FULL PAPER *Parasitology*

Novel Species Specific Antigens of *Trypanosoma congolense* and Their Different Localization among Life-Cycle Stages

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ABSTRACT. Seven monoclonal antibodies (mAbs) were raised against *Trypanosoma congolense* procyclic form (PCF). Localization of the antigens recognized by the mAbs was determined in bloodstream form (BSF), PCF, epimastigote form (EMF) and metacyclic form (MCF) by confocal laser scanning microscopy (CLSM). Two mAbs (10F9 and 20H12) showed different fluorescent patterns among different life-cycle stages of the parasite. The 10F9 recognized a 76 kDa antigen of all life-cycle stages of the parasite and the antigen localization corresponded with that of a mitochondrion. While the 20H12 recognized 119 and 122 kDa antigens of all the life-cycle stages and the antigen localization corresponded with a flagellum in BSF and MCF, tip of a flagellum in PCF, and part of cytoplasm in EMF. Moreover, the 20H12 did not react to *T. brucei gambiense*, *T. b. rhodesiense* and *T. evansi* antigens in both CLSM and immunoblotting. Therefore, the antigens recognized by the 20H12 seem to be *T. congolense* specific. Although, further studies will be required for a full characterization of the *T. congolense* specific 119 and 122 kDa antigens, the mAb 20H12 and the specific antigens may be useful in not only establishment of *T. congolense* specific diagnosis methods but also studies on molecular mechanisms regulating differentiation of the parasite during life-cycle.

KEY WORDS: confocal laser scanning microscope, life cycle, monoclonal antibody, species specific antigen, *Trypanosoma congolense*.

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Trypanosoma congolense, which is a causative agent of Nagana, is biologically transmitted by tsetse flies (*Glossina* spp.). When bloodstream forms (BSFs) were ingested along with blood meals by a tsetse fly, they undergo morphological and biochemical changes, and differentiate to insect forms [17]. Many attempts have been made to identify life-cycle stage and/or species specific antigens in African trypanosomes. However, only a few such specific antigens have been identified in African trypanosomes [1, 2, 6, 12, 13, 15]. In *T. congolense*, a 31 kDa antigen and a glutamate- and alanine-rich protein have been reported as a species specific antigen and a life-cycle stage specific antigen, respectively [1, 2, 13]. Nantulya *et al.* [12] also reported that a monoclonal antibody (mAb) which specifically reacted with *T. congolense* and *T. simiae*. For clinical, epidemiological and biological reasons, it is important to identify such specific antigens. Species specific antigens may help to distinguish one trypanosome species from another. While, life-cycle stage specific antigens may be useful to clarify complicated mechanisms regulating cellular differentiation of tsetse-transmitted trypanosomes during their life cycle. In this study, 7 mAbs including mAbs against novel *T. congolense* species specific antigens and a 76 kDa cytoplasmic antigen were produced, and antigen localization analysis by confocal laser scanning microscopy (CLSM) revealed that the species specific antigens and the 76 kDa antigen were differentially localized among life-cycle stages of *T. congolense*.

MATERIALS AND METHODS

Parasites: Trypanosomes used in this study were obtained from the International Livestock Research Institute, Nairobi, Kenya and maintained in our institute.

In vitro cultivation of T. congolense insect forms: *Trypanosoma congolense* IL3000 insect forms (procyclic form (PCF), epimastigote form (EMF) and metacyclic form (MCF)) were cultured by using the procedure of Hirumi and Hirumi [5]. Briefly, BSFs were obtained from an infected female BALB/c mouse (Japan CLEA Inc., Japan). BSFs were separated from the blood cells by a low speed centrifugation at 100 × g for 7 min. Then the concentration of BSFs was adjusted to 3×10^6 cells/m*l* in Eagle's minimum essential medium supplemented with 20% fetal bovine serum, 2 mM L-glutamine and 10 mM L-proline. Parasite suspension was transferred to 25 cm^2 culture flasks (5 m*l*/flask) and the flasks were kept in an incubator at 27°C for a week in order to transform BSF to PCF. PCFs were maintained by changing medium and making subcultures. After 1 to 2 months, adherent EMFs appeared in the bottom surface of flasks as small clusters. The clusters increased in size and number, and finally covered the whole bottom surface. MCFs also appeared in culture supernatant of the confluent EMF cultures.

