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The Role of an FBS Component in the Trypanocidal Reaction Mediated by Guinea Pig Serum

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Abstract

Heated guinea pig serum (HGPS) exhibits trypanocidal activity against the Wellcome strain of *Trypanosoma brucei gambiense* in the presence of an ultrafiltrate of fetal bovine serum (FBS component; nominal molecular weight <3000) or cysteine. However, the HGPS was not active in the presence of spermidine of which oxidation by a polyamine oxidase is the cause of the trypanosome killing by ruminant sera. Thus, it was confirmed that the spermidine oxidation is not involved in the killing mechanism of HGPS. This study defined the role of the FBS component in the process of trypanosome killing. The role was not the direct attack on trypanosomes, but the activation of the factor in HGPS. The HGPS that had been incubated with the FBS component, then dialyzed or its ammonium sulfate precipitate killed trypanosomes by itself. Cysteine did not produce such an active HGPS and was only effective when coexisted with HGPS. The optimal range of the temperature to activate HGPS with the FBS component was 20 to 37°C. The reaction at 37°C proceeded in a time-dependent manner up to 60 min. The death of trypanosomes started 30 min after the incubation with the activated HGPS. This was the time course of the net killing reaction that was separated from the activating reaction.

Key words: *Trypanosoma brucei gambiense* Wellcome strain; guinea pig serum; fetal bovine serum component; trypanocidal activity; trypanocidal factor.

Introduction

Trypanosoma brucei brucei causes the disease Nagana in animals, but this subspecies of African trypanosomes does not infect humans (Rickman and Robson, 1970; Hajduk *et al.*, 1990). This is due to the presence of a toxic factor in human serum that is specific to this subspecies. The toxicity of human serum against *T. b. brucei* was first identified by Laveran (1902). Rifkin (1978) identified the material responsible for the toxicity as high-density lipoprotein (HDL). Hajduk *et al.* (1989) have shown that the toxicity is localized to a minor subset of HDL, called trypanosome lytic factor (TLF) and that most HDL is nontoxic to trypanosomes. TLF is taken into *T. b. brucei* by receptor-mediated endocytosis and targeted to the lysosome (Hager *et al.*, 1994). The killing is mediated by peroxidase activity (Smith *et al.*, 1995).

Ruminant sera also kill African trypanosomes, but exogenous spermidine is needed for the killing. This is due to polyamine oxidase in the sera (Ferrante *et al.*, 1982). The killing mechanism is the release of cytotoxic products such as aldehyde from spermidine (Suva and Abeles, 1978).

HDL fractions from laboratory animals have no trypanocidal activities to *T. b. brucei* (Rifkin, 1978). The Wellcome strain of *Trypanosoma brucei gambiense* in the blood stream is susceptible to the trypanocidal activity of human serum, but resistant to the sera of other animals. However, we found that heated guinea pig serum (at 56°C; HGPS) exhibits trypanocidal activity against this strain of trypanosome in the presence of 10% fetal bovine serum (FBS) (Funato *et al.*, 1993). We further demonstrated that an ultrafiltrate of FBS (FBS component; nominal molecular weight <3000) or cysteine can

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substitute for FBS (Saeki *et al.*, 1996).

The molecular profile of the factor in HGPS is unclear and its relationship to human TLF and a polyamine oxidase in ruminant sera is unknown. The normal guinea pig serum (NGPS) contains at least two kinds of factors relevant to the trypanocidal activity (Saeki *et al.*, 1996). One is not precipitated by 40% of saturated ammonium sulfate and needs cysteine for the expression of the activity. The other is precipitated by the ammonium sulfate and is trypanocidal in the presence of the FBS component or cysteine after heating. Here, we examined the role of the FBS component and cysteine in the expression of the trypanocidal activity by HGPS. We also studied the effect of spermidine on the expression.

Materials and Methods

Guinea pig sera

Normal guinea pig sera (NGPS) were prepared from blood taken by cardiac puncture from female guinea pigs of the Hartley strain weighing 450–500 g (Japan SLC Inc., Shizuoka, Japan). The upper lipid layer and the precipitate were removed from the sera by a final centrifugation ($1 \times 10^5 \times g$, 4°C, 1 hr). The sera were stored at –20°C until use. NGPS and its ammonium sulfate precipitable fraction in microtubes were heated at 56°C for 45 min in a water bath. The heated guinea pig serum is referred to as HGPS.

