[Jpn. J. Parasitol., Vol. 45, No. 4, 280–289, August, 1996]

Trypanocidal Activity of Guinea Pig Serum and BSA in the Presence of an FBS Component or Cysteine

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(Accepted July 19, 1996)

Abstract

Heated (56°C, 45 min; HGPS), but unheated guinea pig serum (NGPS) exhibited trypanocidal activity in the presence of an ultrafiltrate of fetal bovine serum (nominal molecular weight <3000; FBS component). The activity caused cell swelling and finally lysed trypanosomes, presumably by osmotic pressure. HGPS also generated the activity in the presence of L- or D-cysteine, but in different manner. The trypanocidal activity of HGPS depended on the concentration of the FBS component but not simply upon that of cysteine. It appeared to be generated by cysteine concentrations that counterbalanced that of the HGPS. Saturating NGPS with 40% ammonium sulfate revealed two factors. One precipitated by the ammonium sulfate exhibited trypanocidal activity in the presence of FBS component or cysteine only when heated. The other in the ammonium sulfate-unprecipitable fraction exhibited trypanocidal activity only in the presence of cysteine, regardless of heating. L-cysteine methyl ester, L-cysteine ethyl ester, S-methyl-L-cysteine and glutathione had no effects on the generation of the trypanocidal activity of HGPS. On the other hand, bovine serum albumin (BSA) exerted trypanocidal activity when L-cysteine, but not FBS component, was present. In contrast to HGPS, BSA exhibited the activity in the presence of L-cysteine methyl ester or L-cysteine ethyl ester.

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

Introduction

Trypanosoma brucei gambiense and *Trypanosoma brucei rhodesiense* cause African sleeping sickness in humans. In animals, the disease Nagana is caused by *Trypanosoma brucei brucei*. Although these three subspecies of trypanosomes are morphologically indistinguishable, humans do not become infected with *T. b. brucei* (Rickman and Robson, 1970; Hajduk *et al.*, 1990). This is due to the trypanocidal activity specific to this subspecies in human serum that was first found by Laveran (1902). Rifkin (1978) identified the material responsible for the activity as high-density lipoprotein (HDL). Hajduk *et al.* (1989) have, however shown that the activity is localized to a minor subset of HDL and that the majority of HDL is nontoxic to trypanosomes. Smith *et al.* (1995) have reported that the toxic molecule is the haptoglobin-related protein in the minor subset. The molecule kills trypanosomes via oxidative damage initiated by its peroxidase activity.

T. b. brucei infects many laboratory animals, indicating that animal sera have no trypanocidal activities. HDL in the sera has no activities against this trypanosome (Rifkin, 1978). The Wellcome strain of *T. b. gambiense* in the blood stream is susceptible to the trypanocidal activity of human serum and resistant to animal sera. Therefore, the host range of this strain is similar to that of *T. b. brucei*. We found that guinea pig serum exhibits trypanocidal activity to this strain of trypanosomes in the presence of FBS when heated at 56°C for 30 min (Funato *et al.*, 1993). FBS seemed to be essential to induce the activity to HGPS.

In this study, we clarified that the trypanocidal

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activity of HGPS is generated when more than 6.25% of FBS ultrafiltrate (nominal molecular weight <3,000) is present. The component in FBS can be substituted by cysteine. The trypanocidal activities of HGPS that are generated in the presence of FBS component or cysteine cause similar morphological changes of trypanosomes. We showed that two materials in NGPS are involved in trypanocidal activity. One was and the other was not precipitated by 40% ammonium sulfate. The former exhibited the activity in the presence of FBS component or cysteine when heated, whereas the latter did so only in the presence of cysteine without heating. As BSA exhibits similar trypanocidal activity, the latter may be albumin.

Materials and Methods

Guinea pig sera

Guinea pig sera (NGPS) were prepared from blood taken by cardiac puncture from female guinea pigs of the Hartley strain weighing 450–500g (Japan SLC Inc., Sizuoka, Japan). The upper lipid layer and the precipitate were removed from sera by a final centrifugation ($1 \times 10^5 \times g$, 4°C, 1 hr). The sera were stored at –20°C until use.

Trypanosomes

The Wellcome strain of *Trypanosoma brucei gambiense* was maintained in ICR mice (13 weeks of age, Japan SLC Inc., Sizuoka, Japan) 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).

Heating

NGPS, ammonium sulfate-precipitable and unprecipitable fractions of NGPS in microtubes were heated at 56°C for 45 min in a water bath. The heated guinea pig serum is referred to as HGPS.

Precipitation of NGPS with ammonium sulfate

NGPS was mixed with ammonium sulfate (final concentration 40%; 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 volume of supernate resulted in 1.7-fold increase. The solution and the supernate were extensively dialyzed against the above assay medium at 4°C and used as the ammonium sulfate-precipitable and unprecipitable fractions of NGPS.

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 containing materials with a nominal molecular weight below 3×10^3 was used as the FBS component.

