to the method of Fraker and Speck (1978). The specific activity of the iodinated toxin was 3.25×10^{11} cpm/μmole. For our calculations, 1.0 counts per minute represented 3.25×10^7 molecules of aerolysin.

Preparation of wet blood mounts. Blood was taken from trypanosome-infected animals by cutting the tail 2–3 mm from the end. A drop was placed directly into a small glass test tube, five microlitres of aerolysin (10 μg/ml in PBS) were added and the contents of the tube mixed. Immediately, a drop of the suspension was placed on a microscope slide, covered with a glass coverslip and examined under the microscope.

Microscopy. A Zeiss standard binocular microscope fitted with a 40× phase contrast objective and 10× eyepiece was used for all experiments. Photographs for this publication were however taken using a Zeiss Universal-R microscope fitted with a Neofluor 100× oil immersion phase contrast objective, a Zeiss flash attachment, and an Olympus PM-6 camera loaded with Panatomic-X film.

Results

In separate experiments, aerolysin was purified from *Aeromonas hydrophila* culture supernatants (Buckley et al., 1981) and titrated against whole blood from mice, rats and humans. A final aerolysin concentration of approximately

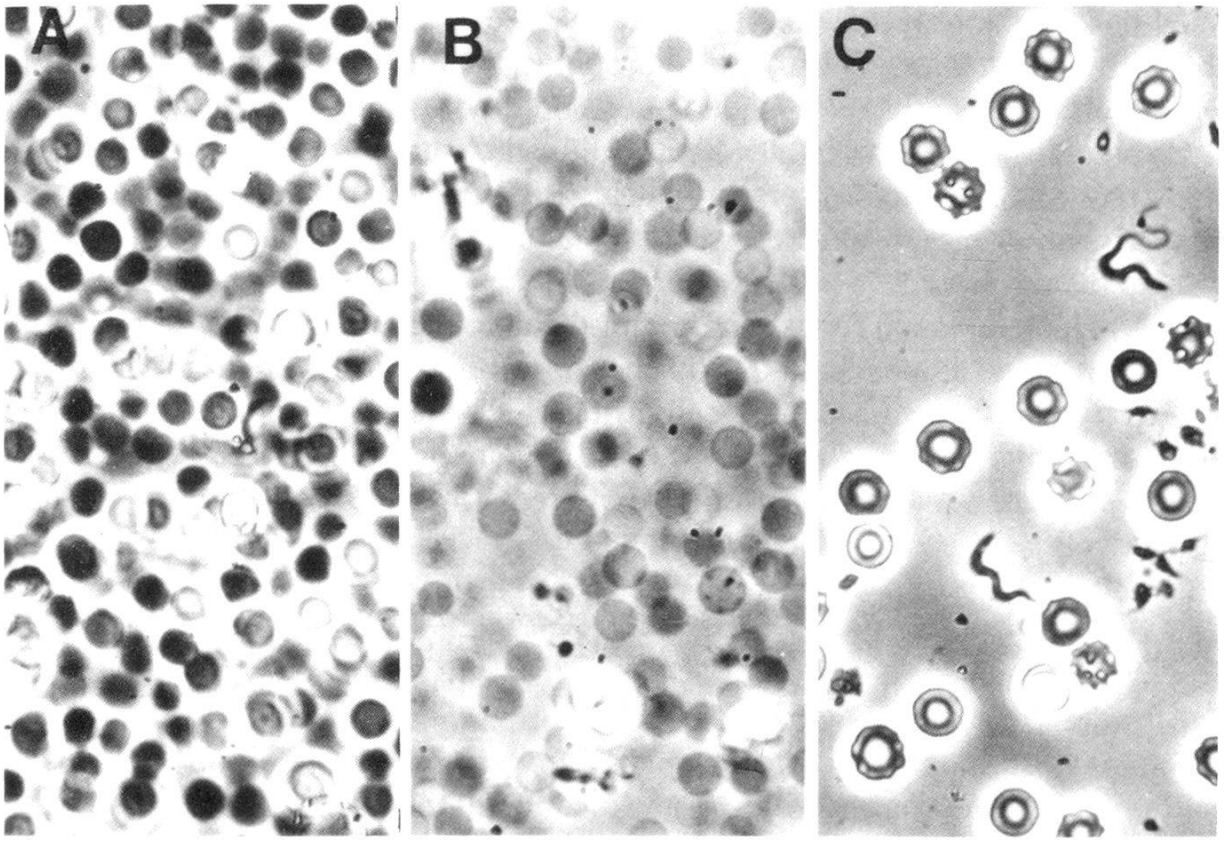

Fig. 1. Photomicrographs of whole blood from rats infected with *T. rhodesiense* before and after addition of aerolysin toxin. (1000× magnification, phase contrast.) A. Before addition of aerolysin. B. Two minutes after addition of aerolysin; erythrocyte ghosts are evident. C. Five minutes after addition of aerolysin.