Isolation of the parasite: BSFs and MCFs were obtained from infected mouse blood and EMF culture supernatant, respectively. BSFs and MCFs were isolated by using a DEAE anion exchange column [9]. PCFs were obtained from culture supernatant by centrifugation at 1,500 × g for 10 min at 4°C. Then PCFs were washed 3 times with phosphate-buffered saline (PBS) by centrifugation as mentioned above. EMFs were collected from confluent culture flasks by scraping. Briefly, the culture flasks were washed 3 times

with 10 m*l* of PBS in order to remove PCFs and MCFs. Then EMFs were removed by scraping from the bottom surface of flasks and suspended in PBS. EMFs were washed 3 times with PBS by centrifugation at 1,500 × g for 10 min at 4°C.

Monoclonal antibody production: PCFs were suspended in PBS at a concentration of 100 mg/m*l* and homogenized by using ultrasonic homogenizer at 4°C (VP-15S, TAITEC Co., Japan). Eight weeks old female BALB/c mice were immunized intraperitoneally with the homogenate (100 μg/head) in Freund's complete adjuvant followed by 3 booster immunizations (14 day intervals) with the homogenate in Freund's incomplete adjuvant (FIA). Three days after intravenous inoculation of the homogenate, hybridoma cells were produced by the procedure of Kühler and Milstein [7]. Hybridoma culture supernatants were screened for antibodies to the homogenate by enzyme-linked immunosorbent assay. Cloned hybridoma cells secreting mAbs were inoculated into BALB/c mice previously injected with FIA to induce ascites formation [11]. Subsequent experiments were performed by using supernatants of hybridoma cell cultures or the ascites. Isotype of each mAb was determined by using kit (Mouse monoclonal antibody isotyping kit, Amersham Pharmacia Biotech Ltd., Japan).

Immunoblotting: The parasites were sonicated in a sample buffer (2% sodium dodecyl sulfate (SDS), 62.5 mM Tris HCl pH 6.8, 5% (v/v) 2-β mercaptoethanol, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue) at a concentration of 1×10^5 parasites/μl and were heated at 100°C for 5 min. Then 5 μl/lane of the samples were subjected to electrophoresis in 10% SDS polyacrylamide gel as described by Laemmli [8]. After the electrophoresis, separated proteins were transferred onto a PVDF membrane (Immobilon-P, Millioipre Ltd., Japan). Then the membrane was blocked in PBS containing 5% skim-milk and normal goat serum for 12 hr at 4°C and incubated with a mAb for 2 hr at room temperature. After 3 times washings with Tween 20 (0.05% w/v)-PBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Bio-Rad Laboratories, U. S. A.) for 2 hr at room temperature. Antigen-antibody reactions were visualized by incubation with 0.25 mg/m*l* of diaminobenzidine and 0.005% H_2O_2.

Indirect Immunofluorescence assay: BSFs, PCFs, EMFs and MCFs were fixed with methanol at 4°C for 10 min. The parasites were incubated with a mAb at 37°C for 30 min, and then stained with fluorescein-conjugated goat anti-mouse IgG (ICN Pharmaceuticals Inc., U. S. A.). After 3 times washings with PBS, nucleus and kinetoplast were stained by propidium iodide (10 μg/m*l*)-PBS. To stain a mitochondrion, live trypanosomes were incubated with 5 μM of MitoTracker® Red CM-H_2XRos (Molecular Probes Inc., U. S. A.) for 40 min. The parasites were fixed with acetone for 5 min at room temperature. Then indirect immunofluorescence staining was performed as described above. Results were observed by using confocal laser scanning microscope (Leica TCS-NT, Leica Microsystems, GmbH).

RESULTS

Characterization of mAbs: Seven clones of hybridoma cells secreting mAb against *T. congolense* were established (Table 1). All mAb reacted with all life-cycle stages of *T. congolense* by CLSM (Table 2) and immunoblotting (data not shown). Thus the mAbs were not life-cycle stage specific antibodies. Nevertheless, antigen localization analysis by CLSM revealed that the antigens recognized by 10F9 and 20H12 were differentially localized in each life-cycle stage

Table 1. Molecular weight and localization of antigens recognized by anti-*T. congolense* monoclonal antibodies

Monoclonal antibody	Isotype	Molecular weight (kDa)[a]	Localization[b]
1C6	IgG1	65	C[c]
4D4	IgG2a	37	PC[d]
4H1	IgG1	41, 44, 54, 61, 66	C[c]
10F9	IgG1	76	M[e]
16C6	IgG1	Ladder	C[c]
20H12	IgG3	119, 122	BSF: F[f]
			PCF: TF[g]
			EMF: PC[d]
			MCF: F[f]
53DC8	IgG2a	62, 64, 66	F[f]

a) Molecular weight (kilodalton: kDa) was estimated by immunoblot analysis on total cell extract of *T. congolense* PCF under reducing condition.
b) Antigen localization was determined by confocal laser scanning microscopy on methanol fixed *T. congolense* bloodstream form (BSF), procyclic form (PCF), epimastigote form (EMF) and metacyclic form (MCF). Except for 20H12, all monoclonal antibody showed the same fluorescent pattern against BSF, PCF, EMF and MCF.
c) Cytoplasm.
d) Part of cytoplasm.
e) Mitochondrion.
f) Flagellum.
g) Tip of a flagellum.