Trypanocidal activity

HGPS, NGPS, the ammonium sulfate fractions, BSA (Fraction V of alcohol precipitation, A-4503, Lot 129F0142, Sigma Chemical Co., MO, USA) and the FBS component were serially diluted with the assay medium. Cysteine or its derivatives were dissolved in the assay medium and the pH was adjusted to 7.0. Trypanosomes (1×10^6) were incubated with each dilution of HGPS, NGPS, the ammonium sulfate fractions or BSA in the presence or absence of various concentrations of the FBS component, L- or D-cysteine, L-cysteine methyl ester, L-cysteine ethyl ester (Wako Pure Chemical Industries, Ltd., Osaka, Japan), S-methyl-L-cysteine (Aldrich Chemical Co., Inc., WI, USA) or glutathione (reduced form, Sigma Chemical Co., MO, USA) in plates with 96 flat-bottomed wells (Corning Glass Works, NY, USA) at 37°C for 1 hr. The final volume in each well was 100 μl. As controls, trypanosomes were incubated in assay medium without additives. After incubation, the viable trypanosomes in the wells were counted using 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. Each test was performed in duplicate and the data are expressed as the average.

Photomicrographs

Trypanosomes (1×10^7) were incubated in assay medium containing 12.5% HGPS and 25% FBS component or 1×10^6 trypanosomes were incubated in assay medium containing 6.25% HGPS and 125 μg/ml of L-cysteine at 37°C for 10 min to 2 hr. After the latter incubation, trypanosomes were washed and concentrated in the assay medium by the centrifugation. A small portion of the trypanosome suspension was dropped onto Blutstan glass microslides (Daiichi Pure Chemicals, Tokyo, Japan) and the trypanosomes were instantly stained for photomicrograph (× 400).

Results

Morphological analysis of the killing process

Trypanosomes incubated with HGPS in the presence of FBS component at 37°C were killed. The morphological changes caused in the process of killing are shown by the photomicrographs (× 400) in Fig. 1. The morphological changes were not completely synchronized, but as follows. Until 15 min of incubation, trypanosomes appeared normal and the effect of HGPS and FBS component seemed reversible. The washed trypanosomes were viable in further incubations in assay medium alone. From 20 min, the cell body rounded up (Fig. 1A) and the effects of these materials became irreversible. The death of trypanosomes that was defined as the complete loss of motility generally started after 30 min. Swelling progressed and cell organelles were biased to one side of the rounding body (Fig. 1B). Cells were maximally swollen around 60 min of incubation (Fig. 1C). At this time, some of the cells were lysed. Dead and maximally swollen trypanosomes appeared as round cell ghosts that had lost many organelles. These cells had free flagella extending out from the remains. The morphological changes were similar when trypanosomes were incubated with HGPS in the presence of L-cysteine (Fig. 1D). On the other hand, trypanosomes were not killed and their morphology did not change when incubated with HGPS, FBS component or L-cysteine alone (Fig. 1E, F and G).

Induction of trypanocidal activity in HGPS by the FBS component

We examined the effect of the FBS component on the generation of trypanocidal activity of HGPS. Trypanosomes (final concentration of 1×10^6) were incubated for 1 hr at 37°C with serially diluted NGPS or HGPS in the presence of the FBS component. As a control experiment, the trypanosomes were incubated in plain assay medium. After the incubation, the number of viable trypanosomes was expressed as the percentage of that of the control. NGPS or HGPS itself did not exhibit the trypanocidal activity (Lines without symbols in Fig. 2A and B). The FBS component itself at final concentrations ranging from 0.78% to 25% neither shows any trypanocidal activities (Fig. 2A and B). When incubated in the assay medium containing NGPS and the FBS component, trypanosomes were not affected (Fig. 2A). However, trypanosomes that were incubated in the assay medium containing HGPS and the FBS component were killed (Fig. 2B). HGPS at concentrations down to a 64-fold dilution exhibited trypanocidal activity when the FBS component was present at final concentrations above 12.5%. The minimal concentration of the FBS component required to induce the activity was 6.25%. At this concentration, HGPS exhibited the activity down to a 16-fold dilution.

Trypanocidal activity of HGPS in the presence of cysteine

HGPS also exhibited trypanocidal activity in the presence of L-cysteine (Fig. 3A). L-cysteine itself did not show any trypanocidal activity. As seen in Fig. 3A however, the generation of the activity in the presence of the amino acid was different from that in the presence of the FBS component (Fig. 2B) in terms of the concentration of both HGPS and L-cysteine. The maximal dilution of HGPS to completely kill trypanosomes in the presence of 7.8 to 62.5 μg/ml of L-cysteine was 64-fold. Although 7.8 μg/ml of L-cysteine was effective at this dilution of HGPS, the concentration of L-cysteine did not effect HGPS dilutions below 16-fold. Similarly, 15.6 μg/ml of L-cysteine had no effects on HGPS dilutions lower than 8-fold. The minimal concentration