1 μg/ml was chosen for the experiments using rat and mouse blood described in this paper since this concentration causes complete lysis of erythrocytes and leukocytes in approximately 5 min at room temperature, a convenient interval for observation under the light microscope.

Cytolysis experiments. Within 1–2 min of addition of aerolysin to trypanosome-infected whole mouse or rat blood, the erythrocytes and leukocytes started to swell and lyse. After 5 min incubation at room temperature the majority of host cells had completely lysed although a few intact cells and erythrocyte ghosts remained (Fig. 1). Trypanosomes were extremely difficult to see in the original wet mounts but soon after addition of aerolysin were clearly visible. This was true for both *T. b. rhodesiense* and *T. b. gambiense* (only *T. b. rhodesiense* is shown). The aerolysin was not toxic to the trypanosomes as they remained motile for more than 60 min even on the slides used for microscopy. Incubation of 5×10^7/ml DE-52 cellulose-purified *T. b. gambiense* parasites in 1 μg/ml (final concentration) of aerolysin was performed to test the effect of the toxin on trypanosomes in the absence of host cells which may otherwise bind all the free toxin. No effect on viability or motility was seen over a 60 min period. Attempts to visualize the trypanosomes by simply lysing host cells under hypotonic conditions (addition of distilled water) failed as the trypanosomes also

lysed or became immobile extremely quickly and were thus difficult to locate and differentiate from debris in the preparation. Because trypanosomes were totally refractory to the toxin, the method could be applied to the blood of other mammals, although it was necessary to adjust the amount of toxin added to take into account differences in erythrocyte sensitivities (Bernheimer et al., 1975). Thus trypanosomes could be visualized in human blood by the addition of 100 μg/ml aerolysin.

Aerolysin binding. To test binding of the aerolysin to *T. b. rhodesiense* or to rat erythrocytes/leukocytes, 200,000 cpm of ^{125}I-aerolysin were incubated with 3×10^6 trypanosomes or with rat cells after which the suspensions were washed three times by centrifugation at $900 \times g$. In two experiments, 0.2% and 0.1% of the counts bound to the trypanosomes, whereas 15% and 16% bound to rat cells respectively.

Discussion

In determination of parasitemias it is often an advantage to use methods which allow simple, direct microscopic observation of whole blood rather than methods requiring concentration or purification of the parasites. Aerolysin is an ideal reagent for selective lysis of host erythrocytes and leukocytes. It is a protein of 51,500 molecular weight which can be purified in large amounts from cell-free culture supernatants of *Aeromonas hydrophila* and purified preparations contain no protease or phospholipase activity (Buckley et al., 1981). The toxin has a characteristic hemolytic spectrum which distinguishes it from other toxins. For example, rat and mouse erythrocytes are much more sensitive than those of humans (Bernheimer et al., 1975) making it an ideal reagent for studying parasitemias in blood of these animals and, by using higher doses of the toxin, in human blood.

The experiments using ^{125}I-aerolysin showed that trypanosomes do not bind the protein to any significant level. This is in contrast to binding of the toxin to rat erythrocytes, where an average of 9.75×10^5 molecules bound per cell. Although it is difficult to determine the difference between nonspecific binding and binding to specific receptors on the trypanosomes, it is clear that the parasites are not affected by the toxin.

It has recently been shown that aerolysin forms holes of discrete size in cell membranes and that binding is facilitated by the presence of a surface glycoprotein (presumably a receptor for the toxin) (Howard and Buckley – unpublished observations). Thus, although all cells with exposed lipid bilayers are susceptible to the toxin, those which contain high amounts of the specific binding component are much more so. Cells which shield their lipid bilayers, such as Gram negative bacteria, including *A. hydrophila* itself and the trypanosome (which is covered with its glycoprotein variable surface antigen) are resistant to toxin action.

The use of specific cytolytic toxins may generally facilitate detection, enumeration and purification of non-susceptible organisms found in cell and tissue preparations. Aerolysin is routinely used in our laboratory for detection of low numbers of African trypanosomes in infected blood and presumably (along with other specific cytolytic toxins) will find application to systems involving other microorganisms including bacteria and intracellular and extracellular parasites.

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