Studies on Vaccine Development and Establishment of Diagnosis against African Trypanosomiasis

（アフリカトリパノソーマ症に対する新規ワクチン法の開発および診断法の確立に関する研究）

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The United Graduate School of Veterinary Sciences, Gifu University

(Obihiro University of Agriculture and Veterinary Medicine)

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Chapter 5

Loop-Mediated Isothermal Amplification (LAMP) for the Detection of African Trypanosomes

Abbreviations

A ABTS: 2,2’-azino-di-3-ethylbenzthiazoline-6-sulfonic acid

AMP: antimicrobial peptide

Abs: antibodies

B *B. bigemina: Babesia bigemina*

B. bovis: Babesia bovis

B. caballi: Babesia caballi

BBB: blood brain barrier

BIP: back inner primer

BSA: bovine serum albumin

BSF: bloodstream form

B3: back outer primer

C CATT: card agglutination test for trypanosomiasis

CCK-8: Cell Counting kit-8

cDNA: complimentary DNA

CNS: central nervous system

Con A: concanavalin A

CSF: cerebrospinal fluid

D DNA: deoxyribonucleic acid

DPI: days post-infection

DPPC: dipalmitoylphosphatidylcholine

DPPE: dipalmitoylphosphatidylethanolamine

dCTP: deoxycytidine triphosphate

E EDTA: ethylenediaminetetraacetic acid

ELISA: enzyme-linked immunosorbent assay

EMEM: Eagle's minimum essential medium

F FCS: fetal calf serum

FIP: forward inner primer

F3: forward outer primer

G GIP: glycosylinosytolphosphate

GPI-PLC: glycosylphosphatidylinositol-specific phospholipase C

H HAT: human African trypanosomiasis

HRP: horseradish peroxidase

I IFAT: indirect fluorescent antibody test

IFN: interferon

Ig: immunoglobulin

IL: interleukin

IMDM: Iscove's modified Dulbecco's medium

i. p.: intraperitoneal

L LAMP: loop-mediated isothermal amplification of DNA

M M3: mannotriose

M3-DPPE: M3 conjugated with DPPE

M5: mannopentaose

N NBD-DPPC: 4-fluoro-7-nitro-2,1,3-benzoxadiazole DPPC

N. caninum: Neospora caninum

ND: not determined

P PARP: procyclic acidic repetitive protein

PBS: phosphate buffered saline

PCF: procyclic form

PCR: polymerase chain reaction

PCV: packed cell volume

PFR A: paraflagella rod protein A

PG: prostaglandin

PI: propidium iodide

P0: P0 subunit ribosomal protein

R RNA: ribonucleic acid

RI: radioisotope

RT: room temperature

RT-PCR: reverse-transcribed PCR

S s.c.: subcutaneously

S. calcitrans: *Stomoxys calcitrans*

SCID: severe combined immunodeficiency

SD: standard deviation

SDS: sodium dodecyl sulfate

SDS-PAGE: SDS-polyacrylamide gel electrophoresis

SE: standard error

SLA: soluble *Leishmania major* antigen

SLA-M3 liposome: SLA encased in M3 liposome

SLA-M5 liposome: SLA encased in M5 liposome

sVSG: soluble VSG

SSH: suppressive subtractive hybridization

STA: soluble *Trypanosoma brucei gambiense* antigen

STA-M3 liposome: STA encased in M3 liposome

T TAE: tris-acetic acid-EDTA

TBS: tris-buffered saline

T. brucei: Trypanosoma brucei

T. b. brucei: T. brucei brucei

T. b. gambiensei: T. brucei gambiense

T. b. rhodesiense: T. brucei rhodesiense

TCA: tricarboxylic acid

T. congolense: Trypanosoma congolense

T. cruzi: Trypanosoma cruzi

T. evansi: Trypanosoma evansi

T. equi: Theileria equi

TGF: transforming growth factor

T. gondii: Toxoplasma gondii

Th1: type 1 helper T cell

Th2: type 2 helper T cell

TNF: tumor necrosis factor

T. orientalis: Theileria orientalis

TRACK protein: *T. b. rhodesiense* activated kinase C protein receptor

homolog protein

T. vivax: Trypanosoma vivax

TS: trans-sialidase

V VSG: variant surface glycoprotein

Unit abbreviation

D ^{o}C: degrees centigrade

H hr: hour

K kDa: kilo Dalton

Kbp: kilo base pair

M M: mol/liter

m*g*: milligram

min: minute

m*l*: milliliter

mmol: millimol

mM: milliM

N n*g*: nanogram

nm: nanometer

P p*g*: picogram

S sec: second

V v/v: volume/volume

W wk: week

μ μ*g*: microgram

μl: microliter

μm: micrometer

General introduction

1. The importance of African trypanosomes

African trypanosomes are extracellular protozoan parasites that cause sleeping sickness and *Nagana* in humans and cattle, respectively. Human African trypanosomiasis (HAT) is a lethal disease caused by infection of the *Trypanosoma brucei* (*T. b*.) subspecies. *Trypanosoma b. rhodesiense* causes acute sleeping sickness in man, and is recognized as zoonosis. In West and Central Africa, sleeping sickness is caused by *T. b. gambiense*, with man as the main host. About 300,000 to 500,000 people living in 36 countries of sub-Saharan Africa are infected with African trypanosomiasis per year, and about 60 million people are at risk of infection (52). On the other hand, *T. b. brucei*, *T. congolense*, and *T. vivax* are the most important causative agents of animal African trypanosomiasis causing vast economic losses to the livestock industry in Africa. Economic losses owing to trypanosomiasis in Africa are US$ 1.4 billion annuallyc (34).

2. Clinical symptoms in sleeping sickness

Trypanosomes transmitted by the bites of tsetse flies. After the infective bite, the parasite is initially confined hemolymphatic system (the first or hemo-lymphatic stage), but as the disease progress, the central nervous system (CNS) is invaded by the parasite, which is referred to as the second or meningo-encephalitic stage. Infection with *T. b. rhodesiense* leads to acute disease with quick CNS invasion (7). Death usually takes place within weeks. The *T. b. gambiense* infection causes

chronic disease that makes progress over several years with slow CNS invasion (7).

There is considerable variation in the clinical picture of African trypanosomiasis. The enlargement of lymph nodes, especially in the neck (Winterbottom's sign), is a cardinal sign of *T. b. gambiense* infection (40). Other clinical signs of the hemo-lymphatic stage are general malaise, anemia, headache, fever, pruritus, oedema, splenomegaly, hepatomegaly, and weight loss. As the disease progresses into the meningo-encephalitic stage, clinical signs of CNS involvement become obvious. Neurological disorders are most extensive in *T. b. gambiense* sleeping sickness, and patients describe symptoms such as sleep disorders, sensory disturbances, endocrine dysfunction, tone and mobility disorders, abnormal movements, mental changes or psychiatric disorders. Finally, the patient stops eating, lapses into a semi-coma and dies (110).

3. Treatments

Drug for treatment of HAT have to be selected by the stage of the disease. Suramin and pentamidine are only effective against the first stage (hemo-lymphatic stage) trypanosomiasis, because those drugs cannot pass through blood-brain barrier (BBB) (45). Treatment of the second stage (meningo-encephalitis stage) trypanosomiasis relies almost exclusively on melarsoprol, an arsenicum derivative, which can infiltrate BBB (110). However, it has been reported that suramin and pentamidin cause the occurrence of resistant parasites (86). Moreover, and melarsoprol is extremely toxic as associated with encephalopathy in about 10% of administered patients (148). Taken together, treatment of African sleeping sickness

has not improved last 50 years.

4. Immunoactivations for vaccine development

African trypanosomes evade the humoral immune responses by antigenic variation of the major surface antigen called variant surface glycoprotein (VSG) (29). Since the repertoire of genes coding for antigenically different VSG is very large (22, 144), vaccination against trypanosomiasis based on the VSG is believed to be impossible. Despite of this issue, the most effective and sustainable way of controlling trypanosomiasis would be a safe and cost-effective vaccine (98). Therefore, vaccine development researches are now focused on identifying unique invariant trypanosome components as potential targets for vaccine (92).

Alternatively, previous studies indicate that type 1 helper T cell (Th1) cytokine responses confer resistance to African trypanosome infection by limiting parasite growth during the early stage of infection (57, 81, 82, 142). The Th1 cytokines, like interferon (IFN)-γ, interleukin (IL)-12, and tumor necrosis factor (TNF), surely contributes to resistance to *T. b. rhodesiense* infection (57, 118) and *T. congolense* infection (141, 142). Thereby, effective vaccine against African trypanosomiasis could need an ability to induce Th1 cytokines.

5. Diagnoses

Correct and rapid diagnosis of African trypanosomiasis is of utmost importance to reduce the risk of progression of the disease as well as to monitor treatment effect. However, the clinical signs and symptoms of the diseases are

largely non-specific, and there is considerable variation in the clinical picture of the disease (76). Therefore, sensitive and specific diagnostic method is clearly needed. Because of high concentration of antigen specific immunoglobulin in infected blood, several serodiagnostic methods, such as enzyme-linked immunosorbent assay (ELISA) and indirect fluorescent antibody test (IFAT) are utilized for diagnosis of trypanosomiasis (75, 101). Recently, the card agglutination test for trypanosomiasis (CATT) - *T. b. gambiense* (83), is currently used in *T. b. gambiense* endemic areas for mass screening of the population at risk. Due to its ease of application and rapidity, the CATT is one of the most commonly used diagnostic tests for African trypanosomiasis.

Although clinical symptoms and serological results can diagnose African trypanosomiasis, the infection should always be confirmed by parasite detection before starting treatment, because of the potentially serious side-effects of anti-trypanosomal drugs (148). For a direct detection of the parasite, classical microscopy has been conducted on a wet blood smear, a Giemsa-stained thick blood smear, and lymph node aspirate. However, the technique has insufficient sensitivity and specially for detection of trypanosomes that tends to show low parasitemia in natural host (76). In order to increase detection sensitivity of microscopy, hematcrit concentration technique, where the parasites are concentrated in the buffy-coat, is utilized even in resource-poor laboratories. More recently, molecular biological techniques have been applied to trypanosome detection (23). Although polymerase chain reaction (PCR) copes with direct and highly sensitive detection of the parasite (68, 109), it is not validated because of need of highly equipped facilities and

expensive reagents. Therefore, cost-effective, simple, sensitive, accurate and rapid diagnostic methods are clearly needed for diagnosis of African trypanosomiasis.

6. Aim of this study

Since all the currently available drugs against trypanosomiasis had been developed over 50 years ago and have severe side-effects, an alternative, effective and safe treatment and/or prophylctic strategies should be developed. Recently, a few reports have shown some potential vaccine candidates (79, 111), indicating the possibility of the vaccine development against African trypanosomiasis. Namely, findings of unique, invariant and immunoactive molecules would give rise to the vaccine development. In addition, to enhance suitable immnoactivations, such Th1 immune responses, an effective adjuvant should be developed. Therefore, this study focused on developing effective vaccine as follows: (I) the assessment of a new adjuvant for Th1 immunoactivation, the mannotriose (M3) coated liposomes, which results in removal of the circulating parasites in blood circulatory system (chapter 1). (II) characterization of immunomodulatory effects of the M3 coated liposmes against the infection (chapter 1). (III) exploration of vaccine candidate molecules from the parasite genes specifically expressed *in vivo* (chapter 3). (IV) the assessment of a vaccine antigen using parasites' trans-sialidase (TS) that might be involved in anemia (102) (chapter 4). Alternatively, since generally a liposome has strong affinity to lipids like cell membranes, the affect of dipaltomitoylphosphatidylcholine (DPPC) liposomes, core of the M3 coated liposomes, were examined to understand their interaction with the membranes of the parasite. The following result was obtained: (V)

Anti-trypanosomal effect of DPPC liposomes against African trypanosomes (chapter 2).

In addition to effective treatment and prophylactic strategies, highly sensitive, easy-to-use, affordable diagnostic methods for African trypanosomiasis have to be established in order to improve current situations in clinical field. Recently, a novel deoxyribonucleic acid (DNA) amplification technique, loop-mediated isothermal amplification of DNA (LAMP) was developed by Notomi *et al*. (103). Since LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions, the method is available even in resource-poor laboratories. Therefore, this study focused on an establishment of highly sensitive and easy-to-use molecular diagnostic method for African trypanosomiasis as the following subject: (VI) establishment of LAMP for the detection of the parasites (chapter 5).

Chapter 1

Vaccine Assessment of Oligomannose-Coated Liposome-Encased Soluble Trypanosome Antigen

1-1. Introduction

Trypanosoma brucei gambiense and *T. b. rhodesiense* cause the chronic *Gambian* and the acute *Rhodesian* sleeping sickness in humans in sub-Saharan Africa, respectively. Like other African trypanosomes, *T. brucei* evades the host's immune responses by constantly modifying its VSG (11, 38). This phenomenon has hampered the design of an effective anti-trypanosome vaccine (15, 16). However, limited reports have shown some potential vaccine candidates, which are dependent on the choice of vaccine molecules (79, 111).

The patterns of cytokine responses during some parasite infections are strongly correlated to the relative susceptibility of the host (122). Most reports indicate that Th1 cytokine responses confer resistance to African trypanosome infection by limiting parasite growth during the early stage of infection (57, 81, 82, 142). Indeed, IFN-γ has been linked with resistance to *T. b. rhodesiense* infection (57, 118). Furthermore, this cytokine, in association with the IL-12-dependent synthesis of immunoglobulin (Ig) G2a antibodies (Abs) and TNF secretion, has been reported to play a protective role against *T. congolense* infection in mice (141, 142). However, sustained secretion of these Th1 cytokines may be harmful to the host, thereby promoting disease progression (80-82, 105). On the other hand, type 2 helper T cell (Th2) cytokines, such as IL-4, have been linked with resistance during the chronic

infection of African trypanosome in cattle (89, 134) and mice (9, 63, 97) via induction of IgG1 Abs.

Since a liposome vaccine strategy has been shown to induce a Th1 cytokine response (46), it might be a suitable method to control the early stage of infection with African trypanosomes. Fukasawa *et al*. (46) developed the liposome coated with a neoglycolipid that consists of mannopentaose (M5) and dipalmitoylphosphatidylethanolamine (DPPE). This liposome, referred to as the M5 liposome, was shown to be incorporated by macrophages via the macrophage mannose receptor (CD206) and induce strong cellular immune responses, such as delayed-type hypersensitivity and cytotoxic T-lymphocyte induction against the encapsulated antigens in the M5 liposome. Furthermore, Shimizu *et al.* (124) showed that the intraperitoneal (i.p.) injection of the soluble *Leishmania major* (*L. major*) antigen (SLA) encased in M5 liposome (SLA-M5 liposome) induced a clear protection against *L. major* infection in BALB/c mice. Recently, it was reported that the liposome coated with mannotriose (M3)-conjugated DPPE (M3 liposome) was more effectively being incorporated in peritoneal macrophages (61), and the critical mechanism behind this protection induced by the SLA-M5 liposome and SLA-encased M3 liposome (SLA-M3 liposome) was proved to be the *L. major* antigen-specific Th1 immune response in mice (Shimizu *et al*., unpublished data). These results indicated that an antigen delivery system with mannose-coated liposomes might also induce protection against the early stage of African trypanosome infection via an antigen-specific Th1 response.

The present study was thus conducted to investigate the prophylactic effect

of a soluble *T. b. gambiense* antigen (STA)-encased M3 liposome (STA-M3 liposome) against *T. b. gambiense* infection in BALB/c mice.

1-2. Materials & Methods

Parasite culture and preparation of the parasite antigen

The bloodstream form (BSF) of *T. b. gambiense* IL2343 was previously obtained from the International Livestock Research Institute, Nairobi, Kenya. The parasites were cultured *in vitro* using an HMI-9 growth medium (59, 60) composed of Iscove's modified Dulbecco's medium (IMDM; Sigma, St. Louis, MI) supplemented with 20% fetal calf serum (FCS; Biological Industries, Ashrat, Israel), 100 U - 100 μg/*ml* penicillin-streptomycin (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Sigma), 0.1 mM bathocuproine (Sigma), 1 mM pyruvic acid (Sigma), 10 ml// HT supplement (0.1 mM sodium hypoxanthine and 0.016 mM thymidine) (Invitrogen), 0.4 mg/m*l* bovine serum albumin (BSA; Sigma), 1 μg/m*l* bovine holo-transferrin (Sigma), 0.2 mM 2-β-mercaptoethanol (Wako, Osaka, Japan), 2 mM L-cysteine (Sigma), and 60 mM HEPES (Sigma) at pH 7.2. The grown parasites were washed with phosphate-buffered saline (PBS) two times by centrifuging at 3,000 x *g* for 10 min at 4°C. After the pellet was lysed mechanically by three-time freeze-thawing and centrifuged at 10,000 x *g* for 30 min at 4°C, the supernatant was collected and filtered through a cellulose acetate membrane (pore size 0.2 μm) (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and then kept at –80°C until use. This solution was defined as an STA solution.

Preparation of liposomes

The DPPE, dipalmitoylphosphatidylcholine (DPPC), and cholesterol were purchased from Sigma. The M3 was purchased from Dextra Laboratories (Reading, UK). The M3 conjugated with DPPE (M3-DPPE) was prepared by conjugating the M3 with DPPE as described previously (90, 91). Liposomes were prepared as described previously (46, 124). Briefly, a chloroform-methanol (2:1, v/v) solution containing 1.5 μmol of DPPC and cholesterol was placed in a conical flask and dried by rotary evaporation. Subsequently, 2 m*l* ethanol containing 0.15 μmol of M3-DPPE was added to the flask and evaporated to prepare a lipid film containing neoglycolipid. Two hundred microliters of PBS containing 5 mg/m*l* STA was added to the dried lipid film, and multilamellar vesicles were prepared by intense vortex dispersion. The multilamellar vesicles were extruded 10 times through a 1 μm pore polycarbonate membrane (Nucleopore, Pleasanton, CA). Liposomes encasing STA were separated from free STA by three successive rounds of washing in PBS with centrifugation (20,000 x *g*, 30 min, at 4°C). The amount of encased antigen was measured using a modified Lowry protein assay reagent (Pierce, Rockford, IL) in the presence of 0.3% sodium dodecyl sulfate (SDS) using BSA as the standard.

Immunization and infection

The 6-wk-old female BALB/c mice (JAPAN CLEA Inc., Shizuoka, Japan, n = 8 per group) were immunized twice subcutaneously (s.c.) with 3 μg STA encased within M3-coated liposomes (STA-M3 liposome group), 3 μg STA encased within uncoated liposomes (STA liposome group), 3 μg STA (STA group) in 100 μ*l* PBS, or PBS (PBS group). A booster immunization was administered after a 7-day interval.

Five mice were each injected intraperitoneally with 1,000 *T. b. gambiense* IL2343 parasites. To monitor parasitemia, blood from the tail was serialy diluted with IMDM, and then the parasite in blood was counted using hemocytometer. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (admission number: 18-15).

Sera collection and cell preparation

Seven days after the second immunization, 3 mice were sacrificed from each group, and a single cell suspension was prepared from the spleen of each mouse as described previously (57). Blood was also collected by heart puncture, and the serum of each mouse was prepared and stored at –80°C until use. Furthermore, serum samples were also obtained from the mice on 4 days post-infection (DPI).

Lymphoproliferation assay

Seven days after the second immunization, the lymphocyte proliferative response of spleen cells (2.5 x 10^5 cells/well) was performed in triplicates in 96-well cell culture plates (Nunc A/S, Denmark) and cultured in an RPMI1640 (Sigma) medium supplemented with 0.1% FCS (0.1% FCS-RPMI) with or without 5 μg/ml concanavalin A (Con A; Sigma) or STA at 37°C in a humidified atmosphere containing 5% CO_2 as previously described (104). Lymphoproliferation was determined using a non-radioactive Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol.

Measurement of cytokines in splenocyte culture and sera

For the evaluation of cytokine secretion from splenocytes prepared from

immunized mice as described above, the cells were adjusted to 5 x 10^6 cells/well in a final volume of 1 m*l* in 24-well cell culture plates (Nunc A/S). After the cells were stimulated *ex vivo* with or without 5 μg/m*l* of Con A or 50 μg/m*l* of STA at 37°C for 72 hrs in a humidified atmosphere containing 5% CO_2, the culture supernatant fluids were collected and frozen at –80°C until use. Cytokines were quantified in the cell culture supernatants or serum samples using specific ELISA kits from Endogen (Rockford, IL) for IFN-γ, IL-4, and IL-12 or BioSource International (Camarillo, CA) for IL-10 following the manufacturers' suggested protocols.

Statistical analyses

P-values were calculated using the Student's *t*-test for statistical analyses of parasitemia, lymphoproliferation, and cytokine levels. Survival rates in the groups of infected mice were plotted according to a Kaplan-Meier method and compared statistically by a log-rank test (Mantel-Cox).

1-3. Results

Anti-trypanosome effect of the STA-M3 liposome

The ability of the STA-M3 liposome to protect against acute *T. b. gambiense* infection in BALB/c mice was tested. Unexpectedly, immunization with the STA-M3 liposome resulted in earlier and significantly higher development of parasitemia throughout the course of infection than that observed in the STA-liposome (*P*=0.013 on 4 DPI, *P*=0.004 on 5 DPI, and *P*=0.046 on 6 DPI), STA (*P*<0.001 on 5 DPI and *P*=0.021 on 6 DPI), and PBS (*P*=0.002 on 5 DPI and *P*=0.038 on 6 DPI) groups (Fig. 1A). Moreover, the STA-M3 liposome group had a significant earlier mortality than

other groups, surviving for about 6.5 days as compared to PBS control group, which survived for about 10.5 days (*P*=0.033, Fig. 1B). These results indicate that vaccination with the STA-M3 liposome does not have an anti-trypanosome effect, but, rather, accelerates the growth of trypanosomes in mice, culminating into early deaths of the infected mice.

Effect of the STA-M3 liposome immunization on lymphoproliferation

The proliferative ability of splenic lymphocytes obtained on day 7 after the second immunization was examined in Con A- or STA-stimulated cultures. Accordingly, Con A stimulation did not show significant differences among the groups, although a tendency towards reduced proliferation was observed in cells from the STA-M3 liposome immunized group as compared with that in cells from other groups (Fig. 2A). Moreover, as expected, stimulation with STA significantly induced increased lymphocyte proliferation in cells from STA group as compared to that of PBS group (*P*=0.048, Fig. 2B). The degree of cell proliferation in the STA liposome group was comparable to that in the STA group. In contrast, the cells from STA-M3 liposome group, like control PBS group, appeared to exhibit an inhibition of STA-induced proliferation as compared to that of STA group (Fig. 2B). These results suggest that immunization with STA-M3 liposomes induces suppression of lymphocyte proliferation in response to parasite-related and -unrelated antigens.

Effect of STA-M3 liposome immunization on cytokine production

The levels of cytokines (IFN-γ, IL-4, IL-10, and IL-12) produced by splenocytes obtained on day 7 after the second immunization were examined following stimulation with or without Con A or STA. Unstimulated splenocytes did not

produce detectable levels of IL-4, IL-10, and IL-12 (data not shown). However, as shown in Figure 3A, the splenocytes from the STA group (*P*=0.011) and, to a greater extent, those from the STA liposome group (*P*=0.008) spontaneously produced markedly higher levels of IFN-γ as compared to cells from control PBS group. Interestingly, unstimulated splenocytes from the STA-M3 liposome group secreted negligible amounts of IFN-γ, which were significantly lower than those in STA (*P*=0.003), STA-liposome (*P*< 0.001), and control PBS groups (*P*=0.004) (Fig. 3A). Furthermore, STA stimulation of splenocytes induced markedly increased levels of IFN-γ in both STA (*P*=0.007) and STA-liposome (*P*=0.008) groups as compared to STA-M3 liposome group (Fig. 3B). Moreover, stimulation with STA resulted in a significant increase in IL-4 production in STA (*P*<0.001) and, to a lesser extent, STA-liposome (*P*=0.038) groups as compared to that in STA-M3 liposome group (Fig. 3C). In addition, the pattern of IL-10 production was very similar to that of IL-4 (Figs. 3C and D).