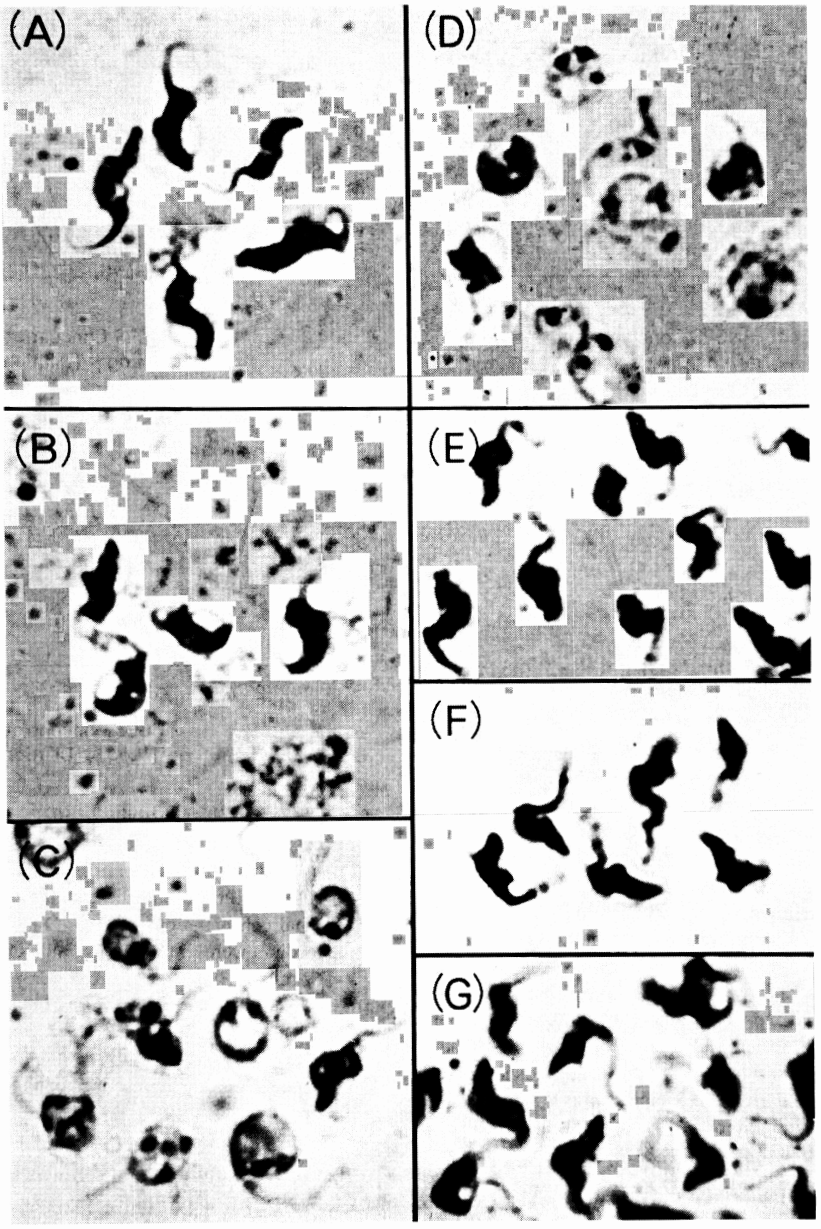

Fig. 1 Morphological changes of the Wellcome strain of *Trypanosoma brucei gambiense* incubated at 37°C with heated guinea pig serum (HGPS) in the presence of the fetal bovine serum (FBS) component or L-cysteine. For photomicrographs (× 400), trypanosomes were instantly stained on Blutstan glass microslides. Trypanosomes (1×10^7) were incubated in assay medium containing 12.5% HGPS and 25% FBS component for 20 (A), 30 (B), and 60 min (C). Trypanosomes (1×10^7) were incubated in assay medium containing 12.5% HGPS (E), or 25% FBS component (F) for 60 min. Trypanosomes (1×10^6) were incubated in assay medium containing 6.25% HGPS and 125 μg/ml of L-cysteine (D) or 125 μg/ml of L-cysteine alone (G) for 60 min.

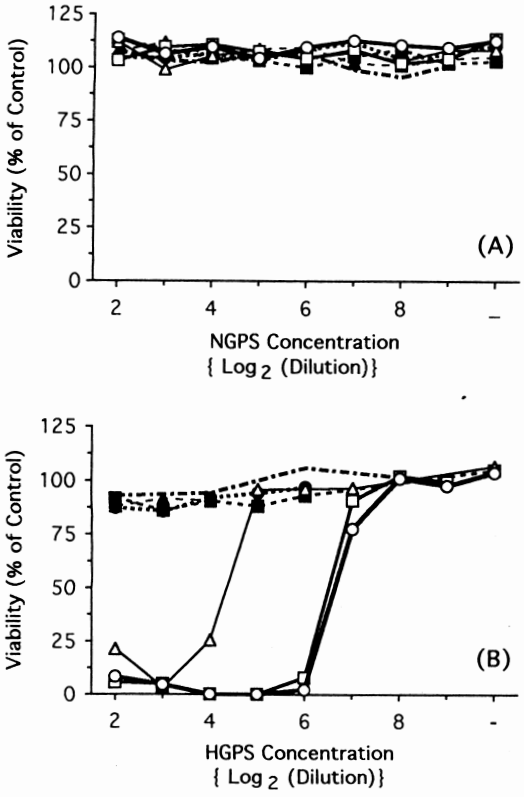

Fig. 2 Trypanocidal activity of HGPS in the presence of FBS component. Trypanosomes (final concentration of 1×10^6/ml) were incubated for 1 hr at 37°C with serially diluted normal guinea pig serum (NGPS) (A) or HGPS (B) in the presence of various concentrations of the FBS component. Control trypanosomes were incubated in the assay medium without sera and the FBS component. The vertical axis expresses the number of viable trypanosomes after incubation as the percentage of that in the control incubation. The horizontal axis indicates the concentration of HGPS expressed as $\log_2$ (dilution). Final concentrations of the FBS component in the assay medium are as follows (%): ○, 25; □, 12.5; △, 6.25; ●, 3.13; ■, 1.56; ▲, 0.78; no symbol, without the FBS component.