Trypanosomes

The Wellcome strain of *Trypanosoma brucei gambiense* was maintained in ICR mice (13 weeks of age, Japan SLC Inc.) by passage at 3-day intervals and used as the target for trypanocidal activity. Trypanosomes were harvested from mice 72 hr after infection, then separated from the blood by passage through a DEAE-cellulose column according to Lanham and Godfrey (1970). After washing, the trypanosomes were suspended in phosphate buffered saline (PBS, pH 7.0) containing 0.1% glucose (assay medium).

FBS component

Heat-inactivated FBS (56°C, 30 min) (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) was passed through an ultrafilter with a nominal molecular weight limit of 1×10^4 (Centriprep-10 Concentrators, Amicon Inc., Beverly, MA, USA), followed by that with a nominal molecular weight limit of 3×10^3 (Centriprep-3 Concentrators). The ultrafiltrate was used as the FBS component.

Reaction between HGPS and FBS component

HGPS and the FBS component (1:1) were reacted in a water bath at various temperatures for the indicated periods, then dialyzed using cellulose tubing (Viskase Sales Corp., Illinoise, USA) against 4 liter (to 1.2 ml of the reaction mixture) of the assay medium for 48 hr at 4°C to remove the FBS component. The reaction product was named activated HGPS.

Precipitation with ammonium sulfate

NGPS, HGPS or the activated HGPS was mixed with ammonium sulfate (final concentration, 40% of saturation; pH 7.0). After stirring for 10 min at room temperature, the precipitate was separated from the supernate by centrifugation ($3{,}000 \times g$, 4°C, 10 min) and dissolved in distilled water. The procedure was repeated twice. The final volume of the solution was adjusted to that of the original serum. The solution was extensively dialyzed using cellulose tubing against the assay medium at 4°C and used as the ammonium sulfate-precipitable fraction.

Trypanocidal activity

HGPS, activated HGPS or their ammonium sulfate-precipitable fractions were serially diluted with the assay medium. The FBS component or the solution containing L-cysteine (pH, 7.0; Wako Pure Chemical Industries, Ltd., Osaka, Japan) or spermidine (pH, 7.0; Wako Pure Chemical Industries, Ltd.) were also diluted with the assay medium. Trypanosomes (1×10^6/ml) were incubated with the diluted activated HGPS or the ammonium sulfate fraction in a 96-well flat-bottomed plate (Corning Glass Works, NY, USA) at 37°C for 1 hr. For comparison, trypanosomes were incubated with HGPS in the presence of FBS component (25%), L-cysteine (250 or 50 μg/ml) or spermidine (250 or 31.25 μg/ml) at 37°C for 1 hr. The final volume in each well was 100 μl. Control trypanosomes were incubated in assay medium without additives. After

incubation, the viable trypanosomes in the wells were determined using a Burker-Turk counting plate and the results are expressed as percentages of the average of the numbers in the control wells. Trypanosome death was defined as the complete loss of motility (Rifkin, 1984). Each test was performed in duplicate and the data are expressed as the average.

Results

The role of the FBS component in a series of trypanocidal reactions

HGPS, the FBS component or L-cysteine individually, are not toxic to trypanosomes (data not shown). In the presence of the FBS component or L-cysteine however, HGPS was toxic to the Wellcome strain of *Trypanosoma brucei gambiense* (Fig. 1A). On the other hand, HGPS was not toxic in the presence of 250 and 31.25 μg/ml of spermidine (Fig. 1A). Although more wider range of the concentration (62.5 to 1000 μg/ml) was tested in this study, spermidine did not affect the generation of trypanocidal activity in HGPS at any concentration (data not shown). To know the significance of the FBS component in the trypanocidal reaction, HGPS was incubated with the same volume of the FBS component for 1 hr at 37°C. The mixture was extensively dialyzed against the assay medium at 4°C to remove the free FBS component. The activity was completely removed from the dialysate (Fig. 1A). Fig. 1B shows that HGPS incubated with the FBS component, then dialyzed, killed trypanosomes without additives. This indicated that the FBS component reacted with and activated HGPS. The FBS component could not activate NGPS (data not shown). HGPS exhibited trypanocidal activity in the presence of L-cysteine as shown in Fig. 1A. However, HGPS incubated with L-cysteine, then extensively dialyzed against the assay medium did not produce active HGPS (Fig. 1B).