Circulating levels of cytokines were also determined in the sera collected on day 7 after the second immunization. There was no detectable IFN-γ (<30 pg/m*l*), IL-4, or IL-10 (<10 pg/m*l*) in the sera from any of the groups. On the other hand, elevated levels of IL-12 were observed mainly in the sera from the STA and STA liposome groups, while the levels of that cytokine tended to be lower in the sera from the STA-M3 liposome and control PBS groups (Fig. 3E). Furthermore, circulating cytokine levels were also quantified in the sera at 4 DPI. There were no detectable levels of IL-4, IL-10, or IL-12 (<10 pg/m*l*) in the sera in any of the groups. In sharp contrast, only mice of the STA-M3 liposome group exhibited a profoundly increased IFN-γ (658

pg/m*l*), while other groups barely had any detectable levels of the cytokine (<30 pg/m*l*). These results suggest that, despite suppression of both Th1 and Th2 cytokine production following immunization with the STA-M3 liposome, a sudden increase in the circulating IFN-γ level shortly prior to death may be associated with the pathogenesis of African trypanosomiasis.

1-4. Discussion

The present study was initially designed to examine the possibilities of using an STA-M3 liposome vaccine against the lethal infection of *T. b. gambiense*. Unexpectedly, however, STA-M3 liposome vaccination resulted in an accelerated multiplication of the parasites, culminating in an earlier and higher development of parasitemia and a significantly greater reduction in the survival rate of infected mice than that observed in the controls. In contrast, mice immunized with either STA or the STA liposome generally exhibited parasitemia dynamics and survival rates that were comparable with those of the PBS-immunized mice.

This study demonstrated that STA-M3 liposome vaccination potently suppresses both Th1 and Th2 cytokine production and, to a lesser extent, STA-elicited lymphoproliferation. It has been demonstrated that immunization of mice with SLA-M3 liposome resulted in the inhibition of Th2 cytokine production, with a concomitant profound enhancement of IFN-γ production in SLA-M3 liposome-immunized mice (Shimizu *et al*., unpublished data). These responses were essential for the protection against murine leishmaniasis. In another study, Shimizu *et al.* (124) reported that vaccination with the SLA-M5 liposome also induced a

protective Th1-like response against *L. major* infection. Based on this background, the observations indicate that the STA may contain unknown trypanosome suppressive factor(s) that require delivery via the mannose receptor to effect immune suppression. Of note, the profound inhibition of spontaneous secretion of IFN-γ in the cells from the STA-M3 liposome group is of particular interest and may partially explain the accelerated development of parasitemia and early mortality in the group. Accordingly, mice of other groups that initially exhibited spontaneously high levels of IFN-γ may retard the parasite multiplication, even though they also equally succumbed later, possibly due to the concomitant increased levels of Th2 suppressive cytokines, including IL-4 and IL-10.

In agreement with previous reports (57, 81, 82, 142), the present observations emphasize that IFN-γ and, to a lesser extent, IL-12 may be crucial to control trypanosome growth during the early stage of infection. Indeed, IFN-γ production has been associated with resistance to *T. brucei* infection in mice (69), possibly through classical macrophage activation that results in their production of trypanotoxic molecules including TNF-α and nitric oxide (132, 142). Furthermore, IL-12-dependent synthesis of IgG2a Abs against trypanosome antigens and increased TNF-α secretion have also been reported to contribute to resistance to *T. congolense* infection in mice (69, 141). Based on this background, it is conceivable that the STA-M3 immunization increased the parasite multiplication through inhibition of effective innate immune mechanisms involved in parasite control.

Although Th1 cytokines appear to be inhibited following immunization with the STA-M3 liposome, this study further provides evidence that serum IFN-γ levels

increased markedly shortly before the immunized mice died. Indeed, whereas pro-inflammatory cytokines may be essential for parasite control in the early phase of trypanosome infection, excessive secretion of such cytokines may cause tissue damage (5). It may, thus, be postulated that the suppressed Th1 cytokines in STA-M3 liposome mice led to massive parasitosis, which possibly culminated in the liberation of excessive parasite antigens that eventually stimulated a strong pro-inflammatory response and caused tissue pathology and death.

There is cumulative evidence that suppressive macrophages, elicited during trypanosome infection (126), are the central effector cells that inhibit lymphoproliferative responses to mitogens and antigens (14, 70, 128). Such macrophages generate immunologically suppressive factors, including prostaglandin (PG) (127), PG inhibitors (131), nitric oxide (32), and IL-10 (97). Moreover, macrophages from trypanosome-infected mice have been reported to have a defective capacity to present antigens in the context of major histocompatibility complex class II molecules (96). It is conceivable that the administration of the STA-M3 liposome, just like live trypanosomes, induces a suppressive phenotype in macrophages via mannose receptors. Since a trypanosome-derived suppressive molecule(s) has not yet been identified, the M3 liposome system could be useful for the identification of the trypanosome-suppressive molecule(s).

1-5. Summary

Since a liposome vaccine strategy has been shown to induce a Th1 cytokine response, it might be a suitable method to control the early stage of infection with

African trypanosomes. The present study examined the possibility of M3-coated liposome-encased STA to induce a protective immunity against *T. b. gambiense* infection in mice. Unexpectedly, vaccination with a STA-M3 liposome led to an earlier appearance of the parasites and subsequent earlier deaths as compared to control groups. This vaccination induced (I) suppression of the proliferation of splenic lymphocytes in response to STA and (II) a significant reduction of cytokine, notably IFN-γ, production *ex vivo* and *in vivo*. These data suggest that the failure of the STA-M3 liposome vaccine to confer protection was partly due to its suppression of anti-trypanosome Th1 cytokine response early in the course of *T. b. gambiense* infection.

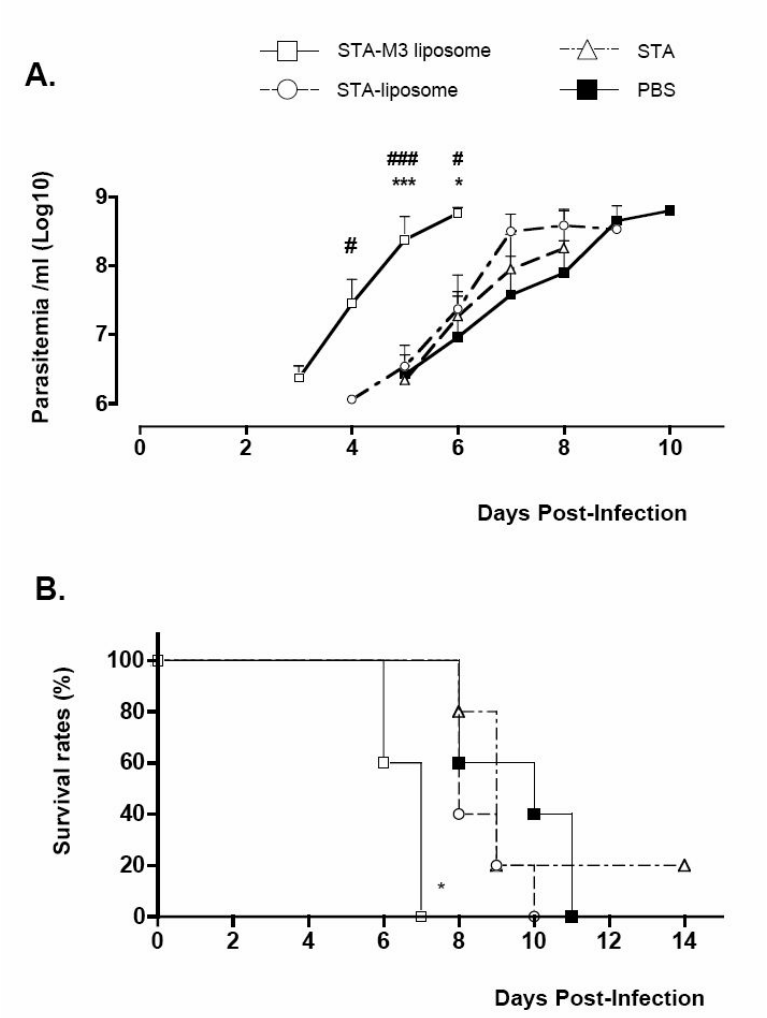


FIG. 1. Anti-trypanosome effect of the STA-M3 liposome. BALB/c mice were immunized subcutaneously twice with 3 μg of the M3-STA liposome, the STA liposome, STA, and PBS at 7-day intervals. Seven days after the second immunization, each mouse was injected intraperitoneally with 1,000 cells of *T. b. gambiense* IL2343. The parasitemia (A) and survival rates (B) were monitored as described in Materials and Methods. Data are presented as the mean ± standard error (SE). Statistical analyses were assessed by the Student's *t*-test (parasitemia) or by a log-lank test (Mantel-Cox) (survival rates). P <0.05 were considered statistically significant. * and *** indicate significant differences between the STA-M3 liposome and PBS groups (P<0.05 and P<0.01, respectively). # and ### indicate significant differences between the STA-M3 liposome and STA-liposome groups (P<0.05 and P<0.01, respectively).

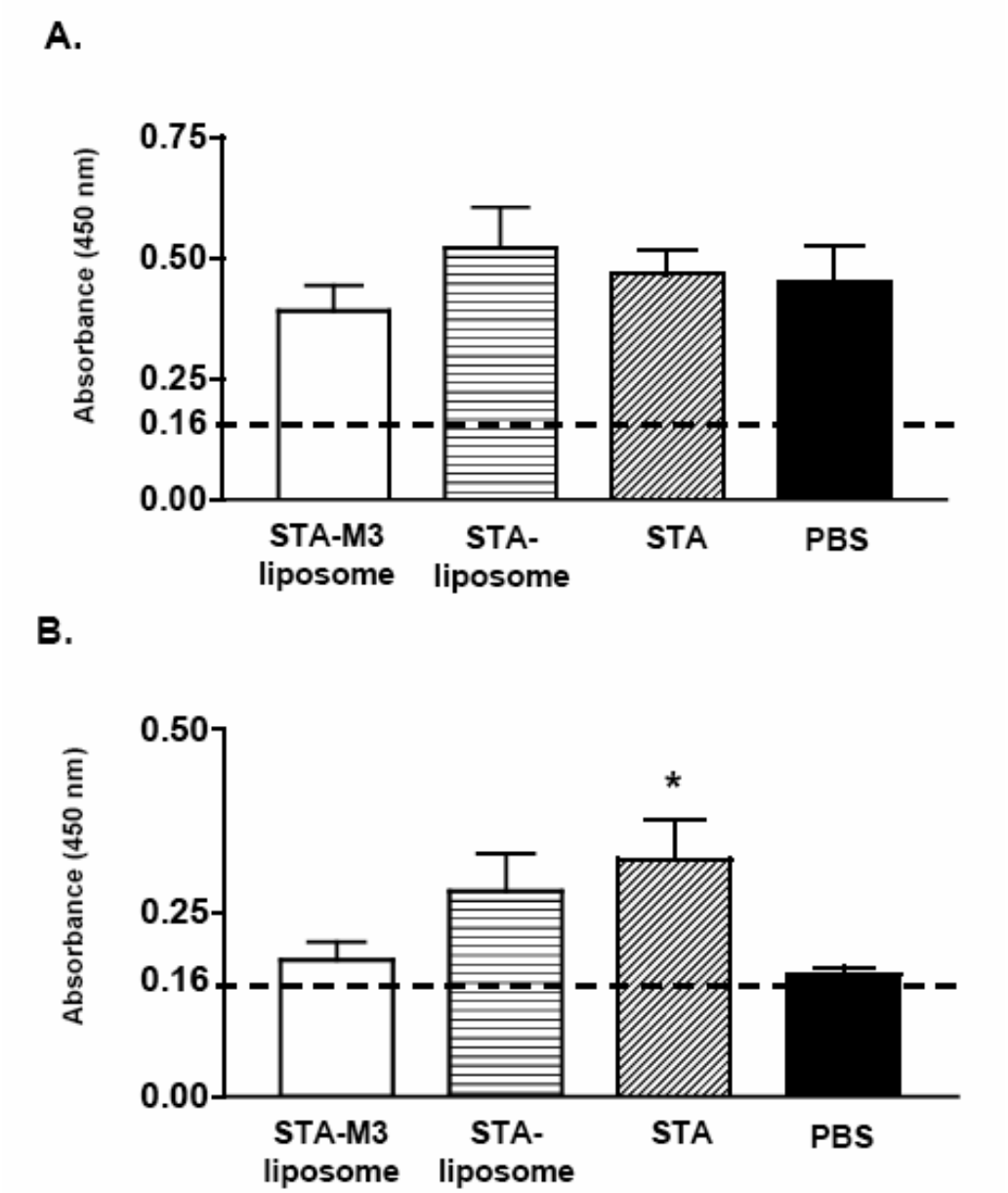
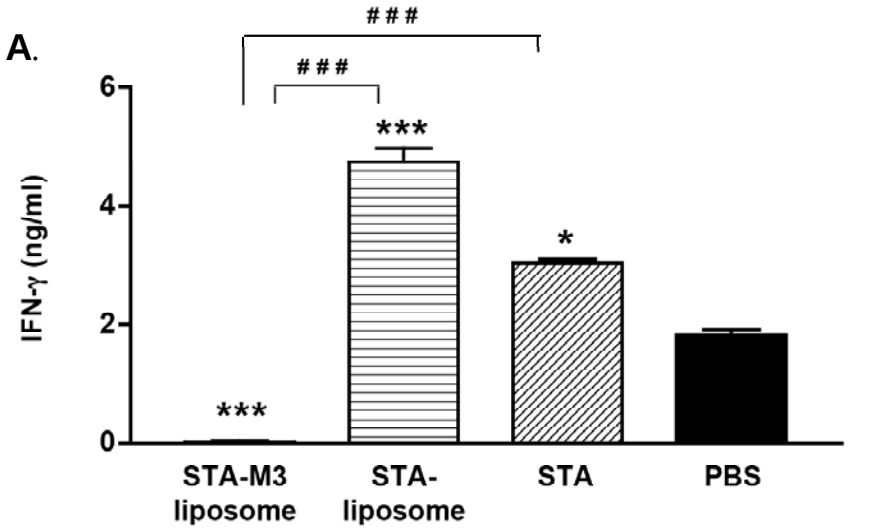
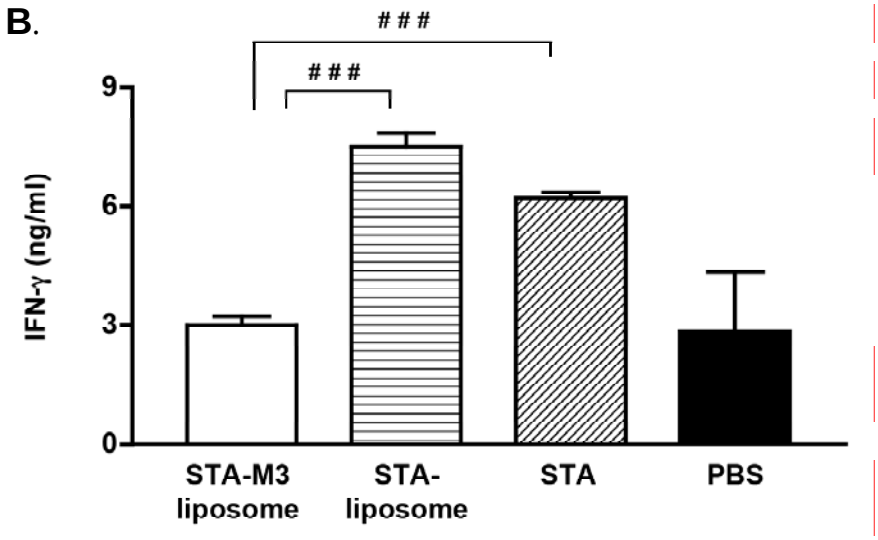
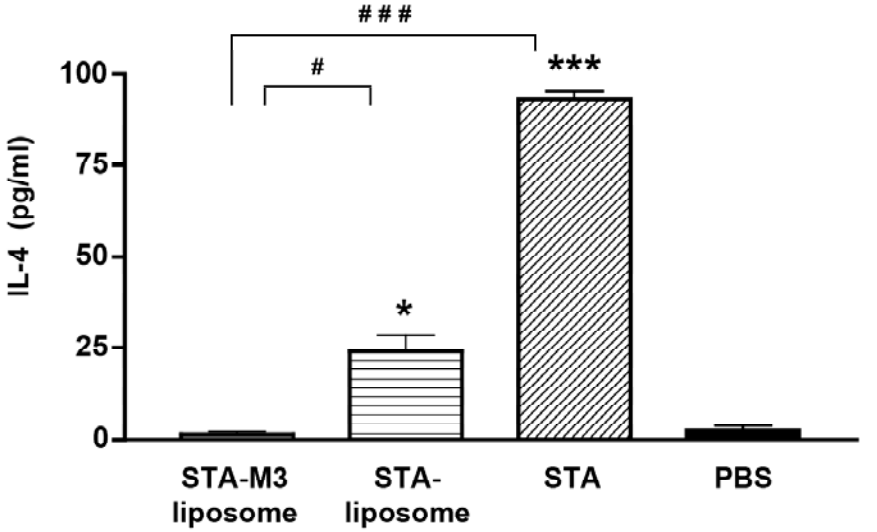


FIG. 2. Effect of the STA-M3 liposome immunization on lymphoproliferation. Splenic lymphocytes were collected 1 week after the second immunization. The ability of cells to proliferate in response to 5 μg/m/ Con A (A) or 50 μg/m/ STA (B) was evaluated using the CCK-8, and the absorbance was measured at 450 nm. *P*-values of <0.05 were considered statistically significant. Data are presented as the mean ± SE. Dashed horizontal lines represent average lymphoproliferation in non-stimulated cells. * indicates significant difference with of PBS group.


A.
###
###
***
*
***
IFN-γ (ng/ml)
6
4
2
0
STA-M3 liposome
STA- liposome
STA
PBS


B.
###
###
IFN-γ (ng/ml)
9
6
3
0
STA-M3 liposome
STA- liposome
STA
PBS


C.
###
#
***
*
IL-4 (pg/ml)
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75
50
25
0
STA-M3 liposome
STA- liposome
STA
PBS

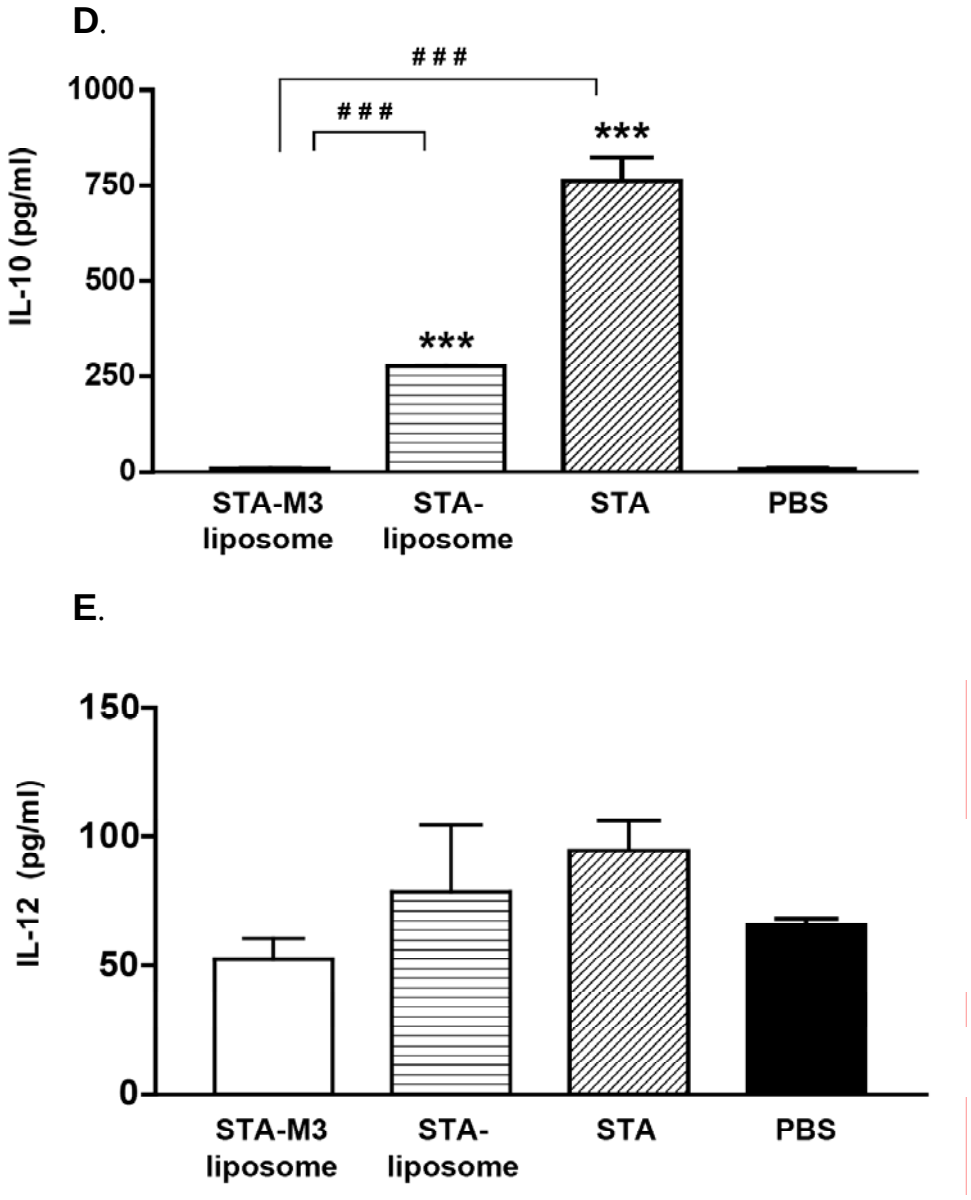


FIG. 3. Effect of the STA-M3 liposome immunization on cytokine production. Splenocytes were collected 1 week after the second immunization and cultured unstimulated (A) or stimulated (B-D) with 50 μg/m*l* STA for the detection of IFN-γ (A and B), IL-4 (C), or IL-10 (D). In addition, sera collected after the second immunization were also used for cytokine detection (IL-12) (E). Data are presented as the mean ± SE. *P*-values of <0.05 were considered statistically significant. * and *** indicate significant differences with the PBS group (*P*<0.05 and *P*<0.01, respectively). # and ### indicate significant differences with the STA-M3 liposome group (*P*<0.05 and *P*<0.01, respectively).

Chapter 2

Efficacy of Dipalmitoylphosphatidylcholine Liposome Against African Trypanosomes

2-1. Introduction

African trypanosomes are tsetse-transmitted protozoan parasites that cause sleeping sickness and *Nagana* in humans and cattle, respectively. About 300,000 to 500,000 people become iinfected per year with African trypanosomiasis in sub-Saharan Africa, and about 60 million people are at risk of infection (52). The currently used drug therapies for sleeping sickness are far from satisfactory (21) because of the increased development of parasite resistance (86) and the economically prohibitive cost. Cysteine protease inhibitors have been proposed as alternatives to the current anti-trypanosomal drugs (100, 120, 140). Additionally, glycolytic pathway inhibitors that target glycerol-3-phosphate dehydrogenase and hexokinase (149, 150), antimicrobial peptides derived from midgut extract of *Stomoxys calcitrans* (17), cathelicidin in mammals (87), and polyphenolic flavonoids in plants (85), have also been recommended as alternative approaches. However, *in vivo* studies must be conducted to elucidate the effectiveness and side effects. Thus, further investigation to develop new chemotherapeutic agents is critical for the future control of African trypanosomiasis (54).

The Dipalmitoylphosphatidylcholine (DPPC), a phospholipid, is made up of choline, phosphoric acid, and glycerol. In the presence of cholesterol, DPPC self-assembles and forms a phospholipid bilayer vesicle, defined as DPPC liposome

(116). The DPPC liposome is stable under storage due to its resistance to oxidation and resists destabilization by plasma proteins and lipoproteins in biological fluids as compared to other types of liposomes prepared from natural phospholipids, such as egg phosphatidylcholine (108). Furthermore, DPPC liposome has been applied as a peptide or drug carrier for the delivery to target cells (46, 66, 67) and also proven to have no toxic side effect against mammalian cells and mice (46, 133).

This study had been designed to develop a drug delivery system for the treatment of trypanosomiasis by using DPPC liposome, but I rather discovered the anti-trypanosomal effect of DPPC liposome itself. Thus, the present study describes here its effect and possible mode of action against trypanosomes.

