Studies on the Surface Component of *Trypanosoma cruzi* Amastigotes: Digestion of the Specific Surface Antigen by Pronase

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Abstract: The first fraction of soluble amastigote antigen through Sephadex G-200 which was determined to contain the specific surface antigen to amastigote in the previous experiment was digested by pronase to examine the antigenic character of the carbohydrate moiety. The digested product was applied to Sephadex G-50 column after deproteinization by 7% TCA. The first peak from Sephadex G-50 showed the precipitin line against antiamastigote serum and the line, of course, shared common antigenicity with the specific antigen with a small spur from the latter to the former. This peak, probably polysaccharide with small peptides, was further purified by DEAE-cellulose column chromatrography.

Key words: *Trypanosoma cruzi*, Amastigote, Surface antigen, Polysaccharide

INTRODUCTION

Three different developmental stages of *Trypanosoma cruzi* possess distinct surface antigens. In amastigotes and trypomastigotes, the surface components play an important role in infection to mamma's, while epimastigotes are lysed by normal fresh sera from various mammals through alternate pathway of complement (Muniz and Borriello, 1945; Nogueira et al, 1975) and fragile to macrophage phagocytosis (Nogueira and Cohn, 1976; Tanaka et al, 1982). Some surface components of trypomastigotes are easily digested by trypsin because trypsin treatment make them susceptible to complement lysis (Kipnis et al, 1981) and also make blood trypomastigotes susceptible to macrophage phagocytosis (Nogueira et al, 1980). On the other hand, those of amastigotes are not affected by trypsin although they are affected by pronase and hyaluronidase (Kanbara et al, 1983). A predominant surface antigen specific to amastigotes was determined in the first fraction of amastigote extract through Sephadex G-200, which is easily precipitated in high concentration (Kanbara et al, 1983). These facts suggest surface antigen consists of large

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amount of sugar. In the present experiment, the first fraction is digested by pronase to examine the antigenic quality of carbohydrate.

MATERIALS AND METHODS

1. *Parasite*

The Tulahuen strain of *T. cruzi*, obtained in 1971 from the National Institute of Health, USA through Keio University, Japan and subsequently maintained in mice and tubes, was used.

2. *Amastigotes*

Blood-form trypomastigotes were isolated from mice on day 10 of infection, and trasferred into the monophasic medium consisting of 9 parts of base solution (1% trypticase, 0.8% NaCl and 0.5% glucose in distilled water) and 1 part of whole rabbit blood, which had been hemolysed in equal volume of distilled water and sterilized by filtration after centrifugation. The mixture was incubated at 25°C. More than 90% of amastigotes were obtained from the culture mentioned above during several passages after the initial transfer (Kanbara et al, 1974). These were used for immunization of rabbits. Amastigotes grown in L-cells cultured in MEM medium containing 10% calf serum, were used for preparation of amastigote antigen (Am-Ag). When about 80% of L-cells were infected with parasites, the medium was changed to TCM 199 medium free from calf serum. After cultivation for 3 days, amastigotes in the overlay medium which had a tendency to agglomerate, were collected by centrifugation at 150g for 5 min and then they were washed three times with saline by centrifugation at 1,300g for 10 min. This step was repeated twice or three times until almost all the host cells had been destroyed.

3. *Antigen preparation of amastigotes*

1) Soluble amastigote antigen (S-Am-Ag)

Amastigotes collected from L-cell culture by the method mentioned above were sonicated at 20kc/s, at 150W for 3 min using Sonore 150 S (Umeda Co.) and centrifuged at 10,000g for 60 min. The supernate was used as S-Am-Ag

2) Fractionation of S-Am-Ag through a Sephadex G-200 column

The first fraction of S-Am-Ag (Fl-S-Am-Ag) was shown to contain a specific antigen to amastigotes as previously reported (Kanbara et al 1983).

3) Digestion of Fl-S-Am-Ag by pronase

Concentrated Fl-S-Am-Ag was incubated in pronase (Pronase E, Kaken chemical Co.) solution (·1 mg/ml in 0.1M Tris buffer, pH 8.0 with 0.05M Ca^{++}) at 37°C for 72hr. The same amount of pronase powder as that in the initial solution was added and pH was adjusted to 8.0 by 1N NaOH every 24 hr. After 72 hr incubation, 50% trichloroacetic acid (TCA) was gradually added to make a final concentration of 7% for the purpose of deprotenization. After centrifugation the supernatent was applied to the next step.

4) Analyzation of digested product of Fl-S-Am-Ag by pronase

The supernatent after TCA treatment was then fractionated through a Sephadex G-50 column. Elution pattern was traced by measuring sugar amount using phenol-sulfuric acid reaction. Immunodiffusion on agar was carried out between each fraction and anti-amastigote serum to find which fraction contained a specific antigen. The fraction containing a specific antigen was further analyzed on DEAE-cellulose using stepwise elution. The following procedure was same as mentioned above.