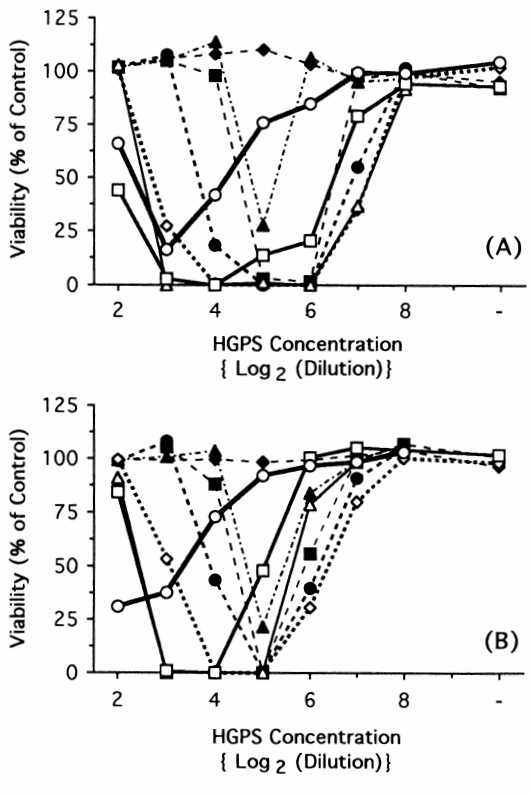

Fig. 3 Trypanocidal activity of HGPS in the presence of cysteine. Trypanosomes (final concentration of 1×10^6/ml) were incubated for 1 hr at 37°C with serially diluted HGPS and various concentrations of L- or D-cysteine. Final concentrations of L- or D-cysteine in the assay medium were as follows (μg/ml): ○, 250; □, 125; △, 62.5; ◇, 31.3; ●, 15.6; ■, 7.8; ▲, 3.9; ◆, 2.0. (A), numbers of viable trypanosomes after an incubation with HGPS and L-cysteine. (B), numbers of viable trypanosomes after the incubation with HGPS and D-cysteine. Other explanations are as described in the legend to Fig. 2.

of L-cysteine required to generate the trypanocidal activity was 3.9 μg/ml, only at a 32-fold dilution of HGPS. Therefore, the generation of trypanocidal activity in the presence of L-cysteine did not linearly depend on the concentration of the amino acid, but the activity was brought about by L-cysteine at concentrations counterbalancing the HGPS concentrations. Although there was a slight difference, the effect of D-cysteine on HGPS was similar to that of L-cysteine (Fig. 3B). The effects of cysteine derivatives such as L-cysteine methyl ester, L-cysteine ethyl ester, S-methyl-L-cysteine and glutathione on 8- and 32-fold dilutions of HGPS were examined. As seen in Fig. 4, these materials had no effects on HGPS.

Effects of FBS component and L-cysteine on ammonium sulfate fractions of NGPS

The effects of cysteine on HGPS was more complex than that of FBS component. To elucidate

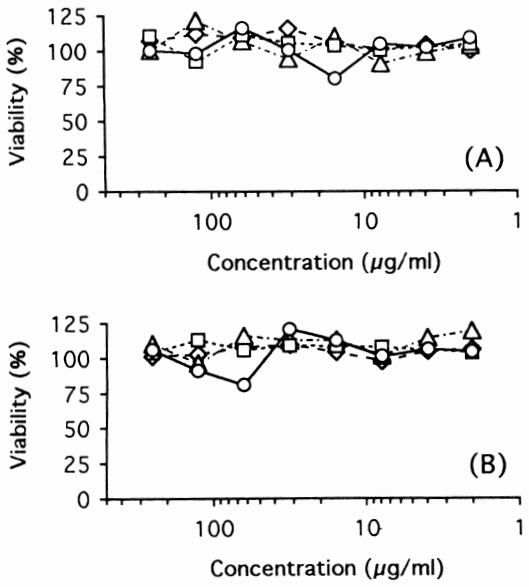

Fig. 4 Effects of cysteine derivatives on the generation of trypanocidal activity of 8-fold (A) or 32-fold (B) diluted HGPS. The horizontal axis (logarithmic scale) indicates the concentration of cysteine derivatives in the assay medium expressed as μg/ml. ○, L-cysteine methyl ester; □, L-cysteine ethyl ester; △, S-methyl-L-cysteine; ◇, glutathione. Other explanations are as described in the legend to Fig. 2.