2-2. Materials & Methods

Parasites

The BSFs of *T. congolense* IL3000, *T. b. gambiense* IL2343, and *T. b. brucei* GUTat 3.1 were previously obtained from the International Livestock Research Institute, Nairobi, Kenya . The *T. congolense* IL3000 was i.p. injected into 6-wk-old female BALB/c mice (JAPAN CLEA Inc.). At the peak of parasitemia, whole blood was collected by cardiac puncture and the parasites were purified from the blood using DE52 anion-exchange column chromatography (Whatman, Brentford, UK) (74). The purified parasites were suspended in PBS containing 0.2 M glucose (glucose-PBS) and used for DPPC liposome-binding assay as described below. The BSFs of *T. b. rhodesiense* IL2343 and *T. b. brucei* GUTat 3.1 were prepared *in vitro* using an HMI-9 growth medium as described in Materials & Methods of chapter 1. The procyclic

forms (PCFs) of *T. congolense* IL3000 and *T. b. brucei* GUTat 3.1 were prepared by transforming the BSFs of the parasites as follows, and then cultured *in vitro* with a TVM-1 growth medium containing Eagle's minimum essential medium (EMEM; Sigma) with 2 mM L-glutamine, 20% FCS, and 10 mM L-proline (Invitrogen) at pH 7.2 (59, 60). For the transformation to PCFs, the BSFs were purified from the infected mice and adjusted to a concentration of 3×10^6 parasites/m*l* in TVM-1. The parasite suspensions were transferred to 25- cm^2 culture flasks (NUNC A/S) and incubated at 27 ^oC for a wk. During the incubation, all BSFs of the parasites transformed to the PCFs (data not shown).

Preparation of DPPC liposomes

The DPPC and cholesterol were purchased from Sigma-Aldrich Japan. 4-fluoro-7-nitro-2,1,3-benzoxadiazole (Benzofurazans; Promega, Madison, WI) was conjugated to the acetylcholine moiety of DPPC to generate a 4-fluoro-7-nitro-2,1,3-benzoxadiazole DPPC (NBD-DPPC). The DPPC and NBD-DPPC liposomes were prepared as described previously (46, 133). Briefly, a chloroform-methanol (2:1, v/v) solution containing 5 µmol of DPPC or NBD-DPPC and 2.5 µmol of cholesterol was prepared in a conical flask and rotary-evaporated to prepare a dried lipid film. Two-hundred µ*l* of PBS was added to the lipid film and multilamellar vesicles were prepared by an intense vortex dispersion. The multilamellar vesicles were extruded 19 times through a 50-nm pore polycarbonate membrane (Nucleopore, Pleasanton, CA) and then washed in PBS by centrifuging at 20,000 ×*g* for 30 min at 4 ^oC. The molar ratios of the lipid components were determined in the liposomes by using high-performance liquid chromatography (123).

The liposomes were adjusted with PBS to the concentration of 2 mg/m*l* of cholesterol and used for further experiments.

Inhibitory effect of DPPC and NBD-DPPC liposomes on the in vitro motility of the BSFs and PCFs of trypanosomes

The BSFs of *T. congolense* IL3000, *T. b. rhodesiense* IL2343, and *T. b. brucei* GUTat 3.1 and PCFs of *T. congolense* IL3000 and *T. b. brucei* GUTat 3.1 were maintained in glucose-PBS, HMI-9, or TVM-1 and added at the volume of 87.5 μ*l* at a concentration of 1.0×10^5 parasites/m*l* into each well of a 96-well plate (MICROTEST 96; Becton Dickinson and Company, Franklin Lakes, NJ). Subsequently, 12.5 μ*l* of 2 mg/m*l* DPPC or NBD-DPPC liposome was added into the wells and incubated at 37 ^{o}C and 27 ^{o}C for the BSFs and PCFs, respectively. After 1 hr incubation, the number of motile parasites was determined with a hemocytometer (Hirschmann Laborgerate, Eberstadt, Germany) under a light microscope (DIAPHOT 300; Nikon, Tokyo, Japan). The percentages of motile trypanosomes were calculated by dividing the number of the motile parasites after the incubation by the number of the parasites before the incubation. The percentages of treated groups with the liposomes were statistically analyzed, comparing with those of the control group without any liposomes. These parasites were fixed in parallel with absolute methanol on glass slides (MATSUNAMI GLASS Ind., Ltd., Kishiwada, Japan) for 1 min at room temperature (RT), stained with Giemsa's solution (Merck Ltd., Tokyo, Japan) for 10 min at RT, and then observed by light microscopy (ALPHAPHOT-2 YS2; Nikon) to examine the morphological changes in the treated parasites.

NBD-DPPC liposome-binding assay

Trypanosoma congolense IL3000 parasites were fixed with absolute methanol on glass slides for 3 min at –30 ^{o}C. After blocking with 3% BSA in PBS, the slides were reacted with 10 μg/m*l* of NBD-DPPC in PBS for 1 hr at RT in darkness and then with 25 μg/m*l* propidium iodide (PI; Molecular Probes, Eugene, OR) containing 50 μg/m*l* RNase A (Roche Applied Science, Mannheim, Germany) for 30 min at RT. The slides were washed 3 times in PBS, and the absorptive phenotype of NBD-DPPC with the fixed parasites was examined with a confocal laser scanning microscope (TCS NT; Leica, Heiderberg, Germany).

Chemotherapeutic effect of DPPC liposome on the experimentally infected mice

The chemotherapeutic effect of DPPC liposome was evaluated in mice infected with the BSF of *T. congolense* IL3000. Briefly, 6-wk-old female BALB/c mice were divided into 2 groups of 5 and i.p. injected with 1×10^3 parasites/mouse. One group was i.p. administered DPPC liposome (20 μg/mouse) on 1, 2, and 3 DPI, while the control group was given the same volume of PBS at the same route. Parasitemia was monitored daily with a hemocytometer following a tail snip. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (admission number: 14-69).

Statistical analyses

P-values were calculated using the Student's *t*-test for the *in vitro* motility assay and evaluation of parasitemia, and *P*-values of <0.05 were considered statistically significant. Survival rates of infected mice were plotted according to a

Kaplan-Meier method and compared statistically between the treated and non-treated groups of mice by a log-rank test (Mantel-Cox).

2-3. Results

Effect of DPPC liposome on the *in vitro* motility of trypanosomes

The inhibitory effect of DPPC liposome was determined *in vitro* by counting the numbers of motile trypanosomes after the incubation with DPPC and NBD-DPPC liposomes (Table 1). The appearance of motile *T. congolense* was significantly suppressed in both of the BSF and PCF, whereas the percentage of motile *T. congolense* BSF (24.6±7.5%) was lower than that of the PCF (69.9±7.5%). The NBD-DPPC liposome was also similarly effective against *T. congolense* (Table 1). The incubation of DPPC liposome showed highly inhibitory effect against the motility of BSFs of *T. b. rhodesiense* and *T. b. brucei* (41.4 ± 6.0 and 41.5 ± 5.2%, respectively), but not against PCF of *T. b. brucei* (83.1±13.8%).

To examine the morphology of trypanosomes after incubation with DPPC liposome, Giemsa-stained thin smears were prepared from the incubated parasites and observed under a light microscope. Morphological changes in parasite shape were observed only in the BSFs incubated with DPPC liposome (Fig. 4A). It appeared to be a disruption of the membrane structure in parasite. In contrast, morphological changes were not observed in the PCFs incubated with DPPC liposome (Fig. 4B). To investigate the binding characteristics of DPPC liposome, the BSF or PCF of *T. congolense* was fixed on glass slides and treated with NBD-DPPC liposome. The fluorescent signal of NBD-DPPC liposome was clearly detected in the BSF (Fig. 5A),

but only very weakly detected in the PCF (Fig. 5B).

Chemotherapeutic effect of DPPC liposome in infected mice

Finally, the *in vivo* efficacy of DPPC liposome was determined in infected mice. As shown in Figure 6A, 20% of the mice treated with DPPC liposome at a dose of 20 μg/mouse were alive on 12 DPI, while all of the mice in non-treated groups died before 10 DPI. Although the survival rates in the 2 groups did not differ statistically, the early development of parasitemia (P=0.038) was significantly reduced on 7 DPI in the treated group (Fig. 6B). However, parasitemia in the treated group peaked (>1.0 × 10^8 parasites/m*l*) on 9 DPI, and 80% of the infected mice treated with DPPC liposome eventually died (Fig. 6A). One of 5 mice in the treated group survived until 30 DPI. In agreement with previous study (46), any difference of weight was not observed between the treated and the control groups, indicating that DPPC liposome itself is not toxic.

2-4. Discussion

The present study demonstrated that DPPC liposome has anti-trypanosomal activity, particularly against the BSFs of African trypanosomes. Because the morphological evaluation revealed that treated parasites lost their intact morphology, DPPC liposome appears to disturb the integrity of the membrane structure of parasites, resulting in cell lysis. Overlay of the methanol-fixed parasites with the NDB-DPPC liposome showed a strong fluorescence signal under confocal laser scanning microscopy in the whole body of the BSF parasite, but not in the PCF. These results suggest that the BSFs express unknown molecules on the surface

membrane or in the cytoplasm that have affinity for the DPPC liposome. Stage-specific expression of the molecule(s) might be related to parasite motility in mammalian hosts.

The BSF and PCF parasites express different membrane glycoproteins and glycolytic metabolism-related proteins, which might be related to selective susceptibility of BSF to DPPC liposome. Surface membrane of the BSF is covered with VSG dimers which protect (as a macromolecular diffusion barrier) the parasite from lysis induced by the host complement pathway (35). Alternatively, the PCF is known to have another diffuse cell-surface coat made up of procyclic acidic repetitive protein (PARP) that forms a highly extended polyanionic rod-like structure (113, 139). The preferable binding of DPPC liposome to the BSF might occur through its specific interaction with the VSGs, which triggers direct cell lysis. In glycolytic metabolism, BSF utilizes glucose to produce energy through the glycolytic pathway. In contrast, PCF shares a more elaborate energy metabolism process involving the tricarboxylic acid (TCA) cycle and the respiratory chain in the mitochondrion followed by the glycolytic pathway (25). The DPPC liposome may also bind to the glycolysis-related, stage-specific proteins, resulting in disruption of energy production, especially in BSF trypanosomes. Interestingly, antimicrobial peptides (AMPs) containing a-ọrb-defensins and cathelicidins (87), and AMP of *S. calcitrans* that is sympatric with tsetse flies (17), were also rather effective against BSF trypanosomes. However, the target molecules of AMPs have not yet been identified. Understanding the anti-trypanosomal mechanism of DPPC liposome may elucidate the mechanism of AMP and identify new drug targets against African trypanosome. I further investigated

the efficacy of DPPC liposome in mice experimentally infected with *T. congolense.* A dose of 20 μg/mouse DPPC liposome resulted in a 20% survival rate and a slight**,** but significant**,** reduction in the early development of parasitemia. Before DPPC liposome can be used clinically as a chemotherapeutic agent, however, further studies are needed to determine the dose regimen, including the optimal injection route, treatment dose, times, and period.

In conclusion, DPPC liposome is selectively effective against BSF trypanosomes. Further studies are needed to elucidate the interaction between the parasite and DPPC liposome and to understand the biological mechanisms by which the liposome kills parasites. This is the first report that liposome has anti-trypanosomal activity.

2-5. Summary

The present study demonstrated here that DPPC liposome has an anti-trypanosomal effect, especially against the BSFs of African trypanosomes (*T. congolense*, *T. b. rhodesiense*, and *T. b. brucei*). The DPPC liposome significantly decreased *in vitro* the percentage of viable and motile BSF African trypanosomes, but only marginally reduced the percentage of viable and motile PCF trypanosomes. The DPPC liposome absorption was much more pronounced to BSF than to PCF trypanosomes. Administration of the DPPC liposome showed a slight but significant reduction in the early development of parasitemia in *T. congolense*-infected mice. These results suggest that parasites were killed by specific binding of the DPPC liposome to the trypanosomes. This work demonstrates for the first time that a

liposome has anti-trypanosomal activity.

TABLE 1. Inhibitory effect on DPPC and NBD-DPPC liposome against the trypanosomes.

Parasites	Stage	The percentage of the motile trypanosomes (mean ± SD)† incubated with		
		DPPC (25 μg / 100 μl)	NBD-DPPC (25 μg / 100 μl)	Control
T. congolense IL3000	BSF	24.6 ± 7.5 %	*22.3 ± 8.4 %*	86.6 ± 2.9 %
T. congolense IL3000	PCF	69.9 ± 7.5 %	*68.0 ± 4.5 %*	86.7 ± 9.5 %
T. b. rhodesiense IL2343	BSF	41.4 ± 6.0 %*	ND**	85.7 ± 11.3 %
T. b. brucei GUTat3.1	BSF	41.5 ± 5.2 %*	ND	86.8 ± 10.3 %
T. b. brucei GUTat3.1	PCF	83.1 ± 13.8 %	ND	83.1 ± 11.4 %

† Each percentage represents the mean±standard deviation (SD) of the motile parasites after 1 hr incubation with DPPC and NBD-DPPC liposome, and without liposome (n=5). Each percentage was calculated by dividing the number of the motile parasites by the number of the parasites that had been detected before the incubation.

* Statistically significant differences (P <0.05) between the treated group with 25 μg of liposome and the control group.

** ND, Not determined.

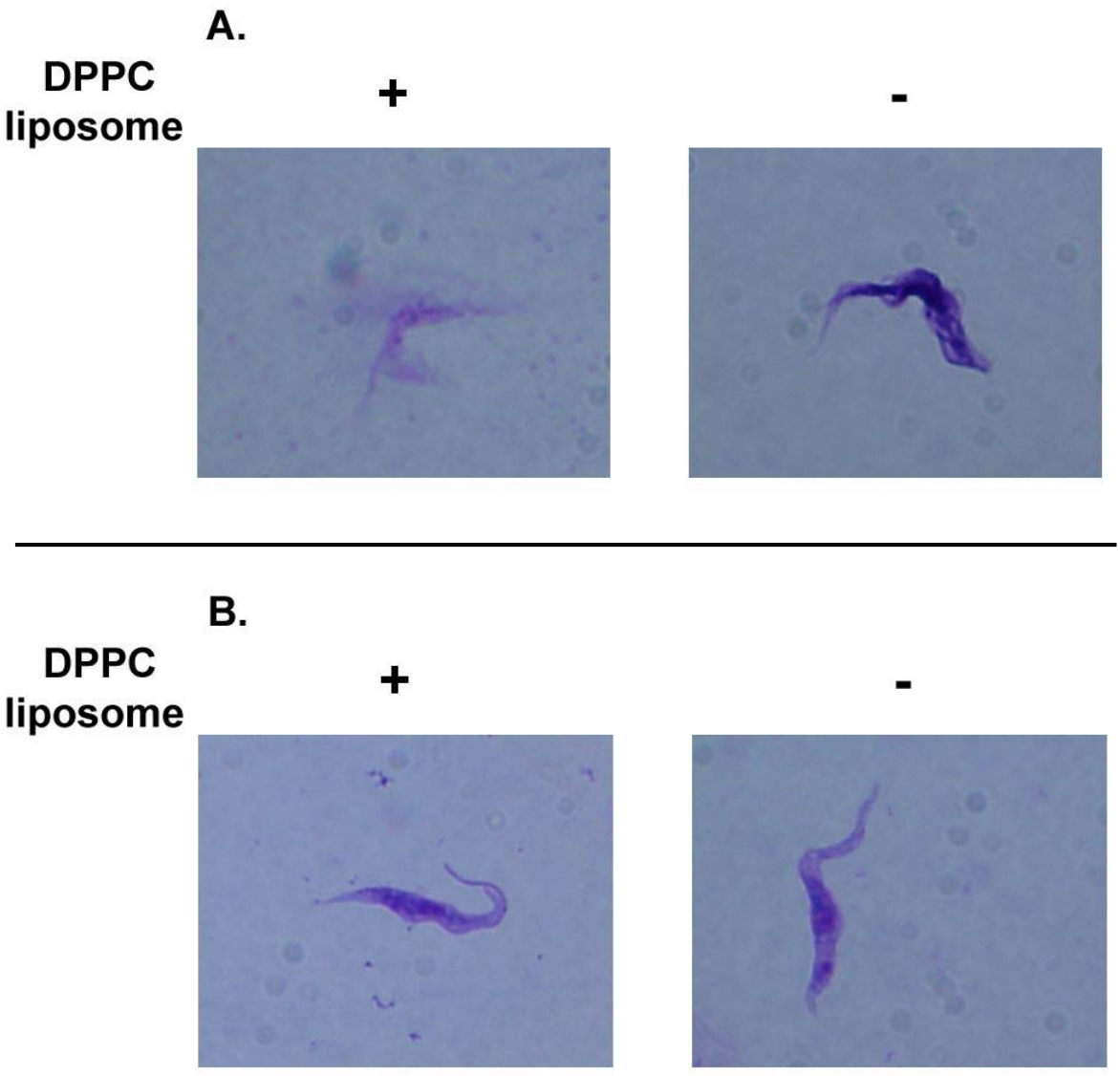


FIG. 4. Morphological changes in *T. congolense* IL3000 stained with Giemsa after incubation with (+) or without (-) DPPC liposome. (**A**) BSF of the incubated parasite. (**B**) PCF of the incubated parasite.

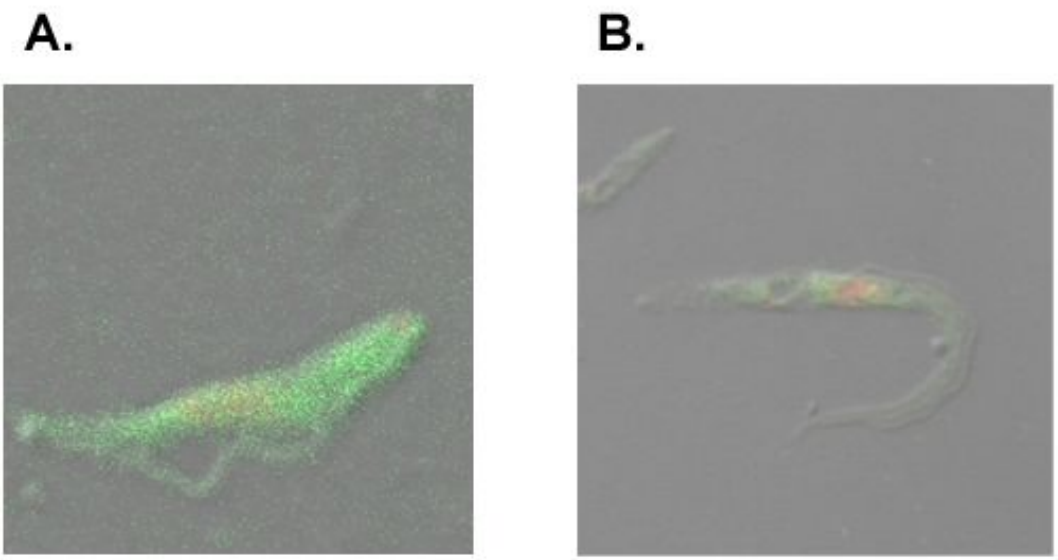


FIG. 5. *T. congolense* IL3000 observed in the NDB-DPPC liposome-binding assay. BSF (**A**) or PCF (**B**) was pre-fixed in absolute methanol and incubated with NBD-DPPC liposome on the glass slide. Green, NBD-DPPC liposome; Red, the nuclei in the parasite stained with PI.

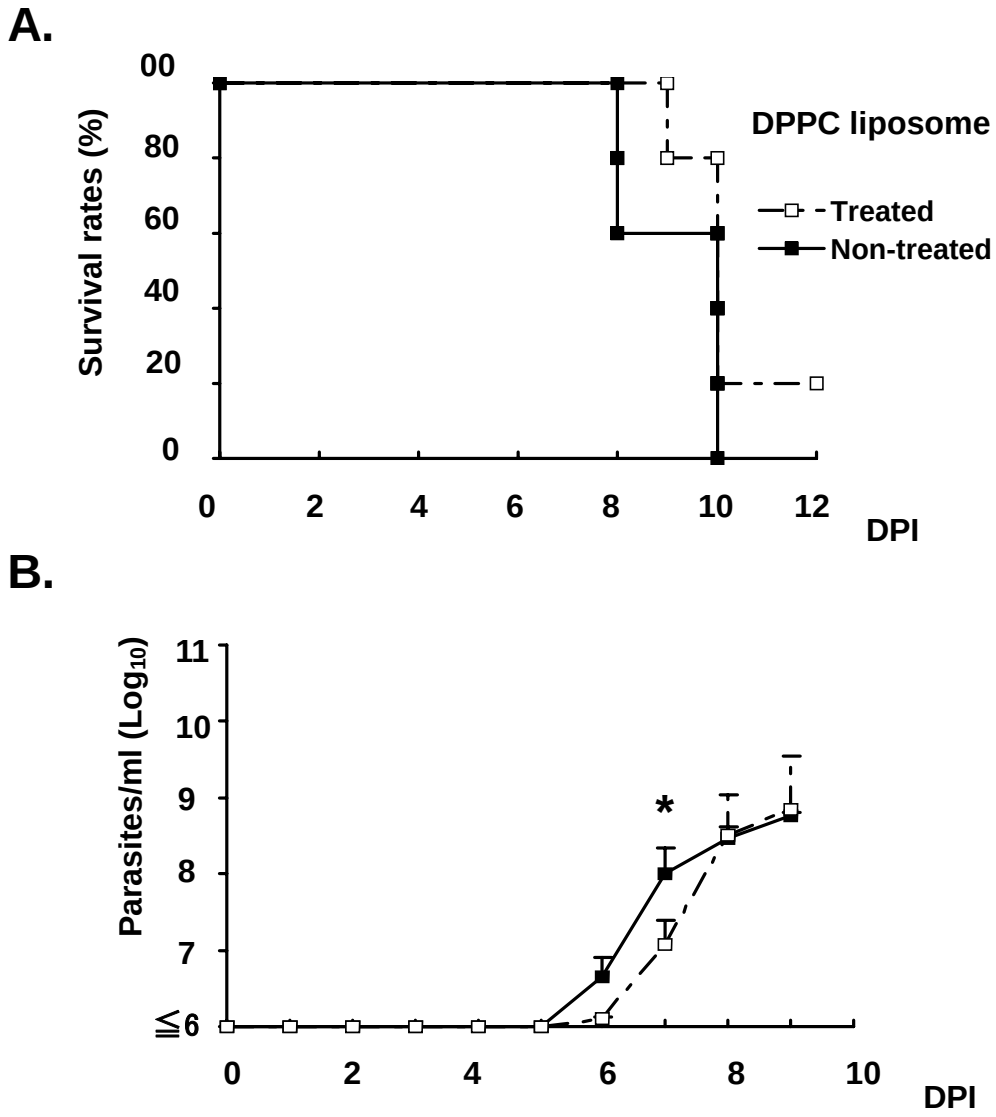


FIG. 6. Chemotherapeutic effect of DPPC liposome on mice infected with *T. congolense* IL3000. The DPPC liposome was administered i.p. to the infected mice on 1, 2, and 3 DPI. Parasitemia of less than 1.0×10^6 parasites/m*l* (detectable limitation) was calculated as 1.0×10^6 parasites/m*l* for the mean and standard error of mean. Asterisk indicates significant differences (P<0.05) between the DPPC liposome-treated (20 μg/mouse) and non-treated groups. Five mice in each group were used for this study. (**A**) Survival (%) after infection with parasites. (**B**) Logarithmic value of the mean and standard deviation of parasitemia (parasites/m*l*).

Chapter 3

Transcriptional Analyses of African Trypanosome from Infected Mice and Culture

3-1. Introduction

African trypanosomes are medically and veterinary important protozoan parasites that cause sleeping sickness in human and nagana in cattle, respectively. Since axenic culture methods for both BSF and PCF of African trypanosomes had been developed (59, 60), these *in vitro* culture system has been contributed to further investigation of African trypanosomes. However, this development has given rise to a question of whether biological characters of *in vitro* propagated parasites are the same as those in host. In fact, prolonged *in vitro* cultivation occasionally results in the loss of infectivity and/or virulence for the host. For example, prolonged *in vitro* growth (14 months) of *Trypanosoma evansi*, evolutionary related to *T. brucei* group trypanosomes, led to reduction or loss of the kinetoplast (dyskinetoplasty), and a loss of infectivity for mice (152). The importance of maintaining trypanosomes (hemoflagellates) is without extensive passages from their original isolate, since characteristic change can frequently occur in populations with prolonged cultivation *in vitro* (119). However, to date, there is no report on examination of transcriptional and expressional differences between serially cultivated BSF of African trypanosomes and the parasites propagated in host animal, although such comparison in PCF of *T. brucei* had been examined and showed no differences (107).