Table 2. Life-cycle stages and species specificity analysis of anti-*T. congolense* monoclonal antibodies by indirect immunofluorescence assay

Parasite	Monoclonal antibody						
	1C6	4D4	4H1	10F9	16C6	20H12	53DC8
T. congolense IL3000 BSF	+	+	+	+	+	+	+
T. congolense IL3000 PCF	+	+	+	+	+	+	+
T. congolense IL3000 EMF	+	+	+	+	+	+	+
T. congolense IL3000 MCF	+	+	+	+	+	+	+
T. evansi IL3960 BSF	+	+	+	+	+	–	+
T. b. gambiense IL3253 BSF	+	+	+	+	+	–	+
T. b. rhodesiense IL2343 BSF	+	+	+	+	+	–	+

BSF, PCF, EMF and MCF indicate bloodstream form, procyclic form, epimastigote form and metacyclic form, respectively.
"+" and "-" indicate positive and negative in indirect immunofluorescence assay, respectively.

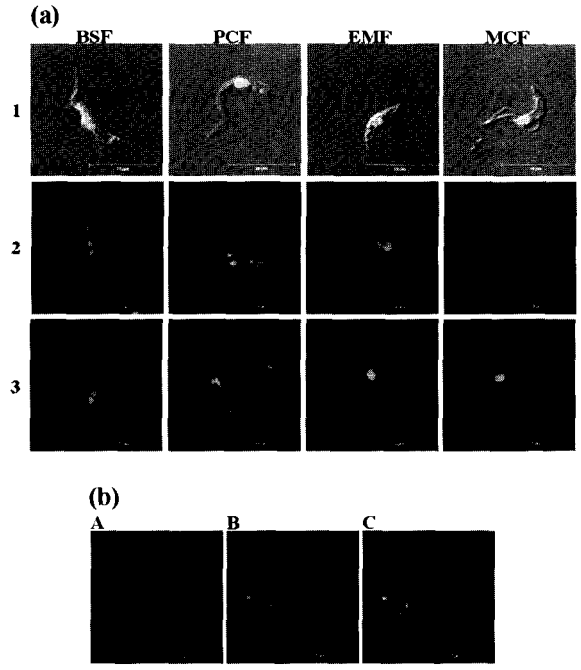

Fig. 1. (a) Confocal laser scanning microscopy using monoclonal antibodies 10F9 and 20H12. Typical parasite of each life-cycle stage was presented in line 1. Methanol fixed bloodstream form (BSF), procyclic form (PCF), epimastigote form (EMF) and metacyclic form (MCF) of *Trypanosoma congolense* were stained by using 10F9 (line 2) or 20H12 (line 3). Nucleus and kinetoplast were stained by propidium iodide. (b) Comparison between immunofluorescent pattern of monoclonal antibody 10F9 and localization of a mitochondrion using confocal laser scanning microscopy. A procyclic form of *T. congolense* was stained by using 10F9 (A) and MitoTracker® Red CM-H_2XRos (B). Panel C is overlay-image of panels A and B.

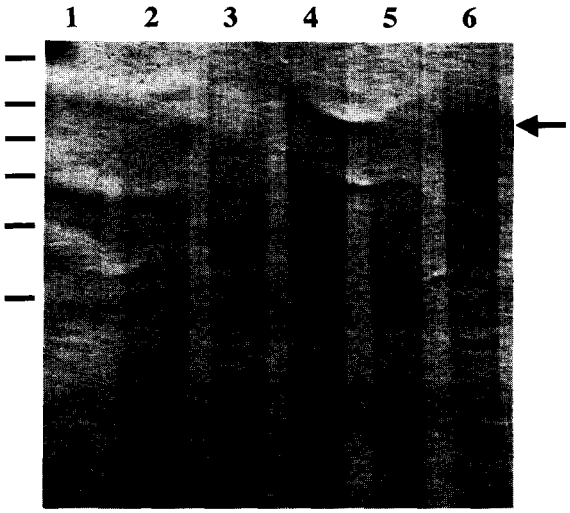

Fig. 2. Immunoblot analysis of species specific antigens recognized by monoclonal antibody 20H12. Whole cell extract of *Trypanosoma evansi* IL3960 bloodstream forms (BSFs) (lane 1), *T. evansi* IL3254 BSFs (lane 2), *T. brucei gambiense* IL3253 BSFs (lane 3), *T. b. rhodesiense* IL2343 procyclic forms (PCFs) (lane 4) and BSFs (lane 5), and *T. congolense* IL3000 PCFs (lane 6) were subjected to immunoblotting. An arrow indicates antigens recognized by 20H12. Molecular weight standards indicated by bars are: 212, 170, 116, 76, 53, 43 kilodalton.