Kinetics of trypanosome killing by the activated HGPS

Trypanosomes (1×10^6/ml) were incubated at 37°C in the assay medium containing 25% of the activated HGPS. Viable trypanosomes were counted every 10 min after the incubation. The trypanosomes started dying from 30 min and were almost all killed

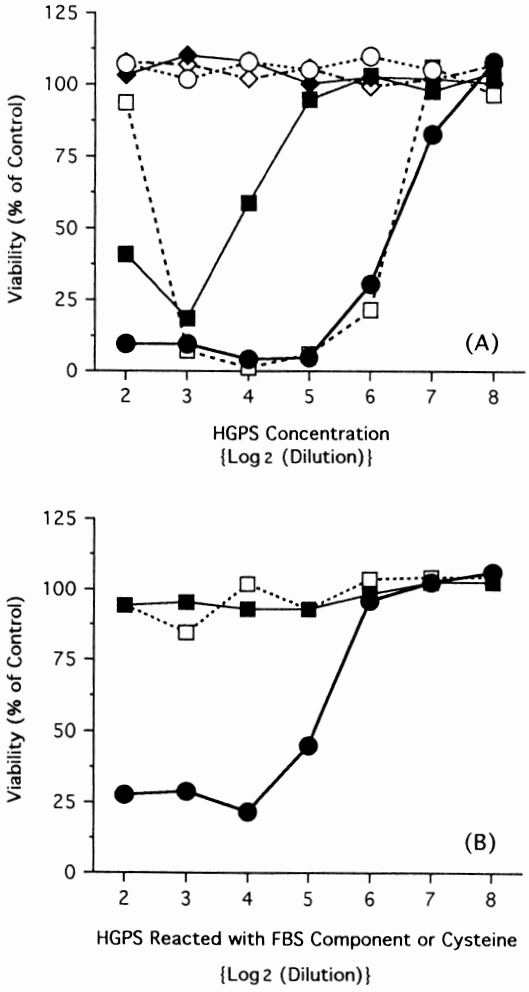

Fig. 1 Trypanocidal activity of HGPS against the Wellcome strain of *Trypanosoma brucei gambiense*. (A), Trypanosomes (1×10^6/ml) were incubated for 1 hr at 37°C with heated guinea pig serum (HGPS) in the presence of 25% of fetal bovine serum (FBS) component (●), 25% of dialyzed FBS component (○), 250 (■) or 50 μg/ml (□) of L-cysteine, or 250 (◆) or 31.25 μg/ml (◇) of spermidine. As the control, trypanosomes were incubated in the assay medium without additives. The vertical axis indicates the numbers of remaining viable trypanosomes and are expressed as percentages of the number in the control incubation. The horizontal axis indicates the concentration of HGPS expressed as $\log_2$(dilution). (B), HGPS was incubated for 1 hr at 37°C with the FBS component (1:1) (●), *500* (■) *or 100* μg/ml (□) of L-cysteine, then dialyzed extensively against the assay medium at 4°C. Trypanosomes (1×10^6/ml) were incubated for 1 hr at 37°C with these HGPS without additives. The horizontal axis indicates the dilution (Log_2) of these HGPS. Other explanations are the same as described in (A).

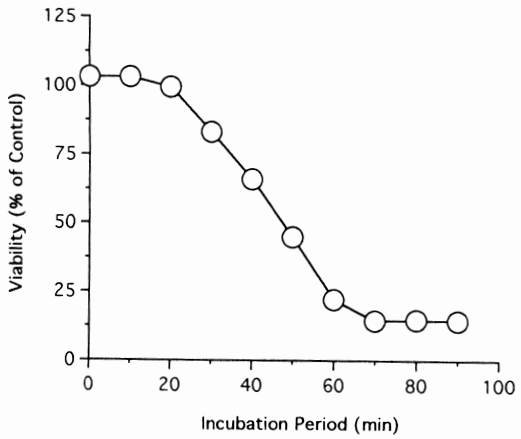

Fig. 2 Kinetics of killing of trypanosomes by the activated HGPS. HGPS was incubated for 1 hr at 37°C with the FBS component (1:1), then dialyzed extensively against the assay medium at 4°C. Trypanosomes (1×10^6/ml) were incubated at 37°C in the assay medium containing 25% of this activated HGPS. Numbers of viable trypanosomes were counted at the indicated times and are expressed as percentages of the control.