It was reported that certain genes of some pathogens were specifically

expressed only after injection to mammalian host. It has been reported that several pathogenic genes in *Salmonella typhimurium* (55, 56, 84, 143), *Yersinia enterocolitica* (33), and *Escherichia coli* (39), such as putative adhesins, lipopolysaccharide core synthesis, and iron-responsive, plasmid- and phage-encoded genes, are specifically expressed in the mammalian host. In case of protozoan parasites, such pathogenic genes also has been reported from *Trypanosoma cruzi* (27), *Entamoeba histolytica* (19) and *Trichomonas vaginalis* (48). These findings imply that African trypanosomes also have *in vivo* specific genes for adaptation and/or pathogenesis to their mammalian hosts. Therefore, the present study was conducted in order to identify the genes specifically expressed and/or up-regulated in host (*in vivo*), but not *in vitro*.

3-2. Materials and Methods

Parasite and experimental animals

The *T. b. gambiense* IL2343 was used for all experiments in this study. The parasite was maintained in HMI-9 medium as mentioned in Materials & Methods of chapter 1. The parasites were harvested *in vitro* culture, and centrifuged at 1,000 $\times$ *g* for 10 min for collection of the pellet. For the purpose of the current study, this pellet is defined as the *in vitro* parasite. For collection of the *in vivo* propagated parasites, female BALB/c mice (6 wks old) were injected i.p. with 5×10^3 cells/head of the parasites derived from the cultivation. At the high peak parasitemia (more than 1.0×10^8 cells/m*l*), whole blood was collected by cardiac puncture and the purified parasites were obtained using DE52 column chromatography as described in Materials & Methods of chapter 2. The purified trypanosome was centrifuged at 1,000

×*g* for 10 min and the pellet was defined as the *in vivo* parasite for the purpose of current study. The pellets were dissolved in TRIZOL (Invitrogen) according to the manufacturer's instructions, and used for extraction of total ribonucleic acid (RNA). All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (admission number: 14-69).

Subtractive library construction

Poly $(A)^+$ RNAs were purified from the RNA samples from the *in vivo* and the *in vitro* parasites using oligo (dT) spin columns (Invitrogen). Using 2 μg poly $(A)^+$ RNA from each preparation, a subtracted library was constructed by using PCR-Selected™ (Clonetech, Mountain view, CA) according to the manufacturer's instructions. The methodology used was based on suppressive subtractive hybridization (SSH) (36). Differential cDNA sequences obtained by this method were inserted into pGEM®-T easy vector (Promega) to prepare the subtracted complimentary DNA (cDNA) libraries. The nucleic acid sequence of the library was determined with the Big Dye terminator cycle sequencing kit (Applied Biosystems Japan Ltd., Japan). Nucleic acid homology searches were performed using the BLAST program through e-mail servers at the DNA Data Bank of Japan (National Institutes of Genetics, JAPAN).

Dot blot analysis

For the dot blot analysis, each insert was amplified by PCR with primers that bind to T7 promoter region (5'-TAATACGACTCACTATA-3') and SP6 promoter region (5'-ATTTAGGTGACACTATAGAAT-3') under the condition as follows. 94°C for 10 min

as an initial denaturation step and then subjected to 30 cycles consisting of 45 sec at 94°C, 1 min at 55°C, and 1 min at 72°C, and finally a terminal elongation for 7 min at 72°C. These amplified products were dotted on the Hybond-N^+ (GE Healthcare, Little Chalfont, UK) nylon with Bio-Dot® microfiltration apparatus (BIO-RAD, Hercules, CA). On the other hand, total RNAs were prepared from the *in vivo* and *in vitro* trypanosomes with TRIZOL (Invitrogen). From these RNAs, ^{32}P-deoxycytidine triphosphate (dCTP)-labeled cDNAs were synthesized with oligo (dT) primer and AMV reverse transcriptase (Invitrogen), and used as probes for dot blot analysis. Hybridization was performed at 42°C for 12 hrs in hybridization solution containing 50 % formaldehyde, 0.6 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.02 M EDTA, 0.5 % SDS and 100 μg/m*l* heat-denatured salmon sperm DNA. Filters were washed with 2 × SSC and 0.1 % SDS three times for 10 min each, and subsequently washed 0.2 × SSC and 0.1 % SDS 4 times for 15 min. Detection was done by autoradiography.

Northern blot analysis

The *in vivo* and *in vitro* total RNAs were electrophoresed in 1.0 % gel and transferred to the Hybond-N^+ nylon membrane with 20 × SSC. From the cDNA clones of the *in vivo* subtractive library, inserts were amplified by PCR and followed by synthesizing ^{32}P-dCTP-labeled inserts with AMV reverse transcriptase. These inserts were used as the probe for the Northern blot analysis. Hybridization was performed under the same condition described as dot blot analysis.

3-3. Results

Construction of the *in vitro* and *in vivo* subtracted libraries

The Poly $(A)^+$ from *T. b. gambiense* IL2343 in culture or mice was used to construct each subtracted library, which supposed to contain the *in vitro* or the *in vivo* specific genes. These clones were arbitrarily picked up from the *in vivo* library (328 clones) and the *in vitro* library (160 clones), and then analyzed following BLAST search and their transcriptions. The outline of this experiment is summarized in Fig. 7.

One hundred thirty-four clones were arbitrarily selected out of 328 clones obtained from the *in vivo* library, sequenced, and the BLAST searches of them were conducted (Table 2). The result showed that the library possesses 38 differential unique genes that share homology (more than e-10). The detected genes consist of ribosomal protein genes (26.9 %), RNA binding / transcription protein genes (4.5%), protease genes (4.5%), other functional genes (10.4%), conserved protein genes (3.7%) and unknown genes (50.0%). On the other hand, the *in vitro* library was also examined to conduct BLAST search analysis of 52 clones out of 160 clones. As a result (Table 3), the library has ribosomal protein genes (7.7%), protease genes (7.7%), other functional genes (32.7%), conserved protein genes (3.8%) and unknown genes (48.1%). As compared to the gene composition of the *in vitro* library, RNA binding / transcription protein genes were only in the *in vivo* library. In addition, both libraries share the same gene, such as *T. b. rhodesiense* activated kinase C protein receptor homolog (TRACK) protein coded gene.

Dot blot analysis on cDNA clones of the *in vivo* library

Even through the subtracted libraries generated by SSH are substantially enriched for differentially expressed mRNAs, they still contain genes that equally exist in the two cDNA populations (37). The present study also showed that there was

TRACK protein gene in the two libraries. Therefore, I screened the subtracted cDNA fragments from the *in vivo* library using *in vivo* or *in vitro* derived cDNA probes by dot blot analysis (Fig. 8). Unexpectedly, as compared to the intensity hybridized with the *in vitro* specific probe against the *in vivo* library, there was no gene that showed more than twice higher intensity than the *in vivo* specific probe (Fig. 8). However, the twice lower intensities were observed in 6 unknown genes (data not shown) and 5 genes (Fig. 8) as follows: *T. brucei* ribosomal protein L1 protein, *L. major* probable 40S ribosomal protein S6 protein (Fig. 8A, 2 and 3), *T. brucei* exosome-associated protein 4 protein, *Trypanosoma rangeli* cathepsin B-like protease protein (Fig. 8B, 12 and 13), *L. major* hypothetical predicted protein P265.14, and unknown function protein (Fig. 8C, 18).

Determination of differentially expressed genes with Northern blot analysis

To confirm *in vivo* or *in vitro* specific expression of the genes selected by dot blot analysis described above, the Northern blot analysis was conducted by hybridizing the total RNA of the trypanosome as summarized in Fig. 8. As an internal standard, α-tubulin gene of *T. brucei* (Accession number: Tb927.1.2360) was used. As a result (Fig. 9A), 2 genes were detected with corresponding probes, and 3 genes were not detected. The detected genes, however, showed almost the same expression levels. On the other hand, since the *in vitro* library consists of 5 unique genes as well as a gene coding TRACK protein that was common in the both libraries, the expression level of the 5 genes was also examined (Fig. 9B). Of the 5 clones, the 3 genes were detected but expressed at almost the same levels. The rest of 2 genes were not detected. In conclusion, differentially expressed genes were not detected in

this study.

3-4. Discussion

To determine whether differentially expressed genes exist in host or in cultivation, the candidates were selected using SSH technology, and then examined at expression levels. The SSH gave rise to the candidate genes, and these genes appeared to be differentially expressed by dot blot analysis. However, the Northern blot analysis showed genes neither specifically expressed *in vivo* nor *in vitro*. The result might indicate no differentially expressed genes between the *in vivo* and the *in vitro* propagated *T. brucei*. However, it is also considerable that the expression levels of the candidate genes might not be able to be differentiated by Northern blot analysis due to its low sensitivity to detect gene expression. It is possible that more sensitive methods, such as run-on assay, real-time PCR or micro array, might reveal differentiation of gene expression.

In case of *T. cruzi*, which also belongs to order Kinetplastida, comparisons of the *in vivo* and *in vitro* parasites have been reported in transcription (147) and protein levels (27). Interestingly, however, the either analysis indicated different genes, which could suggest the importance of comparative analysis of *in vivo* specific factors at protein expression level. In addition, I am not sure whether SSH is suitable method for detecting specific genes in the order Kinetplastida, because their genes are expressed polycistronically and regulated in post-transcriptional level (31).

Taken together, both gene expression and protein level must be examined in order to conclude existence or absence of *in vivo* specific genes in African

trypanosomes. The present study is the first step to examine further comparison between *in vivo* and *in vitro* derived African trypanosomes.

3-5. Summary

African trypanosomes could have *in vivo* specific genes for adaptation of host's environment. The present study here was conducted by using SSH technique to seek the enhanced genes especially in host (*in vivo*). Although total 328 clones from the *in vivo* SSH library and 160 clones from the *in vitro* SSH library were analyzed in their expression levels, no gene showed different expression. Although the result might indicate no trypanosome genes are differentially expressed between the *in vivo* and *in vitro* trypanosomes, further comparative analysis at both gene expression and protein level is necessary to conclude this issue. Taken together, this is the first step to examine gene expression level between *in vivo* and *in vitro* derived African trypanosomes.

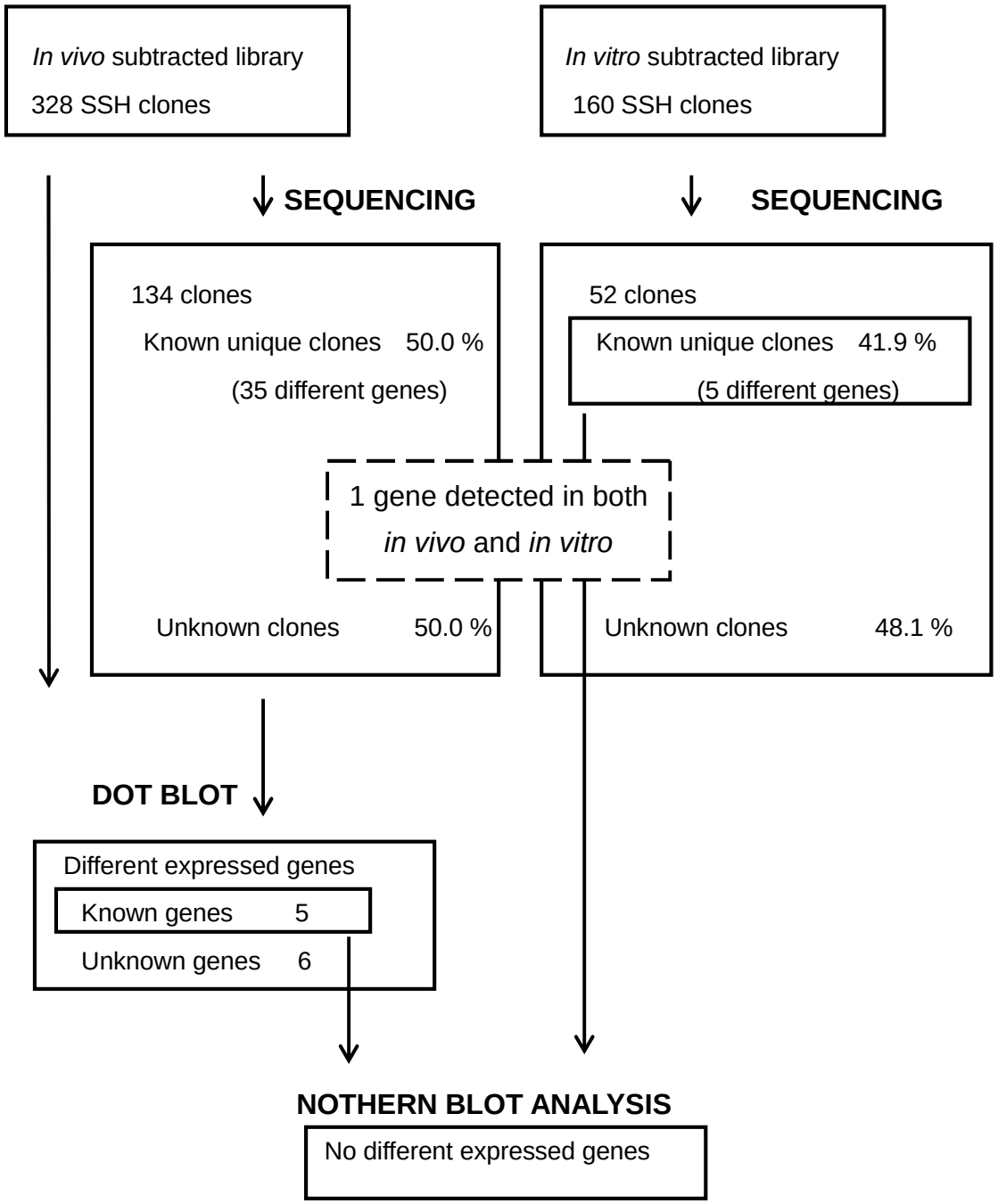


FIG 7. Summary of the SSH strategy.

TABLE 2. The clones in the *in vivo* subtracted library

Gene name	Accession #
Ribosomal L, S and P protein	
Trypanosoma brucei (*T. brucei*) ribosomal protein L1 protein	**Z54340**
T. brucei ribosomal protein L30 protein	**Z54339**
T. brucei ribosomal protein S12 protein	**AF031925**
T. brucei 40S ribosomal protein S13, putative protein	**AE017170**
Trypanosoma cruzi (*T. cruzii*) ribosomal L27 protein	**AY197727**
T. cruzi ribosomal protein P0 protein	**L15558**
T. cruzi ribosomal protein P1 protein	**X65025**
Leishmania major (*L. major*) probable 40S ribosomal protein S6 protein	**AL163505**
L. major ribosomal protein S2 protein	**AF467951**
L. major homologous to rat ribosomal protein S8 protein	**X62942**
L. major 40S ribosomal protein S4, copy 2 protein	**AL365154**
Argopecten irradians ribosomal protein L12 protein	**AF526229**
Myxine glutinosa ribosomal protein L17 protein	**AY130353**
Podocoryne carnea ribosomal protein L18a protein	**AJ009692**
Protease	
T. b. rhodesiense cysteine protease protein	**AJ297265**
T. brucei ubiquitin EP52/2	**X56511**
Trypanosoma rangeli (*T. rangeli*) cathepsin B-like protease protein	**AF400046**
RNA binding protein / transcription	
T. brucei RNA editing complex protein MP24 protein	**AY228168**
T. brucei RNA binding protein	**AF020695**
T. brucei exosome-associated protein 4 protein	**AJ516002**
T. cruzi putative eukaryotic translation factor 6 protein	**AF117890**
Other function	

T. b. rhodesiense activated kinase C protein receptor homolog (TRACK) protein	**AF049901**
Bloodstream-specific protein 1,3-4-*T. brucei*	**B37416**
T. b. rhodesiense prohibitin protein	**AF049901**
T. b. rhodesiense aldolase locus for fructose 1,6 bisphosphate aldolase	**X52586**
T. b.i rhodesiense alpha (complete) and beta tubulin genes in 3.7 kb tandem repeat	**K02836**
T. brucei HSP83 gene	**X14176**
T. brucei malate hydrogenase protein	**AF027739**
Urechis caupo 34/67 kD laminin binding protein protein	**U02370**
Kluyveromyces marxianus S10 protein	**S53430**
Bradyrhizobium japonicum USDA 110 heat shock protein protein	**AP005937**
Schizosaccharomyces pombe SPAC19B12.04,rps30-1,rps3001 protein	**AL390814**
probable ubiquitin-protein ligase (EC 6.3.2.19) fission yeast (*Schizosaccharomyces pombe*)	**T37499**

Conserved protein

T. brucei conserved hypothetical protein	**AL929604**
T. cruzi TC3_70K14.2 protein	**AC116971**
L. major hypothetical predicted protein P265.14, unknown function protein	**AL359716**
L. major hypothetical protein L8342.05 protein	**AL122012**

TABLE 3. The clones in the *in vitro* subtracted library

Gene name	Accession #
Ribosomal protein	
L. donovani 60S ribosomal protein L26 protein	**AF499606**
Protease	
T. brucei proteasome activator protein PA26 protein	**AF085608**
Other function	
T. b. rhodesiense activated kinase C protein receptor homolog (TRACK) protein	**AF049901**
T. brucei hypoxanthine-guanine phosphoribosytransferase protein	**L10721**
T. cruzi laminin receptor precursor-like protein/p40 ribosome associated-like protein protein	**AF133210**
Conserved protein	
T. brucei hypothetical protein, conserved protein	**AE017168**

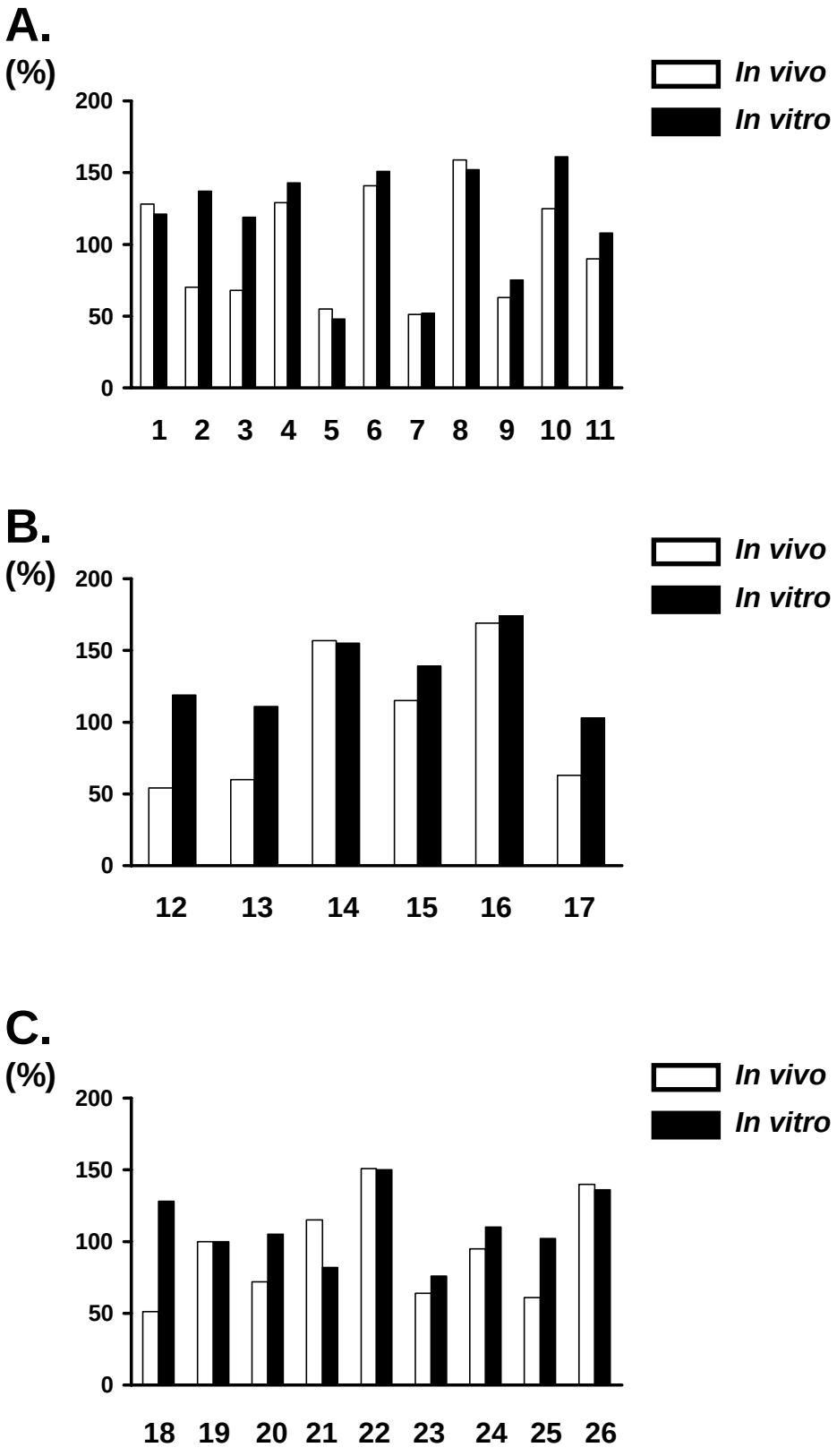

A.
(%)
200
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11
In vivo
In vitro
B.
(%)
200
150
100
50
0
12 13 14 15 16 17
In vivo
In vitro
C.
(%)
200
150
100
50
0
18 19 20 21 22 23 24 25 26
In vivo
In vitro

FIG. 8. Comparison between *in vivo* and *in vitro* RNA expression. Each cDNA clone was hybridized with [α-^{32}P] dCTP-labeled cDNA that was derived from *in vivo* or *in vitro* RNA. (A) 1, *T. cruzi* ribosomal protein L27 protein; 2, *T. brucei* ribosomal protein L1 protein; 3, *L. major* probable 40S ribosomal protein S6 protein; 4, *T. brucei* ribosomal protein L30 protein; 5, *T. brucei* ribosomal protein S12 protein; 6, *T. brucei* 40S ribosomal protein S13, putative protein; 7, *T. cruzi* ribosomal protein P0 protein; 8, *L. major* ribosomal protein S2 protein; 9, *Argopecten irradians* ribosomal protein L12 protein; 10, *Podocoryne carnea* ribosomal protein L18a protein; 11*, Myxine glutinosa* ribosomal protein L17 protein; (B) 12, *T. rangeli* cathepsin B-like protease protein; 13, *T. brucei* exosome-associated protein 4 protein; 14, *T. b. rhodesiense* cysteine protease protein; 15, *T. brucei* ubiquitin EP52/2; 16, *T. brucei* RNA binding protein; 17, *T. brucei* RNA editing complex protein MP24 protein; (C) 18, *L. major* hypothetical predicted protein P265.14, unknown function protein; 19, *T. b. rhodesiense* activated protein kinase C receptor homolog (TRACK) protein; 20, *T. b. rhodesiense* prohibitin protein, 21, bloodstream-specific protein 1.3-4 – *T. brucei*; 22, *T. b. rhdesiensi*e aldolase locus for fructose 1,6 bisphosphate aldolase; 23, *T. b. rhodesiense* alpha (complete) and beta tubulin genes in 3.7 kb tandem repeat; 24, *Schizosaccharomyces pombe* SPAC19B12.04,rps30-1,rps3001 protein; 25, *T. brucei* conserved hypothetical protein; 26, *T. cruzi* TC3_70K14.2 protein.