4. *Anti-amastigote serum (Anti-Am-S)*

About 2×10^8 amastigotes obtained from the monophasic medium were mixed with Freund's complete adjuvant, and injected intramuscularly into a rabbit. They were inoculated three times with intervals of two weeks between inoculation. On day 42 after the first inoculation, about 10^8 amastigotes were injected intramuscularly as a booster and blood was taken on day 49.

5. *Immunological assay*

Immunodiffusion and immunoelectrophoresis were performed with Anti-Am-S and fractionated samples from digested Fl-S-Am-Ag with pronase.

6. *SDS-polyacrylamide gel electrophoresis (SDS-PAGE)*

S-Am-Ag, Fl-S-Am-Ag and the fraction from Sephadex G-50 which was found to contain a specific antigen were applied to SDS-PAGE using 7.5% polyacrylamide gel. Coomassi-blue stain and PAS stain were used to determine protein and carbohydrate respectively.

RESULTS

In the previous experiment (Kanbara et al 1983), the first fraction of soluble amastigote antigen (Fl-S-Am-Ag) was shown to contain the specific surface antigen to amastigotes which was suggested to consist of glycoprotein with large amount of sugar by various results. In the present experiment, Fl-S-Am-Ag was digested by pronase to examine the antigenicity of carbohydrates. The elution pattern through a Sephadex G-50 column of digested products of Fl-S-Am-Ag by pronase, which was deproteinized by 7% TCA before application to the column, was as shown on Fig. 1. Immunodiffusion test proved that the first peak produced the precipitin line against Anti-Am-S which was, of course, shared with Fl-S-Am-Ag except for a small spur from the latter to the former (Photo. 1.). The spur was probably caused by a digested protein part. Therefore, the molecular size of sugar in the first peak was big enough to express antigenicity and had molecular weight more than 10,000. This was so because the fraction was concentrated and dialyzed using a cellulose tube (Union Carbide Co. 8/32), that is, the specific surface antigen to amastigote consisted of at least protein and polysaccharide, both of which possessed antigenic capacity. Further analysis through DEAE-cellulose by stepwise elution

showed that the eluate by 0.4M sodium phoshate (monobasic) contained the precipitin antigen (Fig. 2). Immunoelectrophoresis showed the mobility of the specific antigen was not changed by pronasedigestion; both precipitin arcs of Fl-S-Am-Ag and the first peak of digested product through Sephadex G-50 appeared near the original portion (Photo. 2). On SDS-PAGE, the samples from S-Am-Ag and Fl-S-Am-Ag showed immobile protein containing considerable amount of carbohydrate. This was expected from their high molecular weights estimated by the elution pattern from Sephadex G-200. The nature of digested product could not be determined because it could not be fixed on gel using fixatives such as methanol, acetic acid and TCA (Photo. 3).

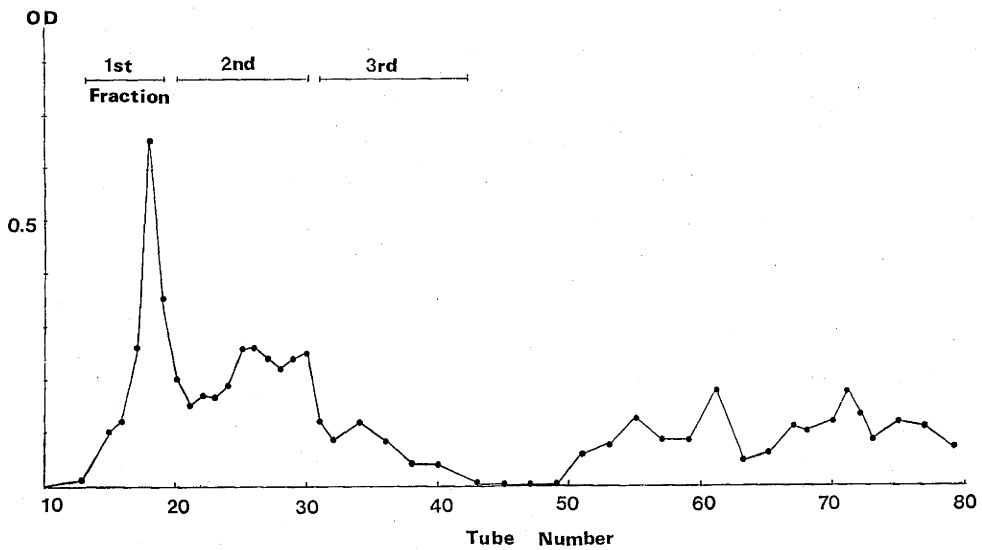

Fig. 1. Column chromatogram of digested product on Sephadex G-50. The first fraction of soluble amastigote antigen through Sephadex G-200 was digested by pronase and applied to a Sephadex G-50 column after deproteinization by 7% TCA. Distilled water was used as eluate. The elution pattern was traced by measuring sugar amount by the phenol-sulfuric acid reaction (480nm). The first fraction contained the specific antigen to amastigotes.