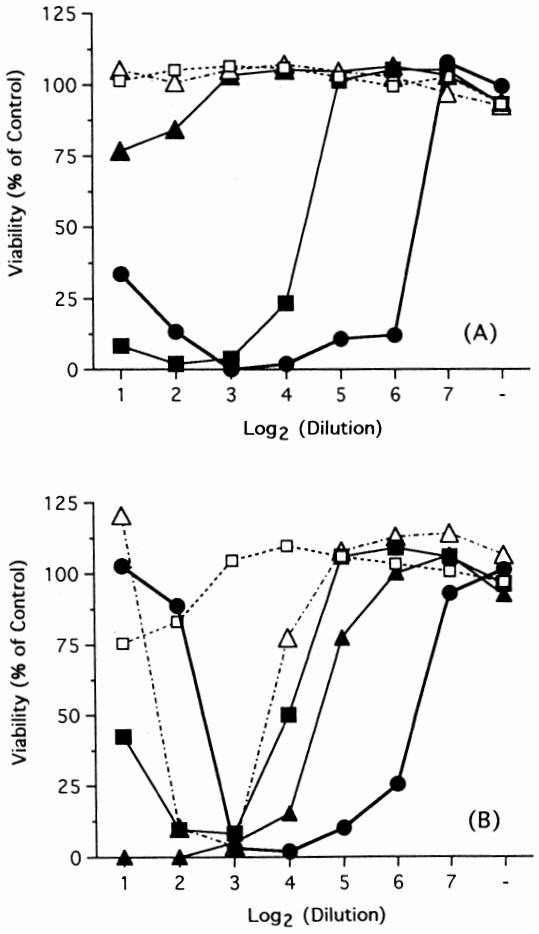

Fig. 5 Trypanocidal activity of the ammonium sulfate fractions of guinea pig serum. The horizontal axis indicates the concentration of HGPS or the fractions expressed as $\log_2$ (dilution). ●, HGPS; □, unheated ammonium sulfate-precipitable fraction (ppt fraction); ■, heated ppt fraction; △, unheated ammonium sulfate-unprecipitable fraction (sup fraction); ▲, heated sup fraction. (A), in the presence of the final concentration of 25% FBS component. (B), in the presence of the final concentration of 31.25 μg/ml L-cysteine. Other explanations are as described in the legend to Fig. 2.

the cause of the difference between their effects, NGPS was fractionated with ammonium sulfate. The ffects of 25% FBS component and 31.3 μg/ml L-cysteine on precipitable and unprecipitable fractions were examined. Only the heated precipitable fraction exhibited trypanocidal activity in the presence of the FBS component (Fig. 5A). The heated fraction also exhibited the activity in the presence of L-cysteine (Fig. 5B). The unheated precipitable fraction did not exert the activity in the presence of both factors. On the other hand, no apparent activities were detected in unprecipitable fractions containing the FBS component (Fig. 5A). Therefore, the factor that generated the trypanocidal activity with the FBS component was located in the precipitate and heating was required for it to become activated. The unprecipitable fraction exhibited trypanocidal activity only in the presence of cysteine, regardless of heating (Fig. 5B). Therefore, we clarified that in NGPS, there are two factors that generate the trypanocidal activity. One exhibits the activity in the presence of the FBS component or L-cysteine when heated, and the other does so, regardless of heating, only in the presence of cysteine.