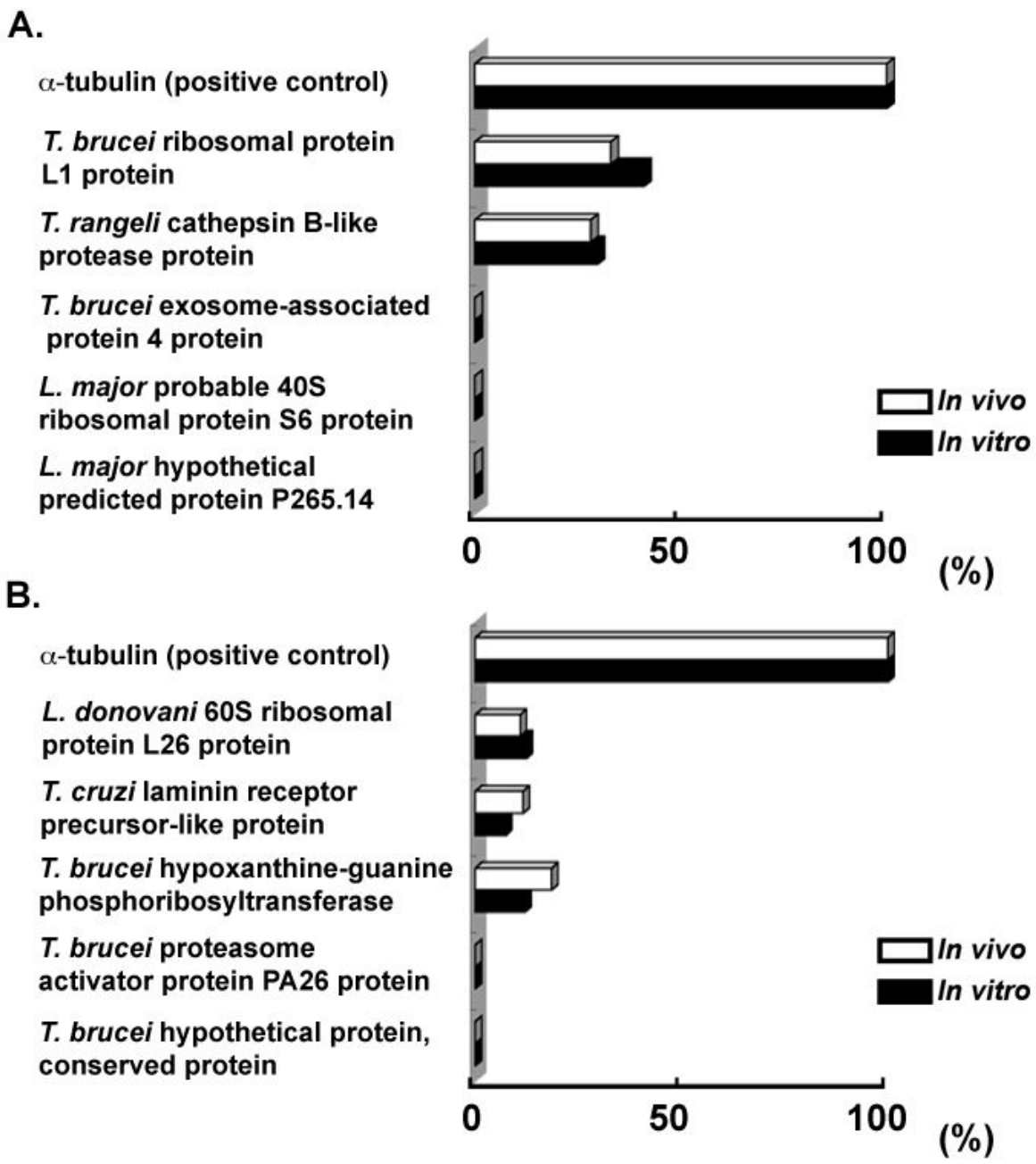


FIG. 9. The mRNA transcriptional levels of genes in subtracted libraries by Northern blot analysis. Total Trypanosome RNAs were transferred on membrane and hybridized with each cDNA clones. (A) The clones of the *in vivo* subtracted library. (B) The clones of the *in vitro* subtracted library.

Chapter 4

Characterization of Trans-Sialidase like Genes in Bloodstream Form of *Trypanosoma congolense*

4-1. Introduction

African trypanosomes are a tsetse-transmitted protozoan parasite that causes sleeping sickness and nagana in human and animals, respectively. In domestic ruminants, severe anemia is a major pathological feature that is caused by infection with *T. congolense*, *T. brucei*, and/or *T. vivax*. The aetiology of trypanosomiasis-associated anemia in cattle is multifaceted, with extravascular haemolysis and noncompensatory erythropoiesis playing a major part (3, 95). Modes of noncompensatory erythropoiesis are marked by proinflammatory cytokines during chronic infection. TNF-α, INF-β, IFN-γ, and IL-1α, work as negative modulators of erythropoesis to cause both decreased production of erythropoietin by the kidney and decreased responsiveness to the hormone in the bone marrow (64, 88). Alternately, extravascular haemolysis has been suggested due to (I) immunological mechanisms, like autogous Abs against red cell (6, 44, 73), or anti-trypanosome Abs against parasites-red cells complex (112), or (II) increased red cell damage by haemolysins, proteases (78), phospholipases (53, 138), and sialidase (10, 43). Considerably, these multifunctional factors would be co-induced during infection and establish its clinical feature.

While sialidase is considered to be a causative factor of the anemia, its existence in BSF of African trypanosomes is under controversy. Engstler *et al*. (42)

examined sialidase and TS activity in the BSF of African trypanosomes, and concluded that only the BSF of *T. vivax* has sialidase activity. On the other hand, as well as in infection of *T. vivax* (10, 43), sialidase activity was observed in sera of infected animals with *T. congolense* (102). Eventually, the parsite's lysates itself possess the activity in the BSF of *T. congolense* (102) and *T. vivax* (20). These accumulated results suggested the importance of sialidase molecule(s) in the BSF of *T. congolense* and *T. vivax*, which accumulates into blood of host and finally contribute to initial and continuous anemia. Interestingly, it is contrast to a preferably mentioned TS activity in the PCF for description of protective strategy from digestion in midgut of tsetse fly (42, 117).

Recently, Tiralongo *et al*. (136) reported that TS-like genes (TS1, Accession number AJ535487; TS2, AJ535488) were transcribed in the PCF of *T. congolense*. This study was designed to examine TS1 and TS2 expressions, and sialidase activity of the lysates from *T. congolense* BSF. Furthermore, it has been evaluated that the association of TS with anemia or parasite pathology and the vaccine potential of TS recombinants against trypanosomiasis.

4-2. Materials & Methods

Parasites

The BSFs of *T. congolense* IL3000 and IL1180, and the PCFs of *T. congolense* IL3000 were previously obtained and maintained as described in Materials & Methods of chapter 2.

DNA extraction

Total DNA was extracted from parasites by published methods (115). Briefly, lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 0.5% sodium dodecyl sulfate, and 100 μg of proteinase K per m*l*) was added to the samples, followed by overnight incubation at 55°C. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol. The purified DNA was dissolved in 100 μ*l* of sterilized distilled water.

Cloning and construction of TS1 and TS2 genes in expression vector

To amplify TS1 and TS2 genes in PCR, the corresponding primers described before (136) were prepared by adding enzyme restriction site as follows: TS1 forward primer, 5'-AGATCTGGTGGGAGAACGTGGAAGAG-3' (TconTS1F, the line below indicates *Bgl*II site); TS1 reverse primer: 5'-GAATTCGAAGCGCTAGCACCACCTGG-3'(TconTS1R, *Eco*RI); TS2 forward primer: 5'-CTCGACCACTTGTTGAGATAGACGGCG-3'(TconTS2F, *Xho*I); TS2 reverse primer: 5'-CTGCAGCACAGTTATGGCAATTGAGCTAC-3'(TconTS2R, *Pst*I). The reaction mixture (50 μ*l*) was prepared by mixing the parasites' genomic DNA with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 2 mM each of the four deoxynucleoside triphosphates, 5 pmol of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Ltd.). The PCR was conducted under the following condition: (i) 94^{o}C for 5 min (denaturation step), (ii) 30 cycles consisting of 94°C for 45 sec, 48°C for 1 min, and 72°C for 2 min, (iii) 72°C for 7 min (extension step). After the PCR products were purified using GENECLEAN III kit (Q-BIO gene, Irvine, CA), the restriction sites were cut using the corresponding enzyme (Roche Diagnostics Co., Basel, Switzerland) as described in the manufacture's suggestion.

Each treated gene was inserted into expression vector (pRSET, Invitrogen) using Takara ligation kit version 2 (Takara Bio Inc., Japan).

RNA isolation

Total RNA of the parasites was prepared as mentioned in Materials & Methods of chapter 3. The RNA was used immediately for Northern blotting or stored at -80ºC until use.

Reverse transcribed (RT)-PCR

Poly $(A)^+$ RNAs were purified from the total RNA using oligo (dT) spin columns (GE Healthcare) following to suggested protocol. The RT reaction was conducted by mixing the Poly $(A)^+$ RNAs and hexaT (GE Healthcare), and incubating them at 95^{o}C for 1min, 60^{o}C for 30 min, and 72^{o}C for 1 min. After the reaction, the product was used as a complementary DNA, and PCR was conducted with TS1 primers or TS2 primers under the same condition as described in Materials & Methods of this chapter.

Northern blotting

TS1 and TS2 transcriptions were examined using the specific probes against total RNAs of the parasites. The radioisotope (RI)-labeled specific primers were prepared in PCR using ^{32}P-dCTP, the specific primers, and the TS genes inserted T/A vector under condition described above. As an internal control for northern blotting, the RI-labeled P0 subunit ribosomal protein gene (*P0*) was synthesized using the forward primer (5'-CGTGGTAAGGGTGAATTGG-3') and the reverse primer (5'-GTGTCCGTCCAACACCTTC-3') in the same condition as described above.

The total RNAs were prepared and electrophoresed in 1.0 % gel and

transferred to a the Hybond-N^+ nylon with 20 × SSC. Hybridization was performed at 42^{o}C for 12 hrs in hybridization solution containing 50 % formaldehyde, 0.6 M NaCl, 0.2 M Tris-HCl (pH 8.0), 0.02 M ethylenediaminetetraacetic acid (EDTA), 0.5 % SDS and 100 μg/m/ heat-denatured salmon sperm DNA. Filters were washed with 2 × SSC and 0.1 % SDS three times for 10 min each, and subsequently washed 0.2 × SSC and 0.1 % SDS 4 times for 15 min each. Detection was done by autoradiography.

Preparation of recombinant TS proteins and ant-TS Abs

The recombinant TS1 and TS2 proteins fused with his-tag were prepared using pRSET expression vector as following to manufacture's suggestion. The proteins were purified with his-tag using ProBond Purification System (Invitrogen) under denature condition, and used for following experiments. For preparation of anti-TS1 and TS2 Abs, five of BALB/c mice were immunized i. p. with 100 μg /head of the corresponding protein mixed with Freund's complete / incomplete adjuvant (Sigma) every two wks, total 4 times. After measuring apparent titers of anti-TS Abs in ELISA described below, the Abs were collected by heat puncture.

ELISA

After 10 μg/m/ of recombinant TS1, TS2 and the parasite's lysates were absorbed on 96-well plates (NUNC A/S), 150 μ/ of 3% skim milk in PBS were put on each well of the plates that were incubated overnight at 4^{o}C. The plates were washed with tris-buffered saline (TBS) at 3 times, reacted with the corresponding sera in the 3% skim milk (× 100 dilution) at 37^{o}C for 1 hr, and again washed with TBS at 3 times. For a titration of IgG, anti-mouse goat IgG conjugated with horseradish peroxidase (HRP) (GE healthcare) in the 3% skim milk (× 1000 dilution) was dispersed into the

well of the plates that were incubated at 37^{o}C for 1 hr. The plates were washed TBS at 3 times, and then reacted with substrate (0.1M citric acid, 0.2M sodium phosphate, 0.003% H_2O_2, and 0.5mg of 2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid [ABTS, Sigma]) at 37^{o}C for 30 min. After reaction, the each absorptive integrity was measured at 415nm using Microplate leader (MTP-500, Corona electric, Ltd., Japan). For titration of IgM, IgG1, IgG2a, IgG2b, and IgG3, anti-mouse goat each Ig subtype conjugated with biotin (GE healthcare) was used as a 2nd antibody. After the plates were incubated with sera as a 1st antibody, the plates were incubated with either Ig subtype in the 3% skim milk (× 300 dilution) as a 2nd antibody, washed with TBS at 3 times, and then incubated with streptoavidin conjugated with HRP at 37^{o}C for 30 min. (Becton Dickinson and Company). After the plates were washed with PBS at 3 times, the following substrate's reaction was conducted as described above.

IFAT

Five μl of blood from the parasite-infected mice was collected and washed with 1 ml of glucose-PBS three times at 4^{o}C. After suspending the blood with 10 μl PBS, and the blood-smear was prepared on slide glass. The smears were fixed with absolute methanol for 1 min at –30^{o}C. After blocking with 3% skim milk (Wako) in PBS, the glasses were reacted with anti-TS Abs in the 3% skim milk (× 100 dilution) at RT for 1 hr, washed with TBS at 3 times. Under darkness, the glasses were incubated with anti-mouse goat IgG conjugated with Alexa 488 (Invitrogen) in the 3% skim milk (× 100 dilution) at RT for 1 hr, washed with TBS at 3 times. The treated glasses were examined with a confocal laser scanning microscope (TCS NT; Leica).

Western blot

The parasites' pellets were suspended in 100 μ*l* of a sample buffer for SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 62.5mM Tris–HCl [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, 2% SDS, 0.01% bromophenol blue) and heated at 100^{o}C for 2 min. The parasites in sample buffer were subjected to SDS-PAGE on 10% ployacrylamide gels and Western blot analysis was conducted (151) using the anti-TS Abs.

Sialidase activity from parasite's lysates

The extract from parasites' lysates was prepared following the previous study (102). Briefly, the collected parasites were suspended in 0.1 M phosphate buffer, pH 6.8, containing 0.2% Triton-X and kept at room temperature (25^{o}C) for 30 min. The lysed cells were then centrifuged at 5000 × *g* for 10 min. The supernatant was retained and the pellet re-extracted twice. Sialidase activity was assayed as described (72). Briefly the substrate 4-methylumbelliferyl-Neu5Ac (4-MU-Neu5Ac: Sigma) at a final concentration of 0.7 mM in 50 mM acetate buffer, pH 5.5, was incubated with 50 μ*l* of supernatant at 37^{o}C for 30 min. At the end of the incubation period, the released methylumbelliferone was then measured at 525 nm of excitation and 490 nm of emission with Fluoroscan Ascent (Thermo Electron Corporation, Waltham, MA).

Vaccination effect of TS1 and TS2 recombinant proteins

The vaccination effect of TS1 and TS2 were evaluated in mice infected with the BSF of *T. congolense* IL3000. Five-wk-old female BALB/c mice were immunized with either TS1 or TS2 s.c. every two wks 4 times. At one week after 4th immunization, the immunized mice i.p. injected 1 × 10^{3} parasites/head of *T. congolense* IL3000.

Parasitemia and packed cell volume (PCV) were monitored every day via a tail snip. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (admission number: 14-69).

Statistical analyses

The titers of total IgG and Ig subtypes were statistically analyzed using the Student's *t*-test. Significance was defined by *P*-values of <0.05 and <0.01.

4-3. Results

Transcription of TS-like genes in the BSF of *T. congolense*

In a preliminary study, RT-PCR was performed using total RNAs of the BSF with TS1 or TS2 specific primers and corresponding bands were confirmed using BLAST search. Next, I analyzed the RNAs transcription levels of TS1 and TS2 in the BSF by conducting Northern blotting. Interestingly, in both the PCF of IL3000m, and the BSF of IL3000 and the BSF of IL1180, TS1 probe clearly showed strong intensity while TS2 probe exhibited relatively low intensity (Fig. 10). TS1 intensity was especially stronger in the PCF of IL3000 and the BSF of IL1180. In contrast, TS 2 transcriptions were also found, but slightly detected in three tested parasites.

Characterization of the BSF of *T. congolense* with anti-TS Abs

The TS1 and TS2 expressions in the BSF were examined using anti-TS1 and anti-TS2 Abs in IFAT. Accordingly, strong fluorescence was observed following staining with either anti-TS1 (Fig. 11A) or anti-TS2 (Fig. 11B) in BSF of *T. congolense*

IL3000. In contrast, both anti-TS1 (Fig. 11C) and anti-TS2 (Fig. 11D) Abs exhibited a weak fluorescence inside *T. congolense* IL1180 BSF parasites. In both cases, anti-TS1 Ab tended to give a stronger reaction than anti-TS2 Ab (Fig. 2A-D).

The reactions of anti-TS serum were also confirmed by Western blotting (Fig. 12). Since the PCF of *T. congolense* IL3000 have been reported to possess about 70 kilo dalton (kDa) of TS1 and TS2 proteins (137), this stage was also added to this analysis. When the anti-TS1 Ab was used, expected band of TS (about 70 kDa) was detected strongly in the PCF IL3000 (Fig, 12A, 1), and relative weakly in the BSF IL3000 and Il1180 (Fig, 12A, 2 and 3). On the other hand, staining with anti-TS2 Ab revealed the expected band in the IL3000 PCF (Fig, 12B, 1)., but not in the BSF IL3000 and Il1180 (Fig, 12B, 2 and 3). In agreement with the IFAT data above (Fig. 11), TS1-Ab reacted more strongly with the IL3000 BSF than the IL1180 BSF.

Sialidase activity in parasite lysates

The sialidase activity in the parasite lysates were measured and compared between the PCF and the BSF. In contrast with previous report (102), the IL3000 PCF exhibited stronger activity (782.7 intensity/mg) compared to In contrast, the activity in the BSF of either IL3000 appeared to that of the BSF (20.3 intensity/mg).

Vaccination effect of TS1 and TS2

The vaccination effects of TS1 and TS2 were evaluated using mice infected with *T. congolense* IL3000. The lysates of the BSF of *T. congolense* IL3000 was also used as a control vaccine molecule (41, 121). Table 4 surmarizes the survivals of vaccinated mice after 30 DPI. Interestingly, partial protective effect was observed in 100 μg TS1 vaccinated group (33.3 %) as well as complete protection in the lysates

group. The effect of 100 μg TS1 was in contrast to that of the other groups that exhibited no survivals.

Parasitemia and PCV were monitored during the course of infection. Accordingly, 100 μg TS1 vaccinated group failed in suppressing waves of parasitemia as well as TS2- and PBS-vaccinated groups. In contrast, the lysates-vaccinated group showed no detectable parasitemia (Fig. 13A). TS vaccinated groups also exhibited no significant difference of PCV as compared to that in the PBS group. The lysate group, having no parasitemia, did not follow the reduction of PCV observed in TS- and PBS-vaccinated groups (Fig. 13B).

To understand the protective effect of TS1 vaccine, Ig responses in the 100 μg of TS1-, TS2-, the lysates-, or PBS-vaccinated group were examined (Fig. 14). The TS1 vaccination induced significantly higher levels of total IgG (Fig. 14A) and IgG2b (Fig. 14C) as compared to those in TS2-vaccinated group. TS1 appeared to induce IgG2a than IgG1, which is similar to those in the lysates-vaccinated groups (Fig. 14B, C and E). In contrast, TS2 vaccination showed lower levels of total IgG (Fig. 14A), and higher ratio of IgG2a/IgG1 (Fig. 14E). The IgM and IgG3 were seldom detected in the groups.

4-4. Discussion

The current study examined TS1 and TS2 gene expression in the BSF of *T. congolense* at transcriptional and protein levels. Interestingly, TS1 transcription and anti-TS1 Ab reaction were stronger than those of TS2. Although TS1 transcriptions were detectable in the BSF as well as the PCF parasites, however, anti-TS1 Ab

sightly reacted the expected TS band in the BSF parasites as compared to the strong reaction to TS in the PCF parasite. On the other hand, TS2 was rarely or not detected in the BSF either at transcriptional or protein level. The results suggest that the BSF parasites posess TS1 molecule, but its expression level is relatively lower than that in the PCF.

Although fuction of TS1 in the BSF is unknown, our study indicated that (i) since the BSF showed no detectable sialidase activity, TS1 might not be related to sialidase activity or pathology of anemia (ii) survival effect of TS1 vaccine indicatates that TS1 might be pathologically important molecule during the course of *T. conogolense* infection. Interestingly, TS1 vaccinated effect was not due to reduction of parasitemia, but release of pathology. Since relatively higher levels of total IgG and IgG1 were induced in TS1 vaccinated group, they could be related to suppress the pathology of trypanosomes. In additon, induction of IgG2b via TS1 vaccine could describe backborne of the protective mechanism. Since class-switching of antibodies to IgG2b needs co-induction of transforming growth factor (TGF)-β (130), this anti-inflammatory cytokine could be induced and involved in survival of TS1 vaccinated group.

Taken together, this study suggests the existence of TS1 in the BSF, and TS1 vaccination contributed to the host's survival, which cold be related to the parasite's pathology.

4-5. Summary

While sialidase was considered to be a causative factor of the anemia, its

existence in BSF of African trypanosomes is under controversy. The current study examined TS1 and TS2 gene expression in the BSF of *T. congolense* at transcriptional and protein levels. Interestingly, although TS1 transcriptions were detectable in the BSF as well as the PCF parasites, anti-TS1 Abs sightly reacted the expected TS band in the BSF parasites as compared to the strong reaction to TS in the PCF parasite. The TS2 was rarely or not detected in the BSF either at transcriptional or protein level. Our study also indicated that (i) since the BSF showed no detectable sialidase activity, TS1 might not be related to sialidase activity or pathology of anemia (ii) survival effect of TS1 vaccine indicatates that TS1 might be pathologically important molecule during the course of *T. conogolense* infection. Taken together, the study suggests the existence of TS1 in the BSF, and TS1 vaccination contributed to the host's survival, which cold be related to the parasite's pathology.

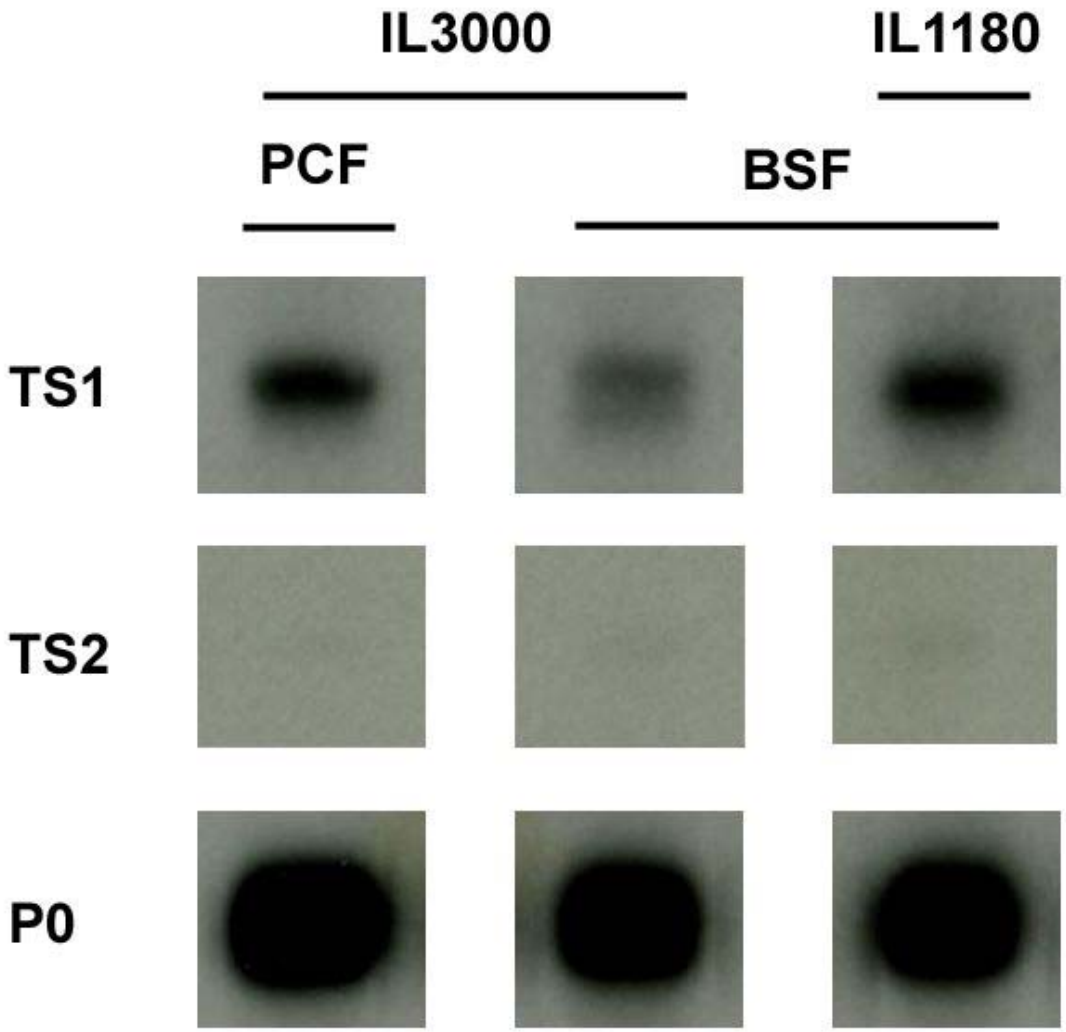


FIG. 10. The transcription of trans-sialidase genes (TS1, Accession number AJ535487; TS2, AJ535488) in *T. congolense*. PCF, procyclic form; BSF, bloodstream form.