(Fig. 1a). 10F9 recognized a 76 kDa antigen and its localization corresponded with that of a mitochondrion by CLSM (Table 1 and Fig. 1b). The fluorescence pattern of 20H12 was also dramatically changed among the life-cycle stages (Fig. 1a). In BSF and MCF, main fluorescence was localized along a flagellum by CLSM, while, 20H12 strongly recognized tip of a flagellum in PCF and part of cytoplasm in EMF. However, 119 and 122 kDa antigens were repeatedly detected by immunoblot analysis using 20H12 regardless of the life-cycle stages (data not shown). Although clones 1C6, 4H1 and 16C6 strongly reacted with whole cytoplasm of the parasite, each of these mAbs recognized different antigens. 4H1 and 16C6 detected broad bands in immunoblotting of total PCF lysates. 4D4 and 53DC8 reacted cytoplasmic and flagellum antigens, respectively (Table 1).

Species specificity of mAbs: Species specificity of all mAb

was assessed by indirect immunofluorescence staining followed by CLSM. Except for 20H12, all mAb showed cross reactivity against *T. evansi, T. b. gambiense* and *T. b. rhodesiense* tested (Table 2), and there were no differences in terms of antigen localization examined by CLSM (data not shown). Immunoblotting was also carried out in order to clarify species specificity of 20H12. As a result, the mAb did not react whole cell lysates of 2 strains of *T. evansi, T. b. gambiense, T. b. rhodesiense* BSF and *T. b. rhodesiense* PCF (Fig. 2). Control reaction using normal mouse serum did not show any bands in all trypanosome lysates tested (data not shown).

DISCUSSION

Salivarian trypanosomes, such as *T. brucei brucei, T. congolense* and *T. evansi*, cause diseases in domestic animals. These species of trypanosomes have been distinguished by morphology, geographical distribution, host range, and other biological criteria. Recently, molecular biological techniques were also used to distinguish different species of trypanosomes [3, 4, 10, 16]. For molecular biological studies on trypanosomes, life-cycle stage specific antigens such as procyclin and variant surface glycoprotein are also useful to clarify molecular mechanisms regulating cell differentiation of tsetse-transmitted trypanosomes during their life-cycle [14]. However, a few trypanosome stage and species specific antigens have been identified [1, 2, 6, 13, 15].

In the present study, we have established 7 clones of hybridoma cells secreting mAbs against *T. congolense* and identified novel *T. congolense* specific antigens recognized by mAb 20H12. Although we can not exclude that the antigens are strain specific, the antigens are not detected by indirect immunofluorescence assay and immunoblotting in *T. b. gambiense, T. b. rhodesiense* and *T. evansi* tested. Therefore we concluded that the antigens are either absent or different among trypanosome species. Immunoblotting revealed that molecular masses of the antigens are approximately 119 and 122 kDa. Interestingly, intracellular localizations of the antigens are different among life-cycle stages of *T. congolense*. To our knowledge, this is the first report on trypanosome species specific antigens localized different part in each life-cycle stage of the parasite. Although the gene cloning of 119 and 122 kDa antigens is under way, we have obtained one cDNA clone encoding a protein recognized by 20H12 (unpublished data). Further analysis of the cDNA clone may help to understand biological feature of the antigens including differential localization among life-cycle stages of the parasite.

The 76 kDa antigen recognized by mAb 10F9 also changes its localization during life-cycle stages, although it is not species specific antigen. The 76 kDa antigen localizes in a mitochondrion and its differential localization during life-cycle of the parasite clearly represents development of a mitochondrion. Because of cross reactivity to several species of salivarian trypanosomes, the 10F9 may be useful mitochondrial marker of trypanosomes.

In this study, 6 mAbs recognize stable antigens, which are well conserved among different species of trypanosomes. We do not examine trypanocidal or growth inhibitory effect of the mAbs yet. Further studies may help us to explore vaccine candidates against trypanosomosis.

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