Trypanocidal activity of BSA

The ammonium sulfate unprecipitable fraction

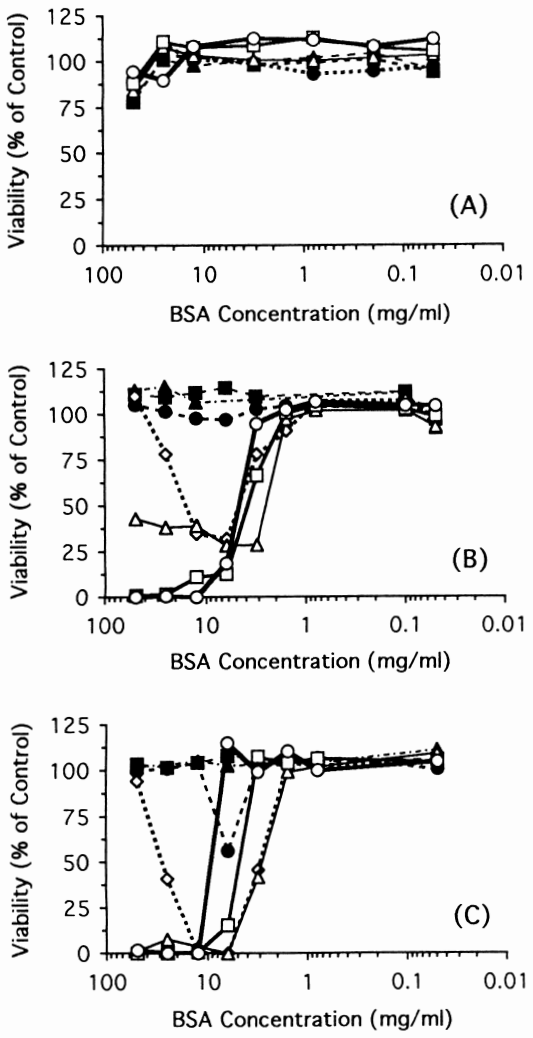

Fig. 6 Trypanocidal activity of bovine serum albumin (BSA) generated in the presence of FBS component or cysteine. The horizontal axis (logarithmic scale) indicates the concentration of BSA expressed as mg/ml. (A), numbers of viable trypanosomes after the incubation with BSA and the FBS component. Final concentrations of FBS component were as follows (%): ○, 25; □, 12.5; △, 6.25; ●, 3.13; ■, 1.56; ▲, 0.78. (B), numbers of viable trypanosomes after the incubation with BSA and L-cysteine. (C), those after the incubation with BSA and D-cysteine. Final concentrations of L- or D-cysteine in the assay medium were as follows (μg/ml): ○, 500; □, 250; △, 125; ◇, 62.5; ●, 31.3; ■, 15.6; ▲, 7.8. Other explanations are as described in the legend to Fig. 2.

contains albumin. We therefore tested BSA to determine whether or not it exhibits the trypanocidal activity. BSA alone did not show the activity, but when L- or D-cysteine was added at concentrations over 62.5 μg/ml, the activity was initiated (Fig. 6B and C). On the other hand, the FBS component had no effect on BSA (Fig. 6A). These findings coincided with those obtained by the above study on the ammonium sulfate unprecipitable fraction of NGPS. However, the trypanocidal activity was also generated in the presence of L-cysteine methyl ester or L-cysteine ethyl ester (Fig. 7A and B). The minimal concentration of these cysteine derivatives required to generate the activity was 7.8 μg/ml. Therefore, these cysteine esters were more effective in producing the activity of BSA than cysteine itself (Fig. 6B and C). S-methyl-L-cysteine and glutathione had no effects on BSA (Fig. 7C and D).

Discussion

The morphological changes of the Wellcome strain of *T. b. gambiense* that occur during the incubation with HGPS and FBS component or ysteine resembled those of *T. b. brucei* caused by human serum in which a cytotoxic factor causes acute, irreversible lethal injury to the plasma membrane (Rifkin, 1984). Rifkin (1984) has described that during the pre-lytic phase, the cell body rounds up and the cell volume increases 1.5- to 2-fold. This swelling is presumably due to a colloid osmotic mechanism that acts as a result of the loss of the selective ion permeability of the plasma membrane. As shown in Fig. 1, *T. b. gambiense* incubated with HGPS and the FBS component started swelling at about 20 min and rounded up at 60 min. Although the morphological changes leading to the final lysis were the same as those of trypanolysis by human serum, the intrinsic mechanism by which HGPS and the FBS component or cysteine kills trypanosomes remains unclear.

Trypanosomes that were incubated with HGPS and the FBS component for 15 min, then washed, were well viable during a further incubation in the fresh assay medium. However, trypanosomes that were incubated with HGPS and the FBS component for more than 20 min were killed during a further incubation after washing (Data not shown). This is