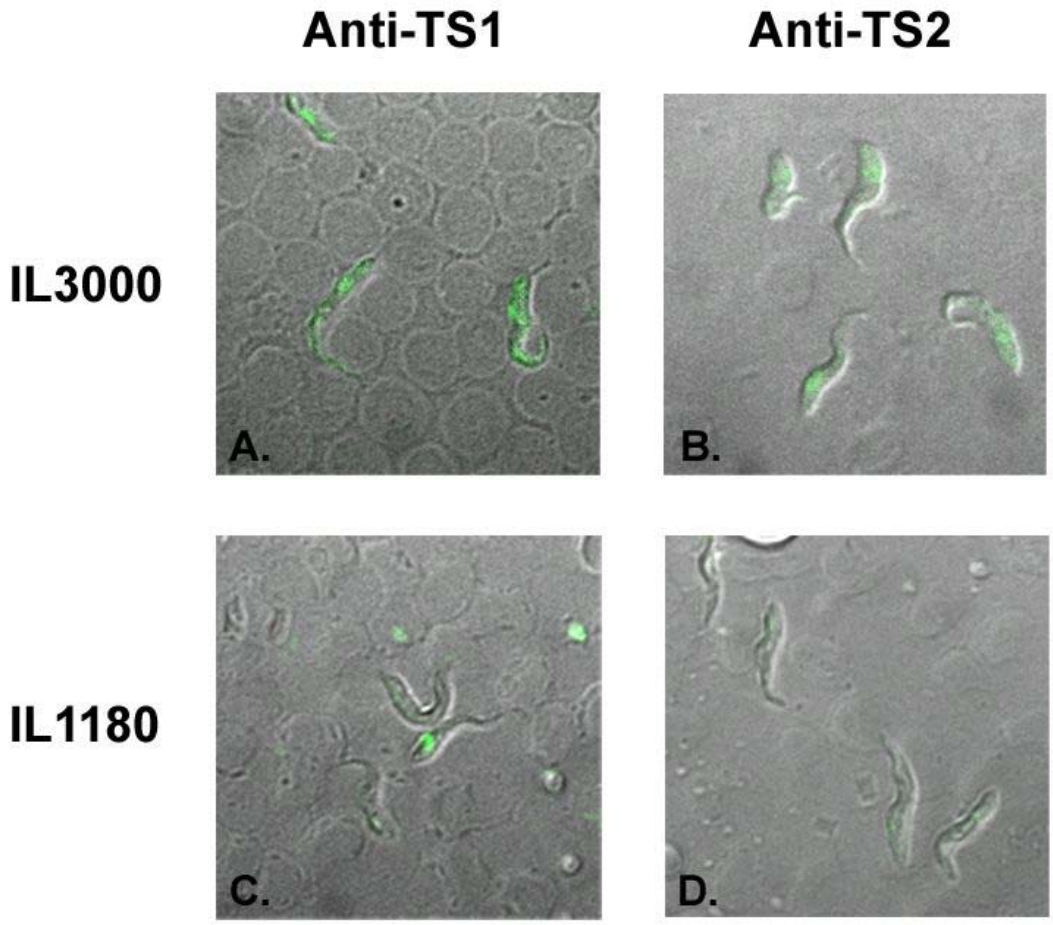


FIG. 11. The reaction of anti-TS1 and TS2 Abs against BSF of methanol-fixed *T. congolense* in IFAT. Green indicated the reaction of anti-TS Abs.

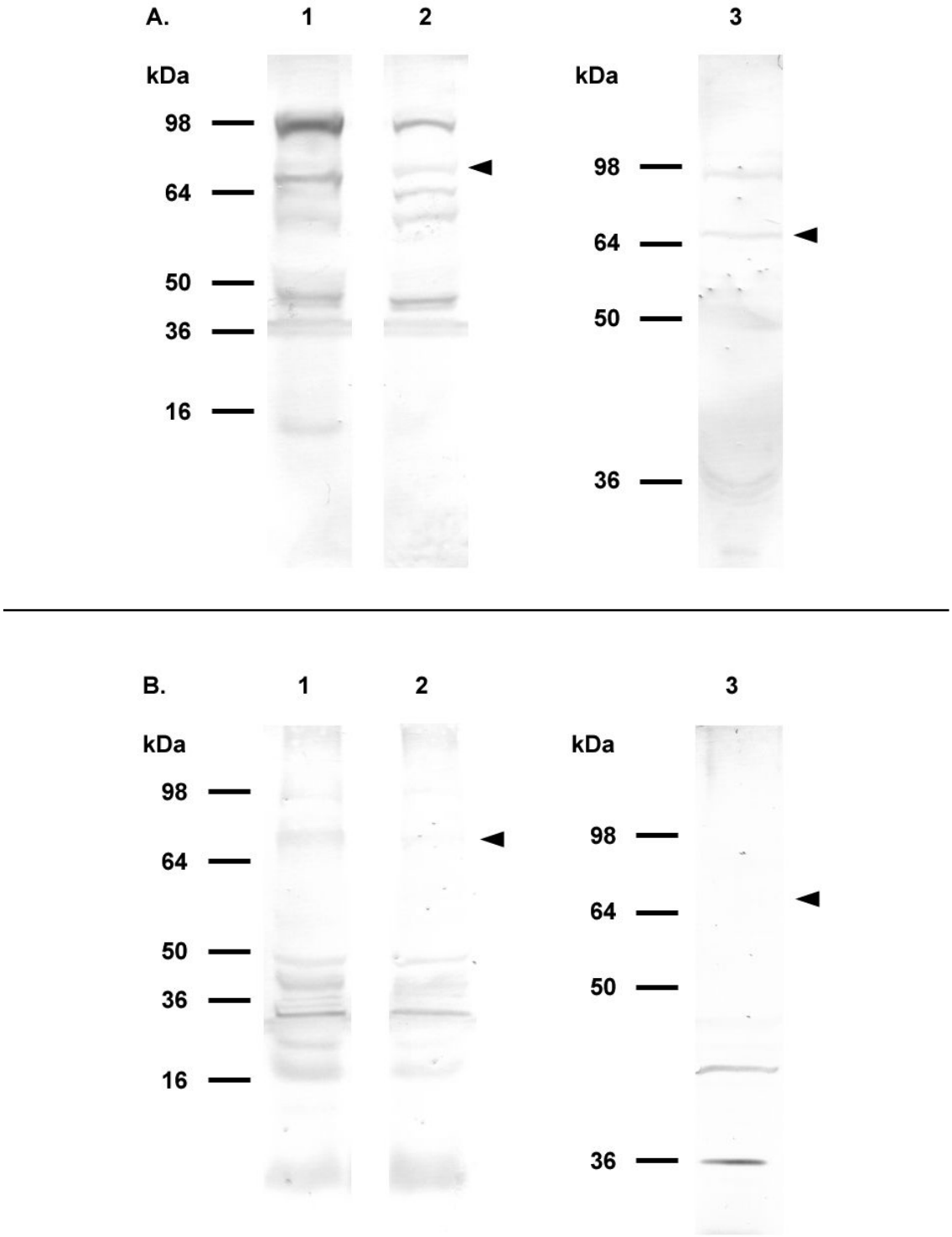


FIG. 12. The reaction of anti-TS1 (A) and TS2 (B) Abs against *T. congolense* in Western blotting. 1, PCF of *T. congolense* IL3000; 2, BSF of *T. congolense* IL3000; 3, BSF of *T. congolense* IL1180. The arrow indicates estimated TS molecules previously reported (Tiralongo *et al.,* 2003).

TABLE 4. Survival rates against *T. congolense* IL3000 infection.

Vaccine molecules (μg/head)*	Survival rates (%)**
The lysates (100)	6/6 (100)
TS1 (100)	2/6 (33.3)
TS1 (20)	0/6 (0)
TS2 (100)	0/6 (0)
TS2 (20)	0/6 (0)
PBS	0/6 (0)

* After total 4 times immunizations, 1,000 cells of *T. congolese* IL3000 was injected the immunized mice, as described in Materials & Methods.

** Rates were determined at 30 DPI after *T. congolese* IL3000 infection.

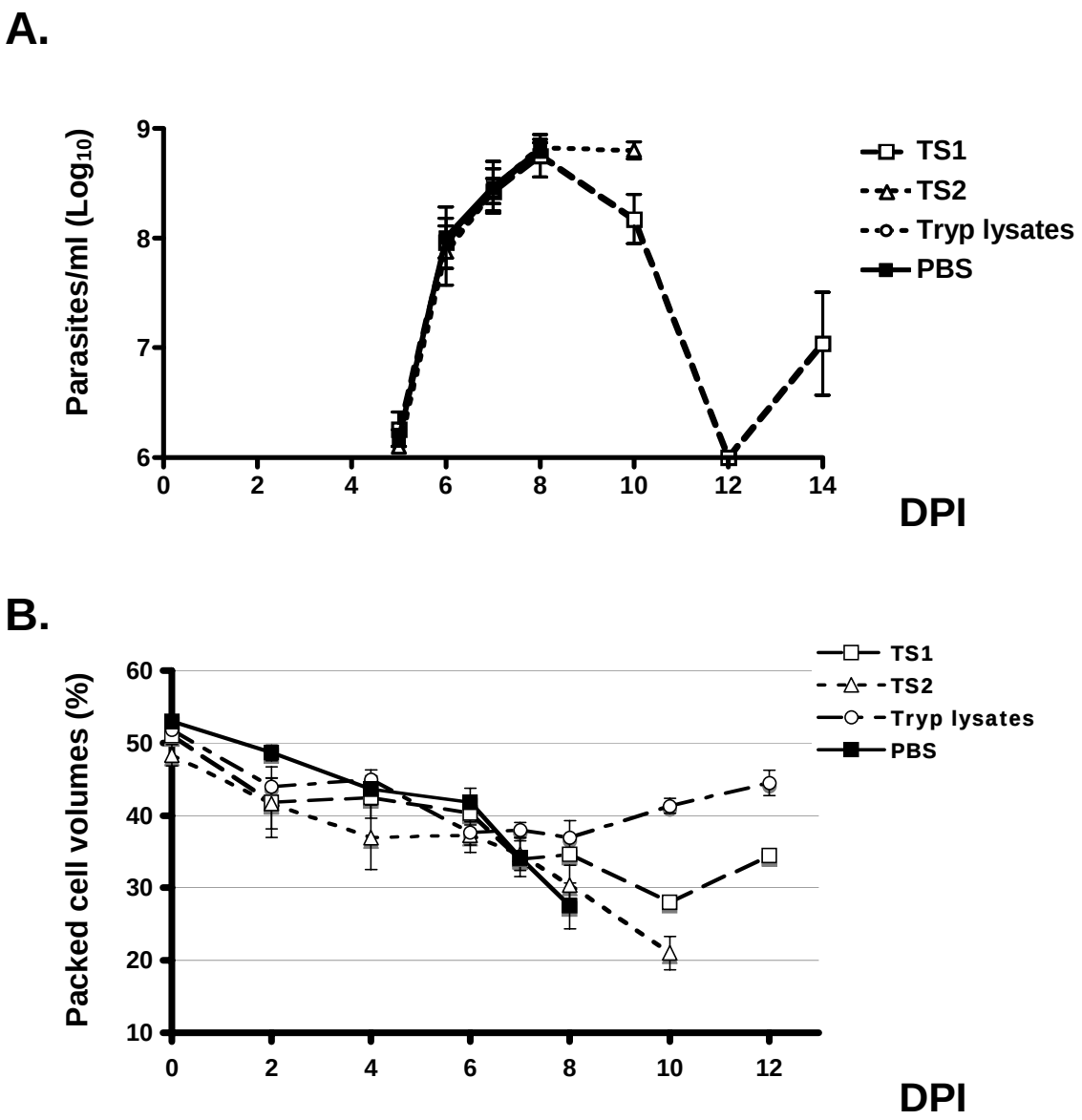


FIG. 13. Efficacies of TS1 and TS2 recombinants as a vaccine against *T. congolense* IL3000 infection. The mice immnuzed with 100 μg of TS1, TS2, and trypanosoma lysates of *T. congolense* IL3000, were infected with 1,000 cells of *T. congolense* IL3000 i.p.. During the course of infection, Parasitemia (A) and Packed cell volumes (B) were monitored. DPI indicates Days Post-infection.

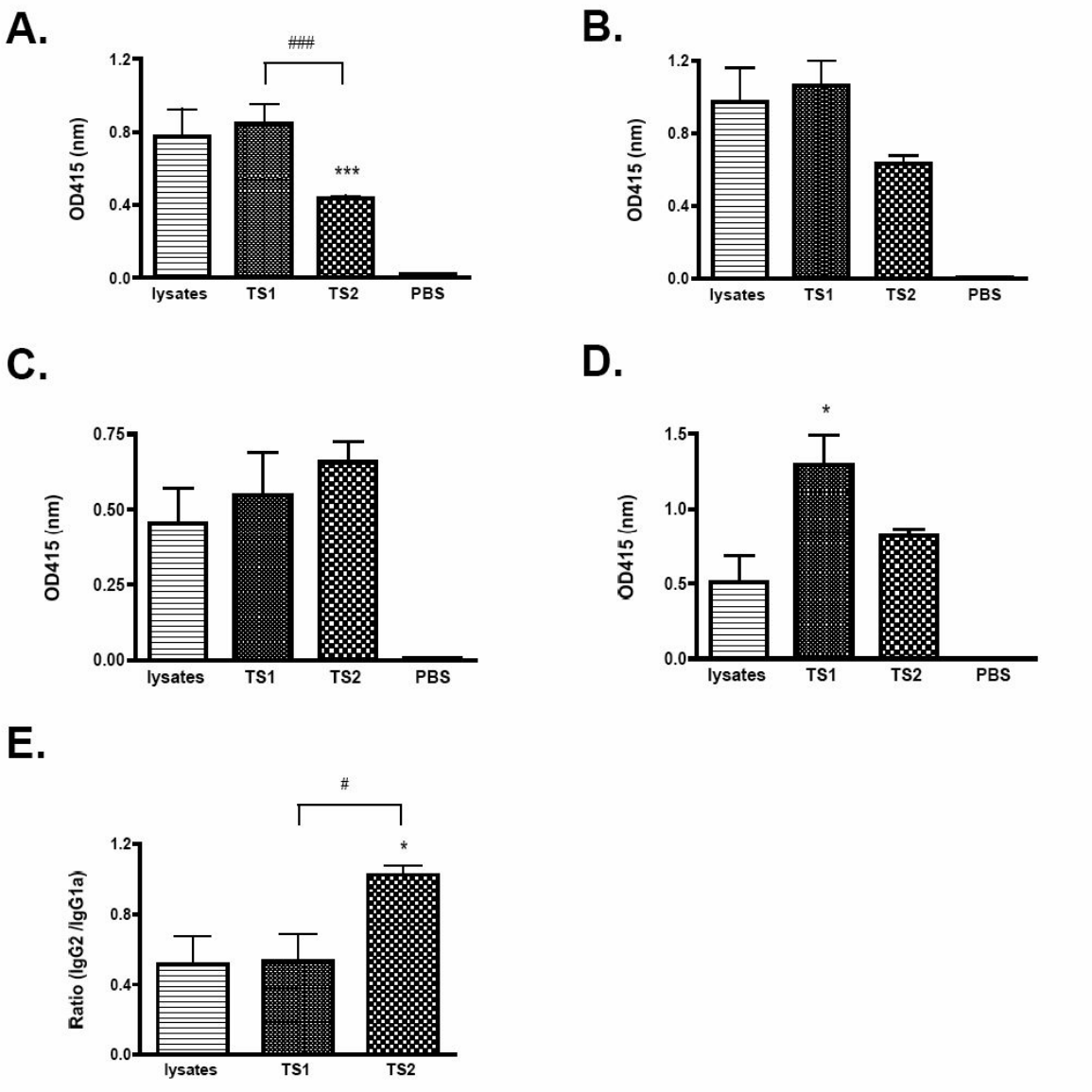


FIG. 14. Inductions of IgM, IgG and subtypes via immunization of TS1 and TS2 recombinants. Each group was immunized with 100 μg of BSF of T. congolense IL3000 (lysates), TS1, or TS2 subcutaneously every 2 wks, total 4 times. At a wk after last immunization, sera from the mice were collected, and subject to titration of IgG Abs in ELISA. A, titration of total IgG; B, titration of IgG1; C, titration of IgG2a; D, titration of IgG2b; E, ratio of IgG2a / IgG1. Statistical analysis was conducted using Student's *t*-test. * indicates statically significance as compared to lysates-immunized group (*, $p<0.05$; ***, $p<0.01$). # indicates statically significance as compared to TS1-immunized group (#, $p<0.05$; ###, $p<0.01$).

Chapter 5
Loop-mediated isothermal amplification (LAMP) for the detection of African trypanosomes

5-1. Introduction

African trypanosomes are medically and agriculturally important protozoan parasites that cause sleeping sickness in humans and nagana in cattle. Since African trypanosomiasis is fatal if left untreated or misdiagnosed, specific and sensitive detection methods are required if early and life-saving treatment for the disease is to be initiated. PCR has evolved as one of the most specific and sensitive methods for the diagnosis of infectious diseases, and many applications of PCR for detecting pathogenic microorganisms have been reported (18, 28, 49, 50, 71, 129). However, problems of reproducibility of PCR diagnosis of human African trypanosomiasis, especially on samples from serologically positive but apparently aparasitemic cases, are also reported (129). Moreover, it has been pointed out that *Taq* DNA polymerase is easily inactivated by tissue and blood-derived inhibitors, such as myoglobin, heme-blood protein complex, and IgG (1, 2, 12, 65). These findings appear to indicate the difficulty in optimizing the reaction conditions in PCR. Recently, a powerful application of PCR, termed real-time PCR, was developed, and applications of a real-time PCR to protozoan parasites have been reported (13, 30, 77, 94). Rapid quantitation and detection of *T. cruzi* and *Leishmania* infections by real-time PCR have been reported, and their application for diagnosis appear to be possible (30, 94).

However, in spite of excellent specificity and sensitivity of PCR and real-time PCR, these methods are not commonly used in the diagnosis of African trypanosomiasis. The reason for this is based more on economics and practicality than need, for in developing nations where African trypanosomiasis is endemic, the automated thermal cyclers and/or real-time quantitative PCR thermal cyclers required for the methods are often not affordable and might work erratically at high ambient temperatures and humidity and/or in dusty environments. Therefore, the identification of African trypanosomes in clinical samples still relies heavily on relatively insensitive microscopic observation of blood smears and cerebrospinal fluid. Therefore, cost-effective, simple, and rapid DNA amplification methods for the diagnosis of early and advanced African trypanosomiasis are clearly needed. The LAMP, loop-mediated isothermal amplification, may provide one answer. The LAMP, a method recently developed by Notomi *et al.* (103), relies on autocycling strand displacement DNA synthesis by a *Bst* DNA polymerase. The LAMP requires two specially designed inner and two outer primers (Fig. 15A); as such, LAMP amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions. Since the LAMP reaction is done under isothermal conditions (63 to 65°C), simple incubators, such as a water bath or block heater, are sufficient for the DNA amplification. Moreover, LAMP synthesizes 10 to 20 μg of target DNA within 30 to 60 min, and the LAMP reaction appears to be limited only by amount of deoxynucleoside triphosphates and primers (51, 103). In the process, a large amount of pyrophosphate ion is produced, which reacts with magnesium ions in the reaction to form magnesium pyrophosphate, a white precipitate by-product (93). This phenomenon allows easy and rapid visual

identification that the target DNA was amplified by LAMP. Therefore, LAMP is a highly sensitive and specific DNA amplification technique suitable for diagnosis of an infectious disease both in well-equipped laboratories and in field situations. In this study, LAMP primer sets specific for either the *T. brucei* group (*T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, and *T. evansi*) or *T. congolense* were designed. A LAMP reaction specific for the *T. brucei* group was evaluated for specificity and sensitivity *in vitro* as well as *in vivo*, and the results were compared with both microscopic observations and classic PCR.

5-2. Materials and Methods

Cells

The protozoan parasites and the mammalian cells used for this study were *T. b. brucei* GUTat3.1, *T. b. gambiense* IL3253 and IL2343, *T. b. rhodesiense* IL1501, *T. evansi* Tansui, *T. congolense* IL3000, *T. cruzi* Tulahuen, *Theileria orientalis* Chitose, *T. equi* U.S. Department of Agriculture, *Babesia bigemina* Argentina, *B. bovis* Texas, *B. caballi* U.S. Department of Agriculture, *Toxoplasma gondii* RH, *Neospora caninum* NC-1, NIH 3T3 (ATCC CRL-1658), HCT-8 (ATCC CCL-244), MDBK (ATCC CCL-22), and Vero (ATCC CCL-81) cells. With the exceptions of *T. b. gambiense* IL3253 and *T. orientalis*, all parasites and cells were maintained *in vitro* (8, 60). *T. b. gambiense* IL3253 was propagated in severe combined immunodeficiency (SCID) mice (62) and purified from infected blood by DE52 anion-exchange column chromatography (74). *T. orientalis* was obtained from infected cattle blood. All animal experiments were conducted in accordance with the Standards Relating to the Care and Management

of Experimental Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine, Hokkaido, Japan (admission number: 14-69).

DNA extraction

The DNA extraction was conducted as described in Materials & Methods of chapter 4. When infected blood was used, total trypanosome (i.e., *T. b. gambiense* IL3253) DNA was extracted as follows. First, the infected blood was blotted and dried on filter paper (FTA card; Whatman, Biometra GmbH, Germany). The blotted blood was cut out with a 2-mm hole puncher (2.0-mm Harris Micro Punch; Whatman). A portion of the blotted blood was then washed three times with 200 μ*l* of FTA purification reagent (Whatman) and twice in 200 μ*l* of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The washed piece of filter paper was used as the source of template DNA for both LAMP and PCR.

Oligonucleotide primers

The LAMP reaction needs four oligonucleotide primers: forward inner primer (FIP), back inner primer (BIP), and two outer primers (F3 and B3) (Fig. 15A) (103). All primer sequences were designed with the software program PrimerExplorer V1 (Fujitsu, Tokyo, Japan). Briefly, the design of the two outer primers, F3 and B3, is the same as that of regular PCR primers, while the design of the two inner primers, FIP and BIP, is different from that of PCR. The inner primers bind both sense and antisense strands of target DNA, and two binding regions within the inner primer (F2 and F1c, or B2 and B1c) are connected by TTTT spacer (Fig. 15A and Table 5). Two sets of 4 primers, named A1 and A2, were designed to hybridize to the gene encoding the paraflagella rod protein A (*PFR A*; GenBank accession number X14819) of *T.*

brucei. Two other primer sets, named P01 and P02, were designed to hybridize to the gene for *P0* (GenBank accession number AB056702) of *T. congolense*. For the PCRs, the outer primers (B3 and F3) were used as the PCR primer pair. All primer sequences are listed in Table 5.