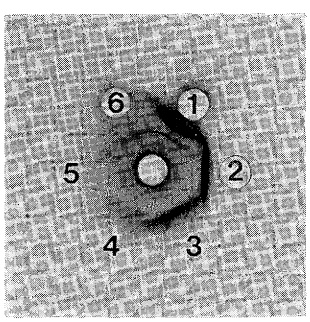

Photo. 1. Immunodiffusion test of soluble amastigote antigen (S-Am-Ag, well 1), the first fraction of S-Am-Ag through Sephadex G-200 (Fl-S-Am-Ag, well 2) and the first fraction (well 3), the second fraction (well 4) and the third fraction (well 5) of digested product of Fl-S-Am-Ag by pronase through Sephadex G-50 and the soluble epimastigote antigen (well 6) against anti-amastigote serum (the center well) which had been absorbed once with epimastigotes.

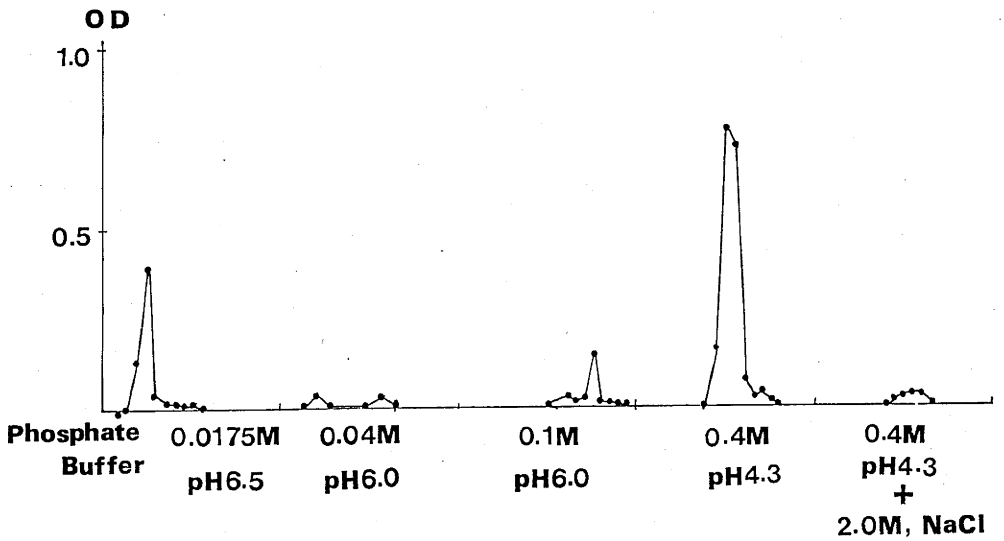

Fig. 2. Column chromatogram of the first fraction from Sephadex G-50 on DEAE-cellulose. The pattern of stepwise elution was traced by measuring sugar. The eluate by 0.4M sodium phosphate (monobasic) contained the specific antigen.

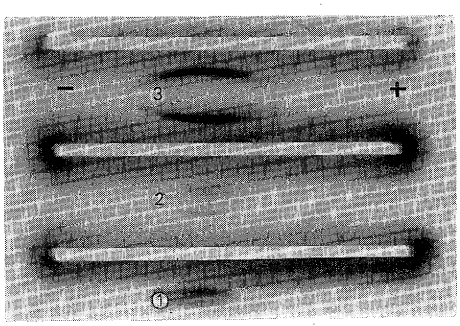

Photo. 2. Immunoelectrophoresis of soluble amastigote antigen (S-Am-Ag, well 1), the first fraction of S-Am-Ag through Sephadex G-200 (Fl-S-Am-Ag, well 2) and the first fraction of digested product of Fl-S-Am-Ag by pronase through Sephadex G-50 (well 3) against anti-amastigote serum which had been absorbed once with epimastigotes.