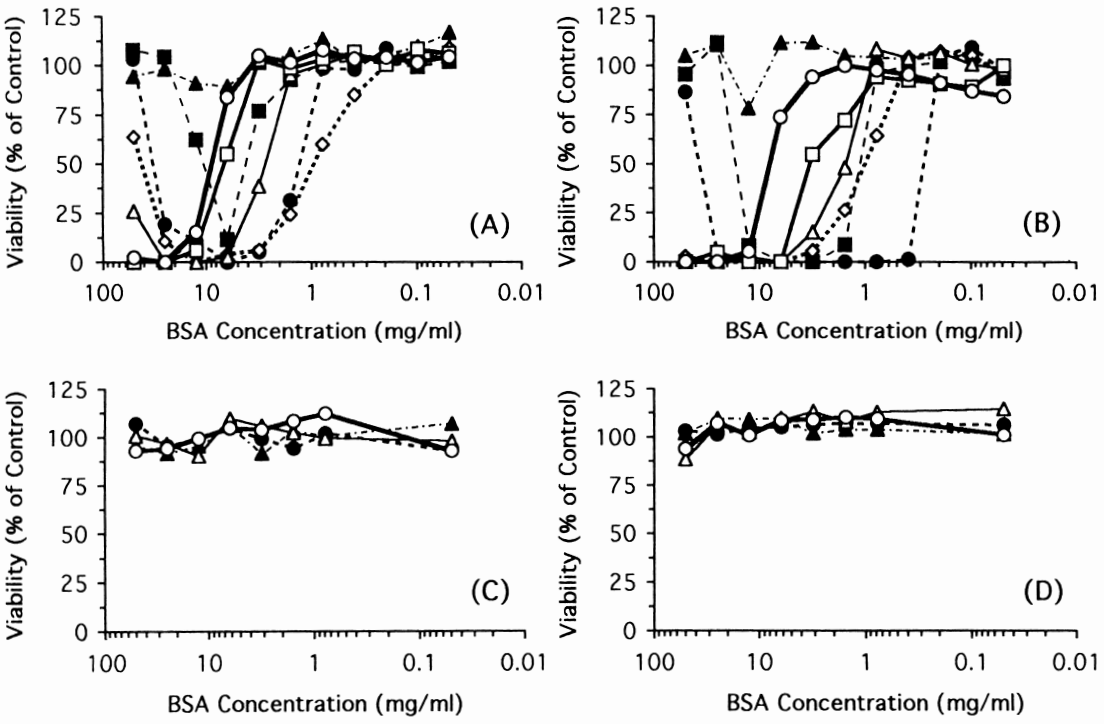

Fig. 7 Trypanocidal activity of BSA in the presence of cysteine derivatives. The horizontal axis (logarithmic scale) indicates the concentration of BSA expressed as mg/ml. Final concentrations of cysteine derivatives in the assay medium are as follows (μg/ml): ○, 250; □, 125; △, 62.5; ◇, 31.3; ●, 15.6; ■, 7.8; ▲, 3.9. (A), L-cysteine methyl ester; (B), L-cysteine ethyl ester; (C), S-methyl-L-cysteine; (D), glutathione. Other explanations are as described in the legend to Fig. 2.

coincident with the trypanolysis caused by human serum (Rifkin, 1984).

In this study, we defined the death of trypanosomes as a complete loss of motility. The trypanocidal activity was evaluated as the viable number of trypanosomes at a 1 hr incubation. Because we examined the activity by electrophoresis in 2% agarose gels, fragmentation of the trypanosomal DNA was not evident (Data not shown). Therefore, it seems that the death is not due to a mechanism such as apoptosis.

The trypanosome lytic factor in human serum contains two apolipoproteins that are sufficient to cause lysis of *T. b. brucei in vitro*. These proteins are human haptoglobin-related protein and paraoxonase/arylesterase. The lytic activity seems to be borne by the former and is due to peroxidase activity (Smith *et al.*, 1995). Whether or not the trypanocidal factor in HGPS exhibits peroxidase activity remains unknown.

Previously, we detected a slight activity of peroxidase in the fraction exhibiting trypanocidal activity in the presence of FBS that was separated using DEAE-Sepharose from NGPS (Funato *et al.*, 1993). Although further studies are required to determine the central cause to mediate the trypanocidal activity, conventional peroxidases in guinea pig serum seems irrelevant to the activity. The activities of such peroxidases are considerably high in NGPS or HGPS. However, the FBS component or cysteine are required to generate trypanocidal activity. The total activities of peroxidase in several lots of whole guinea pig serum were not necessarily parallel to the titers of trypanocidal activity, and the peroxidase activity in HGPS was lower than that in NGPS. In addition, some peroxidases are hemoproteins that are generally inhibited by 1×10^{-3} M of sodium azide (Dixon and Webb, 1964).

However, the trypanocidal reaction mediated by HGPS and the FBS component were not inhibited by doses of sodium azide above 1×10^{-3} M (Data not shown).

HGPS needs the FBS component or cysteine to exert the trypanocidal activity. The guinea pig serum seems to contain two factors. One was precipitated by ammonium sulfate and the other was not. The FBS component has the effect only on the former, whereas cysteine affected both. The FBS component has not yet been identified. In a preliminary experiment using gel filtration, the estimated molecular weight of the FBS component was smaller than that of cysteine if the interaction of the component with the gel is ignored.

Ruminant sera kill African trypanosomes in the presence of exogenous spermidine. Purified polyamine oxidase in the presence of spermidine mediates similar killing of trypanosomes (Ferrante *et al.*, 1982). The killing mechanism seems to be the production of a toxic aldehyde from spermidine. As we examined, spermidine did not affect HGPS (Data not shown). Moreover, trypanosomes that were incubated in the presence of the FBS component were not killed by HGPS that was kept in dialysis tubing. This suggests that the killing of trypanosomes by HGPS and the FBS component is not due to the production of toxic materials with a small molecular mass and that contact of the HGPS with trypanosomes is necessary for the killing.

The role of the FBS component in the process of killing is not that of a direct attack. When HGPS was incubated with the FBS component for 1 hr at 37°C, then extensively dialyzed against the assay medium to remove the FBS component, HGPS exhibited trypanocidal activity without adding the FBS component (Data not shown). Therefore, the FBS component reacts with HGPS and nominally activates it. However, the FBS component does not react with NGPS.

The role of cysteine in the killing process mediated by HGPS is unclear. Cysteine itself did not affect the viability of trypanosomes at concentrations below 500 μg/ml. The morphological anlaysis of the killing process by HGPS and cysteine indicated that trypanosomes were swollen and punctured (Fig. 1D). Therefore, the final result brought about by HGPS and cysteine seems to be the same as that caused by HGPS and FBS component. However, the action of cysteine to HGPS seems to be different from that of FBS component, because incubating HGPS with cysteine did not produce the HGPS with trypanocidal activity (Data not shown). Cysteine seems to play a role when it coexists with HGPS.

The effect of cysteine on HGPS is not solely due to its sulfhydryl group. L-cysteine methyl ester, L-cysteine ethyl ester, the reduced form of glutathione (Figs. 4A and 4B) and DL-homocysteine (Data not shown) did not induce trypanocidal activity in HGPS. We found that L-cysteine methyl ester or L-cysteine ethyl ester rather inhibits the killing of trypanosomes by the heated ammonium sulfate-precipitable fraction of NGPS and the FBS component (Data not shown).