LAMP reaction

The mechanism and expected reaction steps of LAMP were described previously (103). Briefly, inner primer FIP hybridizes to F2c in the target DNA and initiates complementary strand synthesis. Outer primer F3, which is a few bases shorter and lower in concentration than FIP, slowly hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis, releasing a FIP-linked complementary strand, which can form a looped out structure at one end. This single-stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis, leading to the production of a dumb-bell form DNA, which is quickly converted to a stem–loop DNA by self-primed DNA synthesis. This stem–loop DNA then serves as the starting material for LAMP cycling, the second stage of the LAMP reaction. To initiate LAMP cycling, FIP hybridizes to the loop in the stem–loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem–loop DNA with an additional inverted copy of the target sequence in the stem and a loop formed at the opposite end via the BIP sequence. Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem–loop DNA and one gap repaired stem–loop DNA with a stem elongated to twice as long (double copies of the target sequence) and a loop at the opposite end. Both these products

then serve as template for a BIP-primed strand displacement reaction in the subsequent cycles, a part of which is designated the elongation and recycling step. Thus, the final products are a mixture of stem–loop DNAs with various stem lengths and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand.

The LAMP was carried out with the Loopamp DNA amplification kit (Eiken Chemical Co. Ltd., Tokyo, Japan). Briefly, the LAMP reaction mixture (25 μl) contained template DNA, 40 pmol each of FIP and BIP, 5 pmol each of F3 and B3, 8 U of *Bst* DNA polymerase large fragment (New England Biolabs Inc., Ipswich, MA), 1.4 mM deoxynucleoside triphosphates, 0.8 M betaine, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, and 0.1% Tween 20. As a negative control, template DNA was omitted from the reaction. The reaction mixture was incubated at 65°C for 1 hr and heated at 80°C for 2 min to terminate the reaction. The mechanism of LAMP reaction was well explained by Notomi *et al*. (103). In addition, *Hafner et al.* reported that the isothermal in vitro amplification and multimerization of linear DNA targets (linear target isothermal multimerization and amplification) with two primers and *Bst* DNA polymerase (51). The LAMP reaction relies mainly on autocycling strand displacement DNA synthesis that is similar to the cascade rolling-circle amplification reported by Hafner *et al.* (51). However, there is a possibility that linear target isothermal multimerization and amplification also occurs during the LAMP reaction. The minimum LAMP reaction unit consists of two inner primers (FIP and BIP) and target DNA, as shown in Fig. 15B. Each inner primer contains two distinct sequences corresponding to the sense and antisense sequences of the target DNA

and form stem-loop structures at both ends of the minimum LAMP reaction unit (Fig. 15C). These stem-loop structures initiate self-primed DNA synthesis and serve as the starting material for subsequent LAMP cycling reaction. The LAMP products were electrophoresed in a 1.5% Tris-acetic acid-EDTA (TAE) agarose gel. Gels were stained with ethidium bromide solution (1 μg/m*l*).

PCR

The PCRs were carried out under standard and enhanced conditions. Standard conditions of PCR (designated PCR 1) are as follows. The PCR mixture (50 μ*l*) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM each of the four deoxynucleoside triphosphates, 5 pmol of each primer, and 0.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems Japan Ltd.). The reaction mixtures were incubated in a programmable heating block (Whatman) at 94°C for 10 min as an initial denaturation step and then subjected to 30 cycles consisting of 45 sec at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by a terminal elongation for 7 min at 72°C. On the other hand, enhanced PCR (designated PCR 2) was performed as follows. The PCR mixture (50 μ*l*) contained 10 μ*l* of 5 x Ampdirect-D (Shimadzu Biotech Co., Kyoto, Japan), 2 mM each of the deoxynucleoside triphosphates, 5 pmol of each primer, and 0.5 U of *Taq* DNA polymerase (Takara Bio Inc.). The reaction program is the same as that of PCR 1 except thermal cycling was repeated 40 times. Ampdirect-D is a reagent capable of effectively neutralizing the substances that inhibit DNA amplification (99). The PCR products were electrophoresed in a 1% TAE agarose gel and the gels were stained with ethidium bromide solution (1 μg/m*l*).

Cloning and sequence determination of amplified products in the LAMP.

The amplified products in the LAMP were diluted appropriately with distilled water and amplified by PCR with primers that bind to the F2 region (5'-ATC GAC AAT GCC ATC GCC-3') and to complementally strand of B1c region (5'-TTC CCA AGA AGA GCC GTC T-3') shown in Fig. 15A. The PCR product was cloned to pT7Blue-T vector (Novagen Inc.) with Takara ligation kit version 2 (Takara Bio Inc.). The nucleic acid sequence was determined with the Big Dye terminator cycle sequencing kit (Applied Biosystems Japan Ltd.).

Southern blot analysis

Each LAMP product (5 μl) was electrophoresed in a 1.5% TAE agarose gel and transferred to a Hybond-N^+ membrane (Amersham Pharmacia Biotech Ltd.) with 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membrane was probed under stringent conditions with 5'-biotin-labeled synthetic oligonucleotide probe (*PFR A*: 5'- biotin-AAA CTG GAG AAA ATC GAA GAC GAA CTG CGC CGG-3', *P0*: 5'-biotin-TCA GAC AAG CTG TTT CAC CAG ACC TGC GCC GA-3'). The probes do not hybridize to either the inner (FIP and BIP) or outer (F3 and B3) primer binding regions, as shown in Fig. 15B and C in order to confirm target-specific LAMP reactions. Streptavidin-alkaline phosphatase (Roche Diagnostics Co.) and CDP-Star detection reagent (GE healthcare) were used for detection.

Chronically infected mice and blood samples

Five 16 week-old female ICR mice (CLEA Japan, Inc.) were infected intraperitoneally with 10^4 *T. brucei gambiense* IL3253 BSFs. Every other day for 30 days, approximately 30 μl of blood was collected into hematocrit tubes from the tail vein. Then 10 μl of whole blood was centrifuged for 5 min at 10,000 $\times$ *g* to obtain the

buffy coat. A drop of the buffy coat was placed on a glass slide and examined for motile parasites under a light microscope at × 100 magnification. The remaining (20 μ*l*) whole blood was blotted onto filter paper (FTA card, Whatman) for total DNA preparation. The blood blots were air dried and stored at room temperature until DNA extraction.

5-3. Results

Amplification of *PFR A* and *P0* by LAMP

Two sets of primers were designed for the *T. brucei PFR A* and *T. congolense P0* amplifications. To examine whether these sets of primers were able to amplify their target genes, LAMP reactions were conducted and analyzed by agarose gel electrophoresis. All of the primer sets amplified their target sequences in *PFR A* of *T. brucei* or *P0* of *T. congolense*, and the LAMP products appeared as a ladder of multiple bands (Fig. 16). This amplification pattern is characteristic of the LAMP reaction and indicates that stem-loop DNAs with inverted repeats of the target sequence were produced (103).

Sensitivity and sequence specificity of LAMP.

Since the outer primer pair, designated F3 and B3, can also be used for PCR, the same target gene was amplified from serially diluted total trypanosome DNA by both LAMP and standard PCR (PCR 1, see Materials and Methods of this chapter), and the sensitivities of the two methods were compared. Figure 17A shows the results of LAMP and PCR 1 with primer sets A1 and P01. LAMP with primer set A1 successfully amplified *T. brucei PFR A* from 1 pg of total DNA, whereas the detection

limit with PCR1 with primers A1-F3 and A1-B3 was 100 pg. However, LAMP with primer set P01 required 1 ng of total *T. congolense* DNA for detection, and its sensitivity was 10 times less than that of PCR 1 with primers P01-F3 and P01-B3. Likewise, the detection limits of LAMP with primer sets A2 and P02 were the same as PCR 1 (data not shown). The same agarose gel shown in Fig. 17A was used for a Southern blot, and the result clearly indicated both the LAMP products and the PCR products derived from *T. brucei PFR A* and *T. congolense P0*, respectively (Fig. 17B). In this experiment, template DNA was isolated from DE52 column-purified trypanosomes, and no or minimum contamination of blood components that contain several *Taq* DNA polymerase inhibitors (1, 2, 65) was expected. Therefore, we consider that a comparison between the LAMP and standard PCR instead of enhanced PCR is fair.

A LAMP product of a different band pattern was occasionally observed in *PFR A*-specific LAMP (primer set A1) (Fig. 18A), and such a LAMP product did not hybridize to the oligonucleotide probe (data not shown). In order to characterize the LAMP product with the different band pattern, a part of the LAMP products was amplified by PCR, and then the PCR product was cloned into pT7Blue-T vector (Novagen Inc.). The nucleic acid sequence of the PCR-amplified LAMP product is shown in Fig. 18B (Clone 1). Clone 1 contained only LAMP primer and short *PFR A* sequences (*PFR A*512-526: CTT CTG AGA TGG CGC) (Fig. 18B, clone 1). Although the order of each primer in clone 1 was not the same as that of a regular LAMP product (Fig. 18B, LAMP), I concluded that the LAMP reaction of different band pattern (Fig. 18A, lane 4) was not the results of nonspecific amplification but target

DNA specific.

Species specificity of LAMP

Since LAMP with primer set A1 showed 100 times higher sensitivity than PCR, further evaluation of the LAMP reaction was carried out. It has been reported that *T. evans* is evolutionarily closely related to the three subspecies of *T. brucei*, *T. b. brucei*, *T. b. gambiense*, and *T. b. rhodesiense*, and that its genomic DNA is indistinguishable from that of *T. evansi* (4, 58, 106, 145). Therefore, I tested whether LAMP with primer set A1 would give the same positive reactions with 10 ng of template DNA from the *T. brucei* subspecies and *T. evansi*. Total DNA from *T. b. rhodesiense*, *T. b. gambiense*, and *T. evansi* was subjected to LAMP, and all showed a positive reaction. Because the areas of distribution of African trypanosomes and *T. evansi* overlap those of many kinds of protozoan parasites, there is every possibility of mixed infection with trypanosomes and other parasites. Therefore, the specificity of the LAMP was also tested on protozoan parasites such as *T. cruzi*, *T. orientalis*, *T. equi*, *B. bigemina*, *B. bovis*, *B. caballi*, *T. gondii*, and *N. caninum*. Moreover, genomic DNAs of mammalian hosts, namely, human, monkey, bovine, and murine, were subjected to the LAMP in order to examine its specificity. The protozoan parasites and mammalian cell DNAs listed above were all negative in the *PFR A*-specific LAMP (primer set A1). Thus, the results indicate that the LAMP reaction can detect both *T. brucei* and *T. evansi* with high sensitivity and specificity.

Detection of *T. brucei gambiense* DNA from blood samples

The LAMP studies reported above were conducted with purified template DNA. However, to diagnose trypanosomiasis in the field, the method has to be able to

detect parasites in whole blood or cerebrospinal fluid as the most common clinical materials for examination. Therefore, five mice were injected with *T. b. gambiense* IL3253, which has a low virulence in mice. All five mice became infected, and every other day blood samples were collected from the tail vein, and the parasitemia of each mouse was examined by microscopic observation of thin smears obtained from the buffy coat. To simplify DNA extraction procedures, I used commercially available reagents, the FTA card and FTA reagent (Whatman). The FTA card is a chemically treated filter paper that allows the rapid isolation of pure DNA. When samples are applied to the FTA card, cell lysis occurs and high-molecular-weight DNA is immobilized within the matrix. Thus, a small piece of the FTA card can serve as the template DNA source after washing several times with FTA reagent and Tris-EDTA buffer. In the case of mouse 1 (Fig. 19), trypanosomes were detected by microscopic observation at 8, 10, 22, and 26 days post infection, and trypanosome DNA was detected by LAMP reaction at all days post infection. I also tried to detect trypanosome DNA by PCR. At first, PCR was performed under standard conditions (designated PCR 1). However, PCR 1 could amplify trypanosome DNA only at 10 days post infection (data not shown). The same DNA samples from mouse 1 were subjected to PCR 2. As a result, trypanosome DNA was first detected at 6 days post infection, and band intensities of PCR 2 products increased from 6 days post infection to 10 days post infection (Fig. 19). A change in the magnitude of band intensity in the PCR 2 appears to correspond to the result of microscopy. The results for other mice are shown in Table 6. These results clearly indicate the extremely high sensitivity of the LAMP reaction. However, I occasionally observed false-positive LAMP reactions

in negative controls (data not shown). The false-positive reactions were probably due to parasite contamination and/or amplicon cross contamination. Careful precautions against such cross-contamination must be taken during sample collections and preparations for LAMP. Furthermore, the sensitivity of PCR 2 (Fig. 19 and Table 6) was significantly higher than that of PCR 1. Thus, PCR conditions must be carefully optimized.

5-4. Discussion

In this study, sets of LAMP primers for the *T. brucei PFR A* and *T. congolense P0* were designed and tested to show the correct, sensitive and specific amplifications. The sets of LAMP primers for the *T. brucei PFR A* showed 100 times higher sensitivity than PCR primers whilst those for *T. congolense P0* did 10 times lower than PCR primers. It indicated that the well-designed set of LAMP primes have an ability to exert higher sensitive detection than PCR. The analysis of LAMP products hybridized with specific probes indicated that the LAMP truly amplified the target genes.

In analysis of different band pattern of LAMP in *PFR A*-specific LAMP (primer set A1), I concluded that this was not the results of nonspecific amplification but target DNA specific (Fig. 18A, lane 4). It was reported that *Bst* DNA polymerase has two distinct activities, termed linear target isothermal multimerization and amplification and cascade rolling-circle amplification (51). In the same manuscript, target DNA-specific amplification of both linear target isothermal multimerization and amplification and cascade rolling-circle amplification was also proved (51). The

mechanism of the loop-mediated isothermal amplification reaction is similar to that of the cascade rolling-circle amplification. An occasional different LAMP amplification pattern appears to be the result of linear target isothermal multimerization and amplification, because LAMP primers and target DNA seem to be randomly multimerized.

Further evaluation of specificity of LAMP with primer set A1 indicates that the LAMP reaction can detect both *T. brucei* and *T. evansi* with high sensitivity and specificity. A LAMP reaction requires four primers that recognize six different sequences on a target sequence (Fig. 15A) (103). At the first step of a LAMP reaction, *Bst* polymerase synthesizes new DNA strands from the F3 and B3 primers. This reaction is the same as PCR and requires sequence homology between a primer and a target. At the next step, the newly synthesized strands should be recognized by inner primers (FIP and BIP) in order to start loop-mediated autocycling amplification. Therefore, the target sequence specificity of a LAMP reaction appears to be higher than that of PCR (103).

In comparison of diagnostic methods with infected mice blood, PCR 2 clearly showed higher sensitivity than PCR 1 (Fig. 19). The marginal template DNA concentration for the positive reaction in PCR 1 was 100 pg (Fig. 17). With PCR based on the Te664 DNA fragment and ethidium bromide staining, *Ventura et al*. (145) reported that ≈10 pg of total *T. evansi* DNA represents ≈25 cells. Therefore, marginal detection of PCR for *PFR A* is about ≈250 cells. Because I wished to compare the sensitivity of the LAMP and PCR methods targeted to the same gene, I intentionally used PCR primers A1-F3 and A1-B3. It has been shown that microscopy

can detect ≈1,000 parasites in buffy coat material obtained from 1 m*l* of trypanosome-infected blood (146). The template DNA sources for PCR experiments were obtained from less than 5 μ*l* of blood blotted as a 2-mm diameter onto filter paper. In a sample containing ≈1,000 parasites/m*l*, we could expect 5 μ*l* of blood to contain five parasites on the filter paper, an amount that is within the detection limit of light microscopy. Therefore, I conclude that PCR 1 was less sensitive than microscopy, because PCR 1 required at least 250 parasites on the filter paper for detection. On the other hand, PCR 2 could amplify trypanosome DNA with high sensitivity and showed higher sensitivity than the LAMP at 20 DPI for mice 3 and 5. Differences between PCR 1 and PCR 2 are a repeat of thermal cycling and addition of the PCR enhancer termed Ampdirect-D (Shimadzu Biotech Co.).

The result of PCR 2 with Ampdirect-D suggests that the FTA card preparation cannot completely remove blood components that inhibit *Taq* DNA polymerase activity. In fact, it was reported that blood-derived materials such as heme-blood protein complex strongly inhibited *Taq* polymerase activity (1). Thus, I speculate that the lower sensitivity of PCR1 is due to the lower purity of the template DNA, which was extracted with the FTA card. Compared to PCR, LAMP has the advantages of reaction simplicity and detection sensitivity. LAMP does not require complicated thermal cycling steps; an isothermal reaction for a rather short time (≈1 h) is enough to amplify the target DNA to detectable levels.

Another useful feature of LAMP lies in the opportunity for turbidity-based detection of the positive reaction (93). The turbidity of the LAMP reaction mix can be easily judged by the naked eye. In all cases, I could distinguish LAMP-positive

samples from negative samples simply by the turbidity of the reaction mixtures (data not shown). Because PCR and other molecular biological techniques are best conducted only in well-equipped laboratories, these methodologies are often impracticable under conditions requiring a field diagnosis. In contrast, the useful characteristics of LAMP that I have described make it possible to use this highly sensitive DNA amplification method in many places, under field conditions and in local clinics and hospitals where cost and environmental restraints prohibiting PCR are otherwise in effect. While I have taken an important first step, further improvements are still needed, i.e., with the current primers, LAMP detects both *T. brucei* and *T. evansi*. Even so, LAMP will still be useful for the initial screening of suspected infection caused by *T. brucei* species and *T. evansi*, important causative agents of trypanosomiasis in humans and animals.

5-5. Summary

While PCR is a method of choice for the detection of African trypanosomes in both humans and animals, the expense of this method negates its use as a diagnostic method for the detection of endemic trypanosomiasis in African countries. The LAMP reaction is a method that amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions with only simple incubators. An added advantage of LAMP over PCR-based methods is that DNA amplification can be monitored spectrophotometrically and/or with the naked eye without the use of dyes. The current study reported the conditions for a highly sensitive, specific, and easy diagnostic assay based on LAMP technology for the detection of parasites in the *Trypanosoma*

brucei group (including *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, and *T. evansi*) and *T. congolense*. The sensitivity of the LAMP-based method for detection of trypanosomes *in vitro* is up to 100 times higher than that of PCR-based methods. *In vivo* studies in mice infected with human-infective *T. b. gambiense* further highlight the potential clinical importance of LAMP as a diagnostic tool for the identification of African trypanosomiasis.

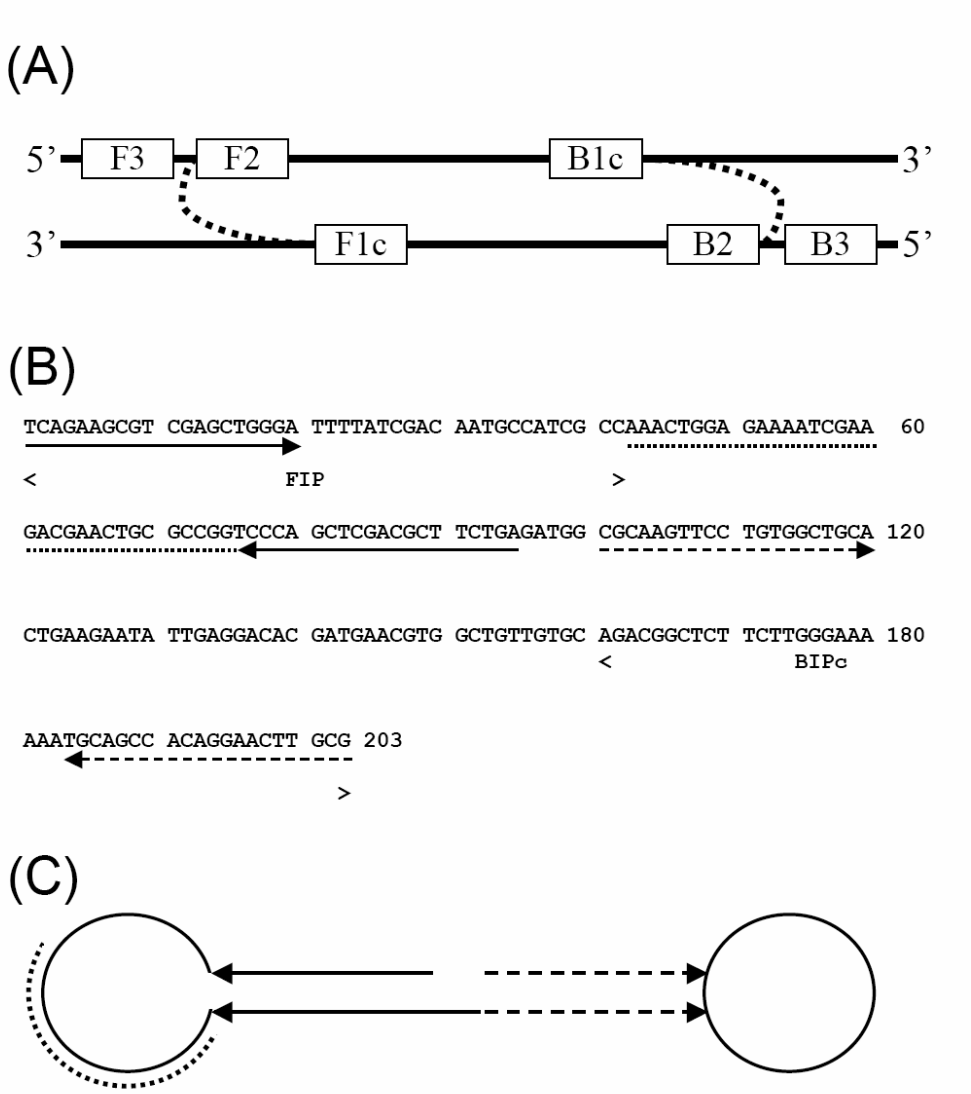


FIG. 15. (A) Schematic presentation of a double-stranded target DNA (solid lines) and LAMP inner (FIP and BIP) and outer (F3 and B3) primer pairs (open boxes). The FIP (BIP) primer consists of F1c (or B1c), a TTTT spacer (dotted line), and F2 (B2). (B) Nucleic acid sequence of minimum *PFR A*-specific LAMP (primer set A1, see Table 1) reaction unit. Two inverted repeats are indicated by solid arrows and dotted arrows. FIP and BIP primers are indicated below the sequence as FIP and BIP, respectively. A probe used for Southern blot analysis of LAMP products is designed to hybridize the region indicated by dotted line. (C) Schematic presentation of the single-stranded minimum LAMP reaction unit. Inverted repeats at both ends (solid and dotted lines) of the fragment form stem-loop structures. A probe used for Southern blot analysis of LAMP products is designed to hybridize the region indicated by the dotted line.

TABLE 5. LAMP primers.