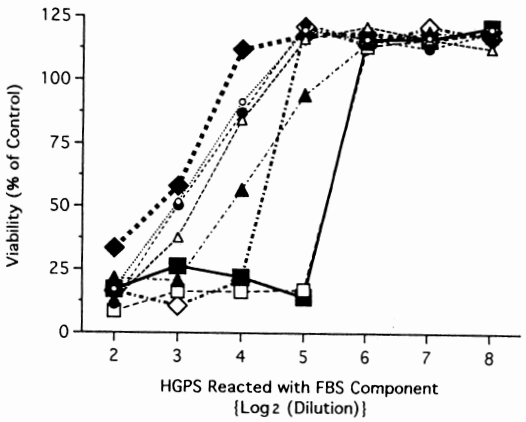

Fig. 3 Kinetics of the reaction between HGPS and the FBS component. HGPS was incubated at 37°C for the indicated periods with the same volume of the FBS component, then dialyzed extensively against the assay medium at 4°C. The incubation periods were as follows: 10 (○), 20 (●), 30 (△), 40 (▲), 50 (□), 60 (■), 90 (◇) and 120 min (◆). Trypanosomes (1×10^6/ml) were incubated for 1 hr at 37°C in the respective HGPS preparation serially diluted with the assay medium. Numbers of viable trypanosomes after the incubation are expressed as percentages of the control. The horizontal axis indicates the dilution (Log_2) of HGPS preparations.

within 60 min. Although about 11% of the trypanosomes remained viable, the kinetics shown in Fig. 2 was similar to that of trypanosomes killed by HGPS in the presence of the FBS component.

Kinetics of the reaction between HGPS and the FBS component

HGPS was incubated with the same volume of the FBS component at 37°C for 10 to 120 min. The mixtures were then extensively dialyzed against the assay medium at 4°C. The toxicity of these HGPS was tested against 1×10^6/ml trypanosomes (Fig. 3). HGPS partially reacted with the FBS component during a 10 min incubation at 37°C. After 30 min, the activity of HGPS increased and the reaction was completed at 50 min at this temperature. The incubation for more than 60 min rather decreased the activity.

The effect of temperature on the reaction between HGPS and the FBS component

HGPS was reacted with the same volume of the FBS component for 1 hr at various temperatures. Thereafter, the reaction mixtures were dialyzed extensively against the assay medium at 4°C. The toxicity of these HGPS was tested against 1×10^6/ml trypanosomes. The results (Fig. 4) demonstrated that the reaction partially proceeded even at 0°C. The activities of the resultant HGPS increased at higher temperatures. The optimal temperatures required to complete the reaction within 1 hr were in the range of 20 to 37°C. The reaction at temperatures above 45°C for 1 hr deactivated the HGPS.

Precipitation of the trypanocidal factor with ammonium sulfate

The ammonium sulfate (40% of saturation) precipitate from NGPS was inactive unless it was heated. As the precipitate exhibited trypanocidal activity in the presence of the FBS component after heating, it was sure that the factor that is responsible for the trypanocidal activity was precipitated by the treatment. The ammonium sulfate precipitate from HGPS was active in the presence of the FBS component without heating (Fig. 5). The recoveries of