The factor in NGPS that is nominally activated by the FBS component was precipitated by ammonium sulfate (Fig. 5A). The factor at least in normal serum is very stable, but when isolated from the precipitate or NGPS by chromatography using DEAE-Sepharose (Funato *et al.*, 1993), it becomes highly unstable. Heating NGPS, the ammonium sulfate-precipitable fraction or the fraction of the above chromatography at 56°C is essential for the generation of trypanocidal activity in the presence of the FBS component (Fig. 5A) or cysteine (Fig. 5B). It is unknown whether or not the heat denatures the factor or inactivates the concomitant inhibitor. The killing of trypanosomes by the heated ammonium sulfate-precipitable fraction and the FBS component is inhibited by NGPS or the unheated fraction (Data not shown). The FBS component also did not react with NGPS. These features again suggested that the trypanocidal factor in the ammonium sulfate-precipitable fraction of NGPS is not the polyamine oxidase identified in ruminant sera (Ferrante *et al.*, 1982), or the haptoglobin-related protein in human serum that has peroxidase activity (Smith *et al.*, 1995) and of which the trypanolytic activity is inhibited by haptoglobin (Smith and Hajduk, 1995).

The other factor in the ammonium sulfate-unprecipitable fraction exhibited trypanocidal activity in the presence of cysteine, but not in that of the FBS component. The unheated fraction also exhibited trypanocidal activity, though to a lesser

extent than that of the heated fraction (Fig. 5B). It therefore seems that the factor is different from that in the ammonium sulfate-precipitable fraction. The most likely candidate is albumin.

We found that BSA exhibited the trypanocidal activity in the presence of cysteine, but not in that of the FBS component (Fig. 6). The trypanocidal activity was also generated in the presence of L-cysteine methyl ester or L-cysteine ethyl ester (Figs. 7A and 7B). The sulfhydryl groups in these molecules seem important for generating the activity, because S-methyl-L-cysteine was not effective (Fig. 7C). However, some molecular feature seems essential to the generation, because the reduced form of glutathione was ineffective (Fig. 7D).

Although the most of the characteristics of the factor in the ammonium sulfate unprecipitable fraction of NGPS seemed to be represented by BSA, there were some contradictions. One was that NGPS did not exhibit trypanocidal activity even in the presence of cysteine when unheated. This was resolved by dialyzing the NGPS. Extensively dialyzed NGPS exhibited apparent trypanocidal activity in the presence of cysteine (Data not shown). Why cysteine esters are ineffective upon HGPS remains to be resolved. The elucidation of trypanokilling mechanisms of serum components is important to controlling trypanosomiasis.

References

1) Dixon, M. and Webb, E. C. (1964): Enzyme inhibitors. In Enzymes, Longmans Green, and Co Ltd, London, 315–359.
2) Ferrante, A., Allison, A. C. and Hirumi, H. (1982): Polyamine oxidase-mediated killing of African trypanosomes. Parasite Immunol., 4, 349–354.
3) Funato, T., Komatsu, T., Saeki, N. and Shinka, S. (1993): Trypanolytic factor and its inhibitor in normal guinea pig serum. Jpn. J. Parasitol., 42, 95–104.
4) Hajduk, S. L., Englund, P. T. and Smith, D. H. (1990): African Trypanosomiasis. In Tropical and Geographical Medicine, 2nd ed., Warren, K. S. and Mahmoud, A. A. F., ed., McGraw-Hill, Inc., New York, 268–281.
5) Hajduk, S. L., Moore, D. R., Vasudevacharya, J., Siqueira, H., Torri, A. F., Tytler, E. M. and Esko, J. D. (1989): Lysis of *Trypanosoma brucei* by a toxic subspecies of human high density lipoprotein. J. Biol. Chem., 264, 5210–5217.
6) Lanham and Godfrey, D. G. (1970): Isolation salivarian trypanosomes from man and other mammalian using DEAE-cellulose. Exp. Parasitol., 28, 521–534.
7) Laveran, A. (1902): De L'action du sèrum humain sur le Trypanosome du Nagana (*Trypanosoma brucei*). C. R. Acad. Sci., 134, 735–739.
8) Rickman, L. R. and Robson, J. (1970): The testing of proven *Trypanosoma brucei* and *T. rhodesiense* strains by the blood incubation infectivity test. Bull. W. H. O., 42, 911–916.
9) Rifkin, M. R. (1978): Identification of the trypanocidal factor in normal human serum: High density lipoprotein. Proc. Natl. Acad. Sci. U. S. A., 75, 3450–3454.
10) Rifkin, M. R. (1984): *Trypanosoma brucei*: Biochemical and morphological studies of cytotoxicity caused by normal human serum. Exp. Parasitol., 58, 81–93.
11) Smith, A. B., Esko, J. D. and Hajduk, S. L. (1995): Killing of trypanosomes by the human haptoglobin-related protein. Science, 268, 284–286.
12) Smith, A. B. and Hajduk, S. L. (1995): Identification of haptoglobin as a natural inhibitor of trypanocidal activity in human serum. Proc. Natl. Acad. Sci. U. S. A., 92, 10262–10266.