Target gene	Set	Sp.	Sequence
PFR A	A1	FIP:	5'-TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC-3'
		F3:	5'-TCACAACAAGACTCGCACG-3'
		BIP:	5'-CGCAAGTTCCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCT-3'
		B3:	5'-GGGCTTTGATCTGCTCCTC-3'
	A2	FIP:	5'-ATGGCGTGACTTGACGGCACTTTTCTGCATGGGTATGCTGGAG-3'
		F3:	5'-TGTGTACAACTGCGACCTTG-3'
		BIP:	5'-TGAGTTGTCTGACCTTCGGCTGTTTTGTTTTGTACAGGCGACGGA-3'
		B3:	5'-GTACACAAGCTGGCCAAGA-3'
P0	P01	FIP:	5'-ATCCGTCGCCTTGCTGTCCTTTTTATGGGGAAGAAGACGCTTCA-3'
		F3:	5'-CGTGGTAAGGGTGAATTGGT-3'
		BIP:	5'-CAAGCAGCTGCTGTGCGGTATTTTTGATCTCCGTAACGTCCTCG-3'
		B3:	5'-GTGTCCGTCCAACACCTTC-3'
	P02	FIP:	5'-ATCATGTGCGGGAGCGTAGCTTTTAGGGCATCAGCAACATCAG-3'
		F3:	5'-CGACGTTGTGGAGAAGTACC-3'
		BIP:	5'-GCATTTAAGACCCTCCTCGGGGTTTTTGTCGCAGGTTCTTACCGT-3'
		B3:	5'-AGCTTGCCTTCCAGAGCA-3'

Sp.: specification, FIP: forward inner primer, F3: forward outer primer, BIP: back inner primer, B3: back primer, GenBank accession numbers: X14819 (*PFR A*), AB056702 (*P0*)

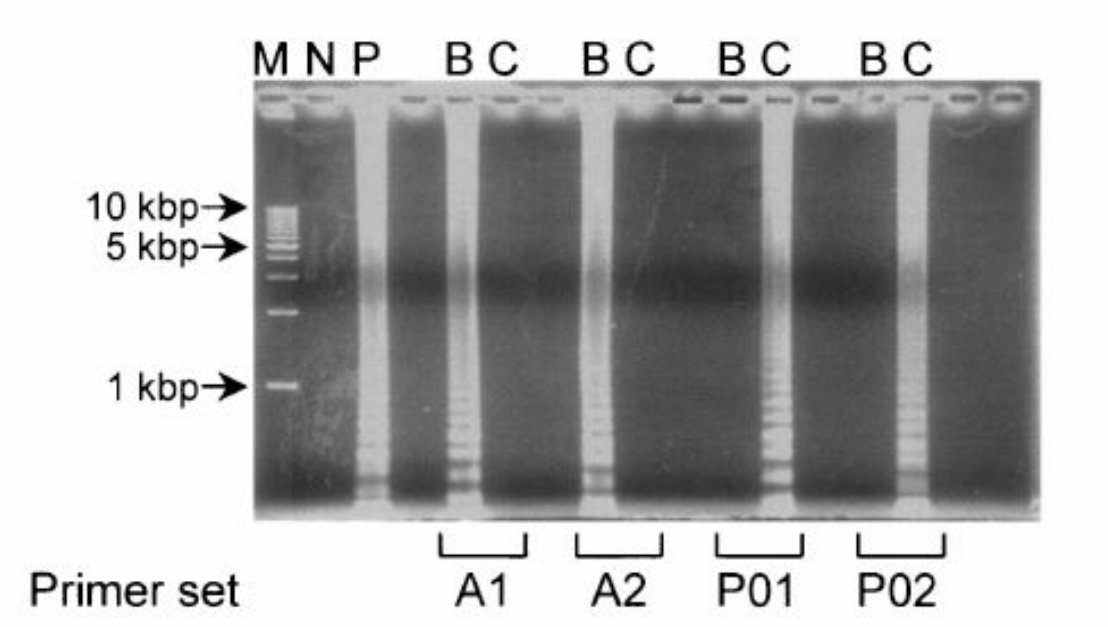


FIG. 16. LAMP reactions for *T. brucei* and *T. congolense*. Four sets of primers were designed to hybridize to the gene encoding *T. brucei PFR A* (A1 and A2) and *T. congolense P0* (P01 and P02). The LAMP products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Template DNAs were obtained from *T. brucei* GUTat 3.1 (B) and *T. congolense* IL3000 (C). Size markers (1 kbp ladder) were electrophoresed in lane M, and their sizes are indicated on the left. Lanes N and P, negative and positive reaction controls, respectively.

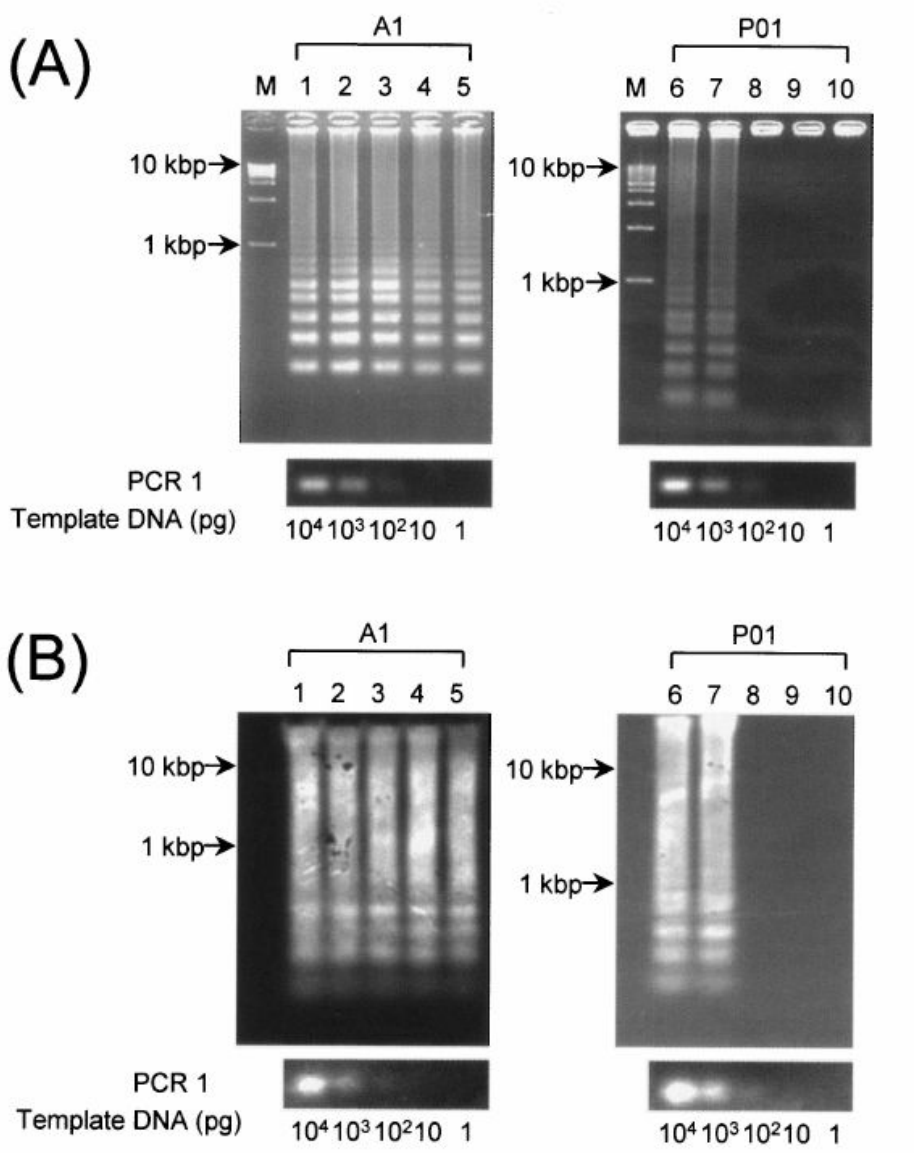
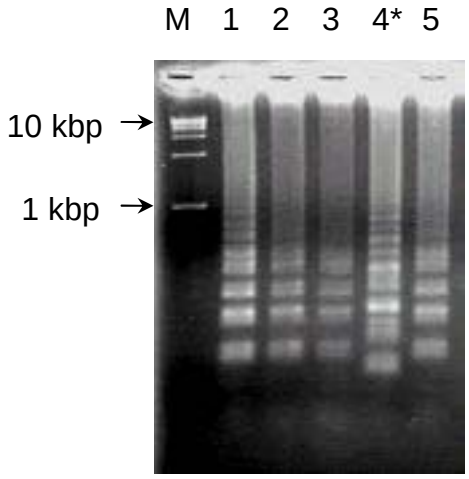


FIG. 17. Comparison of detection sensitivity in LAMP and PCR. (A) Total DNAs from *T. brucei* GUTat 3.1 and *T. congolense* IL3000 were serially diluted from 10 ng to 1 pg and amplified by LAMP and PCR. A1 and P01 are primer sets used in the LAMP reactions. The F3 and B3 primers in each LAMP primer set were used in the PCR. The sizes of the 1-kb size markers in lane M are indicated on the left. (B) Southern blot analyses of the LAMP products. The same LAMP and PCR products shown in A were probed with the synthetic oligonucleotide probes. The probes do not hybridize to either inner (FIP and BIP) or outer (F3 and B3) primer binding regions, as shown in Fig. 15B and C.

A.



B.

```
LAMP    TCAGAAGCGTCGAGCTGGGATTTTATCGACAATGCCATCGCC********  42
        <                  FIP                    >
Clone 1                          ATCGACAATGCCATCGCCCGCAAGTT  26
                                                  <  BIP

LAMP    ************************************************  42
Clone 1 CCTGTGGCTGCATTTTTTCCCAAGAAGAGCCGTCTTCACAACAAGACTCG  76
                BIP                        ><        F3

LAMP    ****AAACTGGAGAAAATCGAAGACGAACTGCGCCGGTCCCAGCTCGACG  88
           <            PFR A466-511                     >
Clone 1 CACG------------------------------------------------  80
           >

LAMP    CTTCTGAGATGGCGC***********AAGTTCCTGTGGCTGCACTGAAG 126
        <                 PFR A512-549                    >
Clone 1 CTTCTGAGATGGCGCAGATCAAAGCCC---------------------- 107
                       <    B3    >

LAMP    AATATTGAGGACACGATGAACGTGGCTGTTGTGCAGACGGCTCTTCTTGG 176
        <                 PFR A550-582      ><      BIPc
Clone 1 ---------------------------------AGACGGCTCTTCTTGG 123


LAMP    GAAAAAATGCAGCCACAGGAACTTGCG 203
                BIPc             >
Clone 1 GAA                        126
```

FIG. 18. Characterization of a different amplification pattern of *PFR A*-specific LAMP. (A) Total DNA from *T. brucei* GUTat 3.1 was serially diluted from 10 ng to 1 pg and amplified by PFR A-specific LAMP. *PFR A* A1 primer sets were used in the LAMP reactions. The band pattern of lane 4 is different from the others (lanes 1, 2, 3, and 5). The sizes of the 1-kb size markers in lane M are indicated on the left. (B) Comparison of nucleic acid sequences between the regular LAMP product (LAMP) and that of lane 4 (Clone 1). Sequence features are described between the and signs. BIPc indicates the complementary strand of the BIP primer. Insertions and deletions found in clone 1 are indicated by asterisks and hyphens, respectively.

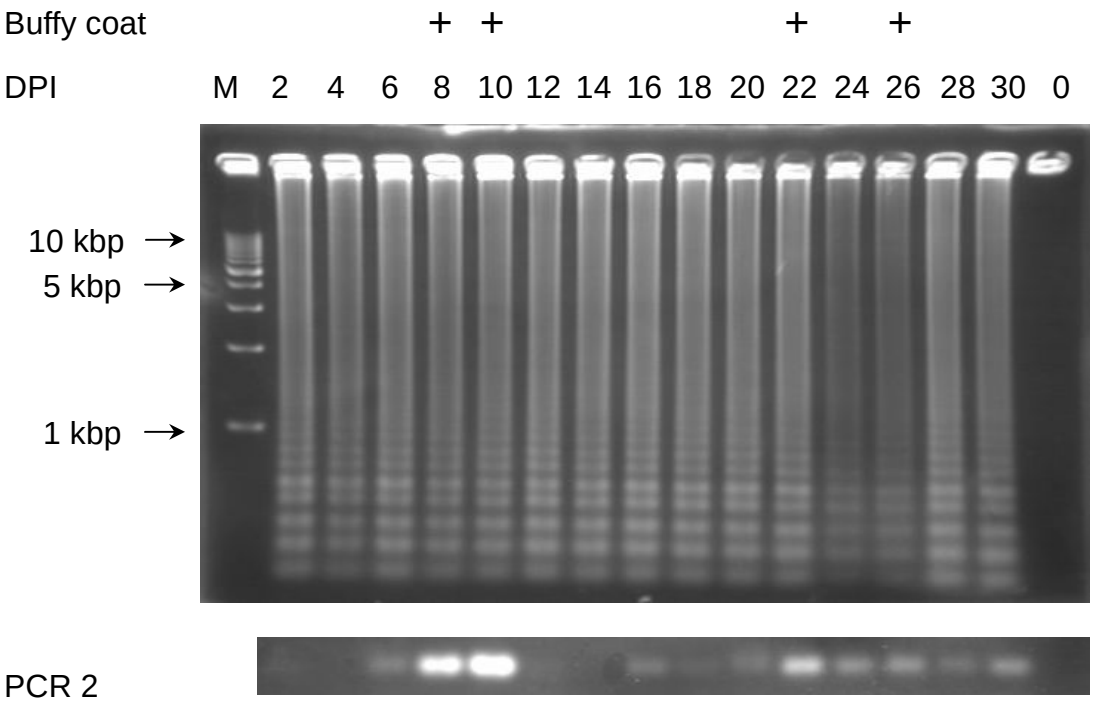


FIG. 19. Sequential analysis of blood samples obtained from mouse 1 infected with *T. brucei gambiense* IL3253. The samples were examined by microscopic observation of buffy coat samples, *PFR A*-specific LAMP with primer set A1, and *PFR A*-specific PCR with primers F3 and B3 of the A1 primer set. PCRs were performed under enhanced conditions as described in Materials & Methods. Numbers above each lane indicate DPI. + indicates the presence of trypanosomes in buffy coat samples observed by microscopy. The sizes of the markers in lane M are indicated on the left.

TABLE 6. Sequential analysis of blood samples from mice infected with *T. b. gambiense* IL3253.

Mouse No.	Method	Results on day postinfection															
		0	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
2	Buffy coat	-	-	+	-	-	-	-	+	-	-	+	+	-	-	-	+
	PCR 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR 2	-	+	+	+	-	+	+	+	+	-	+	+	+	+	+	+
	LAMP	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	Buffy coat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR 2	-	-	-	-	-	+	-	+	+	-	+	+	+	+	+	+
	LAMP	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
4	Buffy coat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PCR 2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	LAMP	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	Buffy coat	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
	PCR 1	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-
	PCR 2	-	+	+	+	-	+	+	+	+	-	+	-	+	+	+	+
	LAMP	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+

+ and - indicate presence and absence of trypanosomes, respectively.

General discussion

The current study consists of vaccine development and establishiment of diagnosis against African trypanosomiasis. For vaccine development, M3 liposomes were evaluated as a Th1 response-induced adjuvant, as well as novel vaccine antigens, such as *in vivo* expressed molecules, and TS were explored.

The M3 liposome, a novel adjuvant that strongly induce Th1 type immune responses, was assessed on its protective effect against *T. b. gambiense* infection. As a result, vaccination of M3 liposome containing STA led to immunosuppression in the mice, which subsequently turned in earlier death of the infected mice. Although it was reported that even M3 liposome encased with BSA has an ability to induce Th1 cytokines (46), M3 liposome might stimulate immne responses by different pathway depending on antigen(s) encased in the liposome. It is expected that STA contains preferable soluble VSG (sVSG) shed from disrupted trypanosomes by the activity of endogenous glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) as described previously (118). Since the glycosylinosytolphosphate (GIP), component of GPI anchor of VSG, possibly inactivate macrophages of the innate immune system (26), it could occur that delivery of GIP components to macrophages via M3 liposome targeted to the mannose receptor preferentially induces macrophage inactivation. Therefore, to improve an ability of M3 liposome as an adjuvant for protective vaccine against trypanosomiasis, a novel encased antigen should be discovered in order to induce macrophage activation and induction of Th1 type protective immune responses.

Protective vaccine candidates were searched by SSH method, and by testing recombinant TS proteins as vaccine antigens. As a result, *in vivo* specific genes were not identified. This indicates that expression control of pathogenic genes in African trypanosmes is quite different that of bacteria those pathogenic genes expression is most likely up-regulated after infection. However, in the view of search for vaccine candidate, the *in vivo* specific molecules in the parasite, if they exists, must be attractive antigens as a protctive vaccines. Therefore, further experiments have to be done for discovering *in vivo* specific genes from trypanosomes. On the other hand, TS1 vaccination allowed a certain level of protection in the immunized mice against *T. congolense* infection. Since TS1 vaccination resulted in longer survival than the control mice, the protective effect might be due to modulating excessive inflammation causing tissue damages and death. Otherwise, anti-TS1 antibodies in the immunized mice inhibited pathological function(s) of TS. Since it has been reported that TS in *T. cruzi* itself directs neural differentiation (24), stimulates IL-6 secretion from normal human endothelial cell (114), as well as potentiating T cell activation through antigen-presenting cell (47), TS in *T. congolense* also could have pathological effect(s) on the cells and/or the tissues in the host.

Even though it is not clear what kind of trypanosome antigen is suitable for an encased antigen for M3 liposome-based vaccine, antigen(s) that induce Th1 type immune responses through macrophages activations, might be good candidates of an encased antigen. However, there is no information about macrophage activated factors in African trypanosomes except for VSG and parasite DNA (125). Although STA contained VSG, which has ability to macrophages activations by itself, STA-M3

liposomes did not show protective effects in this study. However, to date, vaccine candidates for trypanosomiasis, such as tubulin (79) or microtubule-associated protein (111), TS1 in this study (chapter 4), might be able to improve by using M3 liposomes, which can effectively activate macrophages. Thus, further experiments have to be done in order to examine vaccine effects of those candidates in combination with M3 liposomes. Since the current study also showed anti-trypanosomal effects of DPPC liposome that is a platform of M3 liposome, application of M3 liposome would exert trypanolytic activity as well as vaccine adjuvant. Thereby, further studies of DPPC liposome are needed to determine the dose regimen, including the optimal injection route, treatment dose, times, and period.

For development of a simple and sensitive diagnostic method against African trypanosomiasis, the study optimized LAMP for detection of African trypanosomes. An application of LAMP for diagnosis of the trypanosomes gave rise to highly sensitive and specific detection of the parasite as compared to conventional PCR. The LAMP detected the parasite with high sensitivity from infected blood. Moreover the LAMP reaction was not interfered by inhibitory molecules from the blood, which affect for PCR. The results indicate that LAMP has a potentiality to introduce a new molecular diagnostic method into clinical field. However, the performance of the LAMP needs to be improved for establishment of the field-side molecular diagnostic methods. As the accuracy, sensitivity and specificity of the LAMP had been examined in previous study (135), the following studies should be conducted for field application of LAMP; (I) optimal condition for each primer. (II) simple and sensitive determination

of positive amplification without applying the LAMP product to the agarose gel electrophoresis. (III) simplification of template DNA preparation.

Thereby, the study suggests that, TS, if combined with effective adjuvant like M3 liposome, might be applied for establishment of anti-trypanosomal vaccine. In addition, LAMP method for African trypanosomiasis with its advantages of cost-effective, simple and high sensitivity is a prospective DNA amplification method for diagnosis in the field.

Conclusion

Trypanosomes are transmitted by the bites of tsetse flies. After the infective bite, the parasite is initially confined in the hemolymphatic system (the first or hemo-lymphatic stage), but as the disease progresses, the CNS is invaded by the parasite, which is referred to as the second or meningo-encephalitic stage. There is considerable variation in the clinical picture of African trypanosomiasis. Clinical signs of the hemo-lymphatic stage are the enlargement of lymph nodes (Winterbottom's sign), anemia, fever, pruritus, edema, splenomegaly, hepatomegaly, and weight loss. As the disease progresses into the meningo-encephalitic stage, clinical signs of CNS involvement are neurological disorders, such as sleep disorders, sensory disturbances, endocrine dysfunction, tone and mobility disorders, abnormal movements, mental changes or psychiatric disorders. Finally, the patient stops eating, lapses into a semi-coma and dies.

Although treatment of HAT absolutely relies on chemotherapy, it is problematic because the drugs cause the occurrence of resistant parasites, or extreme toxicity as associated with encephalopathy in administered patients. African trypanosomes evade the humoral immune response by constantly modifying VSG, which has hampered the design of an effective anti-trypanosomal vaccine. However, few reports have shown some potential vaccine candidates, which are dependent on the choice of vaccine molecules. Namely, findings of unique, invariant and immunoactive molecules would give rise to the vaccine development. In addition, to enhance suitable immnoactivations, such Th1 immune responses, an effective adjuvant should

be developed. Therefore, the present study focused on developing an effective vaccine against trypanosomiasis by conducting the following subjects: (I) development of a new adjuvant for Th1 immunoactivation, the M3 coated liposomes, which results in for removal of the circulating parasites in blood circulatory system. (II) characterization of immune responses of a new adjuvant against the infection (III) exploration of vaccine candidate molecules from the parasite genes specifically expressed *in vivo*. (IV) assessment of a vaccine antigen using parasite's TS that could be involved in anemia. Alternatively, since generally a liposome has strong affinity to lipids like cell membranes, the affect of DPPC liposomes, core of the M3 coated liposomes, were examined to understand their interaction with the membranes of the parasite. The following result was obtained: (V) Anti-trypanosomal effect of DPPC liposomes against African trypanosomes

On the other hand, since the clinical signs and symptoms of the diseases are largely non-specific and variable, sensitive and specific diagnostic method is of utmost importance. Several serodiagnostic methods, such as ELISA, IFAT, and the card agglutination test for CATT, are commonly used. However, direct parasite detection is also needed because of the potentially serious side-effects of anti-trypanosomal drugs. The microscopic detection is useful, but has insufficient sensitivity. Although PCR copes with direct and highly sensitive detection of the parasite, it is not validated because of the need for highly equipped facilities and expensive reagents. Recently, a novel DNA amplification technique, loop-mediated isothermal amplification of DNA (LAMP) was developed, which amplifies DNA with high specificity, efficiency, and rapidity under isothermal conditions. The LAMP would

be able to introduce DNA diagnosis even in resource-poor laboratories. Therefore, the study also focused on the following subject: (VI) establishment of LAMP for the detection of African trypanosomes.

Since most reports indicated that Th1 immune responses confer resistance to African trypanosome infection by limiting parasite growth during the early stage of infection, vaccine strategy of M3 liposome might be a suitable method. The M3 liposome, which consists of mainly DPPC liposome covered with M3, is effectively taken up by antigen-presenting cells, such as macrophages in the spleen, via the mannose receptor (CD206), which can eventually induce Th1 immune responses. The phenomenon indicates that application of M3 liposome, in which a specific antigen is encased, would lead to an effective vaccine development. Therefore, in chapter 1, vaccine effect of M3 liposome was examined by encasing soluble *Trypanosoma brucei gambiense* antigen (STA) that itself can work as a vaccine antigen. Unexpectedly, however, the vaccination of STA-M3 liposome led to earlier death of the infected mice that showed immunosuppression. It indicated that adjuvant effect of M3 liposome is far from expectation, but M3 liposome could be useful for the identification of the trypanosome-suppressive molecule(s) related to the pathology. Alternatively, since generally a liposome has strong affinity to lipids like cell membranes, the affect of DPPC liposome was examined to understand its interaction with the membranes of the parasite (chapter 2). As a result, DPPC liposome lysed the bloodstream form (BSF) of the trypanosomes stage-specifically. Because the high density of a VSG covers the cell membrane of the BSF, the binding of DPPC liposome to VSGs could be a trigger of the cell lysis.

In bacteria, some of pathogenic factors are induced only after the host's infection. If the factors were also in the parasites, it could be interesting for application of novel vaccine antigens. Therefore, in chapter 3, by conducting suppressive subtractive hybridization to compare mRNAs of parasites from blood of infected mice and from cultivation, the parasite genes specifically expressed *in vivo* were explored. However, no *in vivo* specific genes were identified, which indicates that expression control of pathogenic genes in African trypanosomes is quite different from that in bacteria.

To explore TS as a vaccine candidate, in chapter 4, expression patterns of TS in both the BSF and the procyclic form (PCF) of *Trypanosoma congolense* were characterized, and the vaccine effects against anemia and parasitemia were monitored in mice vaccinated with TS recombinants. As a result, TS is expressed in the BSF as well as in the PCF. The TS vaccination did not affect anemia and parasitemia at all, but contributed to partial protective effect (33.3% survival). The result suggests that, if TS vaccination were modified, for example, if administered in combination with an effective adjuvant, then it would be a good candidate for establishment of trypanosomal vaccine.

To develop more simple and sensitive diagnosis, in chapter 5, an application of LAMP was tested for diagnosis of the trypanosomes. As a result, it gave rise to highly sensitive and specific detection of the parasite as compared to conventional PCR. The LAMP method also detected the parasite in blood from infected mice with high sensitively, without being affected by inhibitory molecules in the blood which inhibit PCR.

The study was summarized as follows: (I) vaccination of STA-M3 liposome aggravated the infection, which was in contrast to TS vaccination that induced partial protection against the infection. (II) DPPC liposome lyses the BSF trypanosomes in stage-specific manner. (III) In African trypanosomes, there was no difference in expression pattern of mRNA between the parasite in blood of infected mice and in culture, however, it differs to that in bacteria. (IV) the LAMP diagnosis for African trypanosomes was established as a cost-effective, simple and sensitive detective system.

Thereby, the study suggests that, TS, if combined with effective adjuvant like M3 liposome, might be applied for establishment of anti-trypanosomal vaccine. In addition, LAMP method for African trypanosomiasis with its advantages of cost-effective, simple and high sensitivity is a prospective DNA amplification method for diagnosis in the field.

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