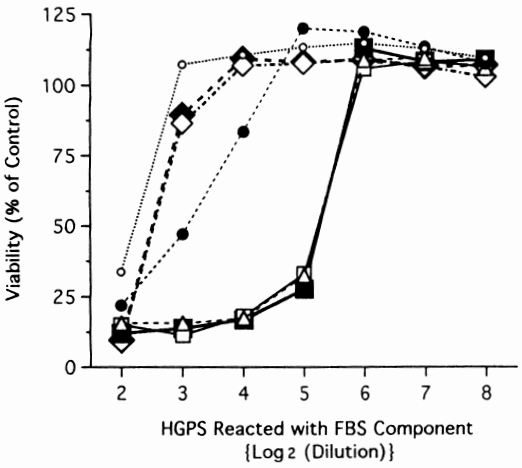

Fig. 4 The effect of temperature on the reaction between HGPS and the FBS component. HGPS was incubated for 1 hr at indicated temperatures with the same volume of the FBS component, then dialyzed extensively against the assay medium at 4°C. The incubation proceeded at 0 (○), 10 (●), 20 (△), 30 (□), 37 (■), 45 (◇) and 50°C (◆). Trypanosomes (1×10^6/ml) were incubated for 1 hr at 37°C in the respective HGPS preparation diluted serially with the assay medium. Other explanations are as described in the legend to Fig. 3.

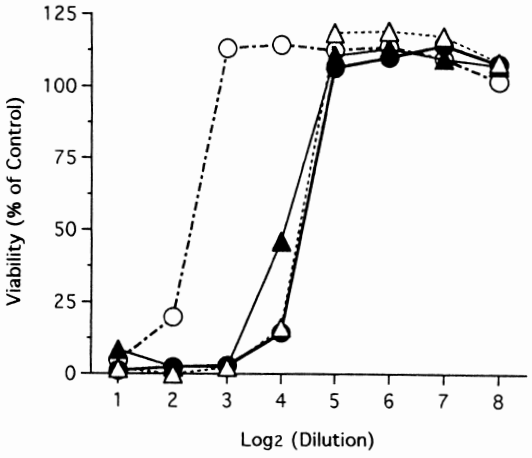

Fig. 5 The trypanocidal activity in the ammonium sulfate-precipitable fraction of activated HGPS. HPGS was incubated for 1 hr at 37°C with the same volume of the FBS component, then dialyzed extensively against the assay medium at 4°C. The dialysate of this activated HGPS was precipitated at room temperature with 40% of saturated ammonium sulfate. The precipitate was finally dissolved in the same volume of distilled water as that of original serum, then extensively dialyzed against the assay medium at 4°C. As controls, ammonium sulfate precipitates from NGPS and HGPS were also prepared. The trypanocidal activities of these preparations were assayed as described in the legend to Fig. 3 and are expressed as viabilities of trypanosomes after the treatment with these preparations. The horizontal axis indicates the dilution (Log_2) of preparations. Trypanocidal activity (without additives) of activated HGPS (●); that of ammonium sulfate-precipitable fraction from this activated HGPS (○); trypanocidal activity (in the presence of 25% FBS component) of heated ammonium sulfate-precipitable fraction from NGPS (△); that of the unheated ammonium sulfate-precipitable fraction from HGPS (▲).

activity in both precipitates were equivalent and 25% of original sera. In the ammonium sulfate supernates, the activities were not detected (data not shown). The ammonium sulfate precipitate from the HGPS that had been incubated with the FBS component at 37°C for 1 hr, then extensively dialyzed killed trypanosomes by itself without the additional FBS component. Thus, the factor bearing trypanocidal activity that was produced in the HGPS by the FBS component was recovered into the ammonium sulfate precipitate. The activity in the precipitate was 25% of that in the activated HGPS.

Discussion

Human, ruminant and heated guinea pig sera have similar effects on blood stream trypanosomes. The killing mechanisms mediated by human and ruminant sera have been elucidated, whereas that mediated by HGPS remains unclear. HGPS can not kill trypanosomes by itself. Our previous studies demonstrated that the trypanocidal activity appears in the presence of the FBS component or cysteine. This is also shown in figure 1A. The FBS component and the used doses of cysteine themselves have no effects on trypanosomes.

Ruminant sera kill African trypanosomes in the presence of exogenous spermidine. This is due to polyamine oxidase in the sera (Ferrante *et al.*, 1982). The killing mechanism is the release of cytotoxic products such as aldehyde from spermidine (Suva and Abeles, 1978). Spermidine is a popular polyamine in animals. In rodent, a polyamine oxidase that degrades spermidine to putrescine and 3-aminopropionaldehyde also exists (Hölttä, 1977).

These suggest that polyamine oxidase that catalyzes the oxidation of spermidine may be involved in the killing mechanism mediated by HGPS. In addition, the possibility is also proposed by the biochemical base that polyamine oxidase needs exogenous thiols for the full enzyme activity (Hölttä, 1977).