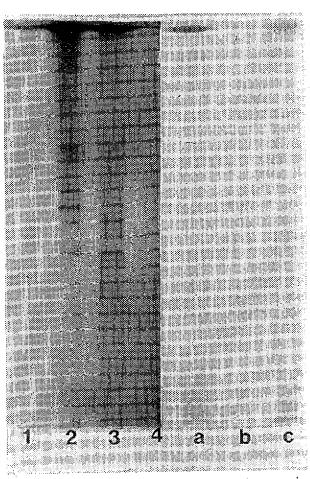

Photo. 3. SDS-PAGE of soluble amastigote antigen (S-Am-Ag, lane 2 and a), the first fraction of S-Am-Ag through Sephadex G-200 (Fl-S-Am-Ag, lane 3 and c) and the first fraction of digested product of Fl-S-Am-Ag by pronase through Sephadex G-50 (lane 1, 4 and b). Lane 1, 2, 3 and 4 were stained with Coomassie Brilliant Blue and lane a, b and c were stained with PAS. Both S-Am-Ag and Fl-S-Am-Ag showed PAS positive in immobile part, whereas the digestive product showed nothing because of its inability of fixation.

DISCUSSION

Amastigotes of the Tulahuen strain of *T. cruzi* are adhesive and tend to agglomerate in both culture with and without host cells. Thick surface layer of PAS positive materials was shown on amastigotes by histochemical electronmicroscopy (Kanbara et al, 1983). At the same time, the predominant surface antigen specific to amastigotes was determined as high molecular component through Sephadex G-200, which was easily precipitated in high concentration. The present result showed that this specific antigen consisted of protein-polysaccharide complex. The polysaccharide had a molecular weight of more than 10,000 and shared common antigenicity with the specific antigen. Considering the general character of protein-polysaccharides the various characters of the cell surface of amastigote found in the Tulahuen strain may be well explained.

It is clear that the antigenic polysaccharide originated from amastigotes and not from host-cells ; This was so because the Anti-Am-S was obtained from a rabbit immunized with amastigotes grown in cell-free medium and also this antibody was predominant in rabbit infected with trypomastigotes (Kanbara et al, 1983). The isolated polysaccharide itself, however, failed to induce precipitating antibody from immunized rabbits, therefore protein complex is important for effective immunization.

It is doubtful whether these findings apply to other strains of *T. cruzi*. Some strains didn't show amastigote growth in tube after transfer from infected mice as the Tulahuen strain (Brener and Chiari 1965). Electronmicroscopic observation also showed different carbohydrate existence on tissue stage parasites from ours ; Mehlhorn et al (1977) demonstrated a fuzzy layer of carbohydrates on the late epimastigote and the trypomastigote forms within the muscle fiber of experimentally infected mice with Ecuador strain, while the amastigote and the early epimastigote forms were not provided with it. Souza and Meyer (1975) showed a polysaccharide surface coat in the various developmental stages of *T. cruzi* maintained in tissue culture for more than 15 years after isolation from a patient in Brazil.

Binding of lectins to cell surface of *T. cruzi* also showed different character both among strains and among developmental stages (Araujo et al 1980).

Araujo and Remington (1981) reported that demonstrable differences among three strains were not observed in the amastigote or epimastigote stages but in the trypomastigote using radioiodination of surface protein, and that serum from rabbits infected with trypomastigotes or epimastigotes and a serum from a human chronically infected with *T. cruzi* precipitated a significant amount of surface antigens of amastigotes. Although the specific antigen to amastigote detected by us was characteristically quite different from those by Araujo and Remington, both caused strong antibody formation in the infected rabbits or human. If the hypersensitivity to parasite antigen play an important role in pathogenesis of Chagas'disease as suggested by some authors (Teixeira et al, 1975 ; Tafuri, 1979), surface antigens on amastigotes may relate with variety of pathological

lesions. Besides protective role of surface component for amastigotes to survive in hosts, the relationship between surface antigen and pathological cause should be investigated.

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Trypanosoma cruzi, Amastigote の細胞表面構成々分について：特異表面抗原のプロナーゼによる消化実験

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先の実験により *Trypanosoma cruzi* の Amastigote の特異表面抗原が Amastigote 可溶性抗原を Sephadex G-200にて分画した第1分画に認められることが示された．多くの特徴より多量の糖が含まれることが予想されたので，この第1分画をプロナーゼ処理して蛋白部分を消化分解し，残存する糖部分についてその抗原性を追求した．プロナーゼ消化後，7％ TCA にて除蛋白を行い Sephadex G-50 にて分画した．この分画による第1のピークに Amastigote 特異抗原性を認めた．この分画は更に DEAE-cellulose によって純化された．この蛋白分解による糖ペプチド部分が抗原性を示す十分な大きさであるところから Amastigote 特異表面抗原は少くとも蛋白，多糖の複合体であることが示され，そのどちらにも抗原性のあることはプロナーゼ処理前の抗原が処理後のものと共通抗原性をもち，しかも小さな Spur を形成することから理解できる．

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