The result in figure 1A however, shows that spermidine is ineffective in the induction of trypanocidal activity. This indicates that the oxidation of spermidine is not involved in the killing mechanism. In addition, the inefficaciousness of thiols other than cysteine on the induction of trypanocidal activity (Saeki *et al.*, 1996), the inability of pyridoxal phosphate for the induction (data not shown) that is a stimulator of polyamine oxidase (Hölttä, 1977) and the inability of a sulfhydryl reagent, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) for the suppression of the induction (data not shown) that should block sulfhydryl groups essential for the enzyme activity of polyamine oxidase (Hölttä, 1977) also support this.

The FBS component is different from cysteine considering from reactivities to DTNB, optical absorption spectra and gel-filtration patterns (unpublished data). This study defined the role of the FBS component in the process of trypanosome killing with HGPS. The role is the production of active factor from HGPS and is not the direct attack on trypanosomes, because the HGPS that has been incubated with the FBS component, then dialyzed kills trypanosomes by itself (Fig. 1B). The comment on the retention of free FBS component in the final preparation is completely excluded by the data in figure 1A. As cysteine does not produce such an active HGPS and is only effective when coexists with HGPS (Fig. 1B), the processes of HGPS activation mediated by the FBS component and cysteine are different.

The time course of trypanosome killing in wells containing HGPS and the FBS component is therefore, the gross of time courses of separate reactions. The result in figure 2 shows the net kinetics of killing reaction that is isolated from the reaction for the activation of HGPS. However, this is similar to the previous observation on the trypanosome killing in wells containing both HGPS and the FBS component. The similarity is due to the relatively rapid progress of the activating reaction (Fig. 3), indicating that the killing phase is the rate-determining step.

The activation of HGPS by the FBS component proceeds even at nonphysiological temperatures, but the optimal range to produce the active factor is 20 to 37°C (Fig. 4). Although the reaction proceeds in a time-dependent manner up to 60 min at 37°C, the recovery of active product decreases after reactions for over 60 min (Fig. 3). Together with the observation that the recovery also decreases after reactions at temperatures over 37°C, the active product is considerably unstable. The effects of photons on the product are unknown, but all reactions proceeded in the dark.

Our previous studies demonstrated that NGPS and its ammonium sulfate precipitate do not exhibit trypanocidal activity even in the presence of the FBS component unless they are heated. The results in Fig. 5 show that the ammonium sulfate precipitate from HGPS exhibits the activity in the presence of the FBS component without heating. The final activities in both precipitates from NGPS and HGPS are equivalent and the recoveries of activity in both precipitates are generally 25% of those in original sera (Fig. 1A). As the activity is not detected in the ammonium sulfate supernate (data not shown), the loss of activity is not due to the insufficient precipitation. The loss of activity is also seen in the activated HGPS preparation (25% of original HGPS activity). The constant recovery rate of activity in each preparation indicates common causes for the loss, such as denaturalization, aggregation or adsorption that may occur during procedures. As the dialysis is a common procedure to each preparation, most of the activity loss may occur during dialyses. We succeeded in the precipitation of factor bearing trypanocidal activity. The ammonium sulfate precipitate from the HGPS that has been activated by the FBS component can kill trypanosomes by itself (Fig. 5). The activity of the precipitate is again about 25% of that in the activated HGPS.

The mechanism by which the active factor kills trypanosomes is still unknown. The spermidine oxidation has been excluded from the mechanism by this study. The relevancy to the killing mechanism of human serum remains to be examined. The factor in human serum that is toxic to *T. b. brucei* is the haptoglobin-related protein in TLF (Smith *et al.*,

1995). TLF containing this factor is taken into trypanosomes by receptor-mediated endocytosis and targeted to the lysosome (Hager *et al.*, 1994). Trypanosomes are killed by the oxidative damage initiated by peroxidase activity of the factor. The bloodstream forms of the African trypanosomes do not express catalase (Baernstein, 1963). This results in high intracellular concentrations of hydrogen peroxide, making the cells susceptible to oxidative damage (Meshnick *et al.*, 1977, Meshnick *et al.*, 1978). In following studies, the participation of peroxidase activity in the trypanosome killing mediated by the active factor will be examined.

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