Establishment of *In Vivo* and *In Vitro* Systems for Propagation of Salivarian Trypanosomes, and Their Application for Molecular Biological and Immunological Studies

（トリパノソーマ原虫の大量増殖法の確立ならびに
分子生物学および免疫学的応用研究）

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ABBREVIATIONS

A

ABP: Adult bovine plasma

ABS: Adult bovine serum

B

BSF: Bloodstream form

BALB/c: BALB/c/A-+/+

C

CATT: Card agglutination test for trypanosomes

CSF: Cerebrospinal fluid

E

ELISA: Enzyme linked immunosorbent assay

F

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

H

HoP: Adult horse plasma

HoS: Adult horse serum

HPD: Highest population density

HuP: Human plasma

HuS: Human serum

I

IFN-γ: Interferon γ

IL: Interleukin

ILRI: International Livestock Research Institute

IMDM: Iscove's modified Dulbecco's MEM

IMDM-HoS: IMDM Hybri-Max® supplemented with horse serum

IMDM-HuP: IMDM Hybri-Max® supplemented with human plasma

I.p.: Intraperitoneally

K

kDNA: Kinetoplast DNA

M

MAb: Monoclonal antibody

MAEC: Miniature anion-exchange centrifugation

N

NTTAT: Non-tsetse transmitted animal trypanosomosis

Nude: BALB/c/A-nu/nu

P

PARP: Procyclic acidic repetitive protein

PCR: Polymerase chain reaction

PCV: Packed cell volume

PDT: Population doubling time

PFGE: Pulsed-field gel electrophoresis

PI: Post-infection

PSG: Phosphate-buffered saline containing 1% glucose

R

rRNA: Ribosomal RNA gene

RFLP: Restriction fragment length polymorphism

RT-PCR: Reverse transcription polymerase chain reaction

S

SCID: Severe combined immunodeficient

SDS: Sodium dodecyl sulfate

SPDT: Shortest population doubling time

T

TBE: Tris-borate-EDTA

Th1: T helper 1-type

Th2: T helper 2-type

TNF: Tumor necrosis factor

V

VAT: Variable antigen type

VSG: Variable surface glycoprotein

GENERAL INTRODUCTION

I: Salivarian trypanosomes

I-1: The disease

Introduction

Salivarian trypanosomosis is one of the most important and widespread diseases of domestic animals and man in the world. The causative agents of the disease are obligate extracellular parasites which occur in blood, celebrospinal fluid (CSF) and tissue fluids. The salivarian trypanosomosis is further divided into tsetse-transmitted African trypanosomosis and non-tsetse transmitted animal trypanosomosis (NTTAT).

Taxonomy and morphology

Pathogens of the salivarian trypanosomosis are members of the section Salivaria and belong to the genus *Trypanosoma*, that is within the order Kinetoplastida and the class Zoomastigophora (Table 1). The organism is a spindle shape and flagellated protozoa, which is about 8 to 39 μm in length. It has a centrally located nucleus and a single unbranched mitochondrion which extends along the length of the cell. The flagellum starts in the posterior part of the cell and run forward along the outer margin of the undulating membrane. Where the flagellum emerges from the cell, there is a flagellar pocket. This is where most of the excretion and pinocytosis take place. The mitochondrial genome of the organism is known as the kinetoplast, which is located beneath the flagellar pocket (Fig. 1) (152).

Table 1. Classification of the section Salivaria

Phylum: Protozoa
Subphylum: Sarcomastigophora
Superclass: Mastigophora
Class: Zoomastigophora
Order: Kinetoplastida
Suborder: Trypanosomatina
Family: Trypanosomatidae
Genus: *Trypanosoma*
Section: Salivaria
Subgenera: *Duttonella*
Species: *T. vivax*
Species: *T. uniforme*
Subgenera: *Nannomonas*
Species: *T. congolense*
Species: *T. simiae*
Subgenera: *Trypanozoon*
Species: *T. evansi*
Subspecies: *T. e. evansi*
Subspecies: *T. e. equinum*
Species: *T. brucei*
Subspecies: *T. b. brucei*
Subspecies: *T. b. gambiense*
Subspecies: *T. b. rhodesiense*
Species: *T. equiperdum*
Subgenera: *Pycnomonas*
Species: *T. suis*

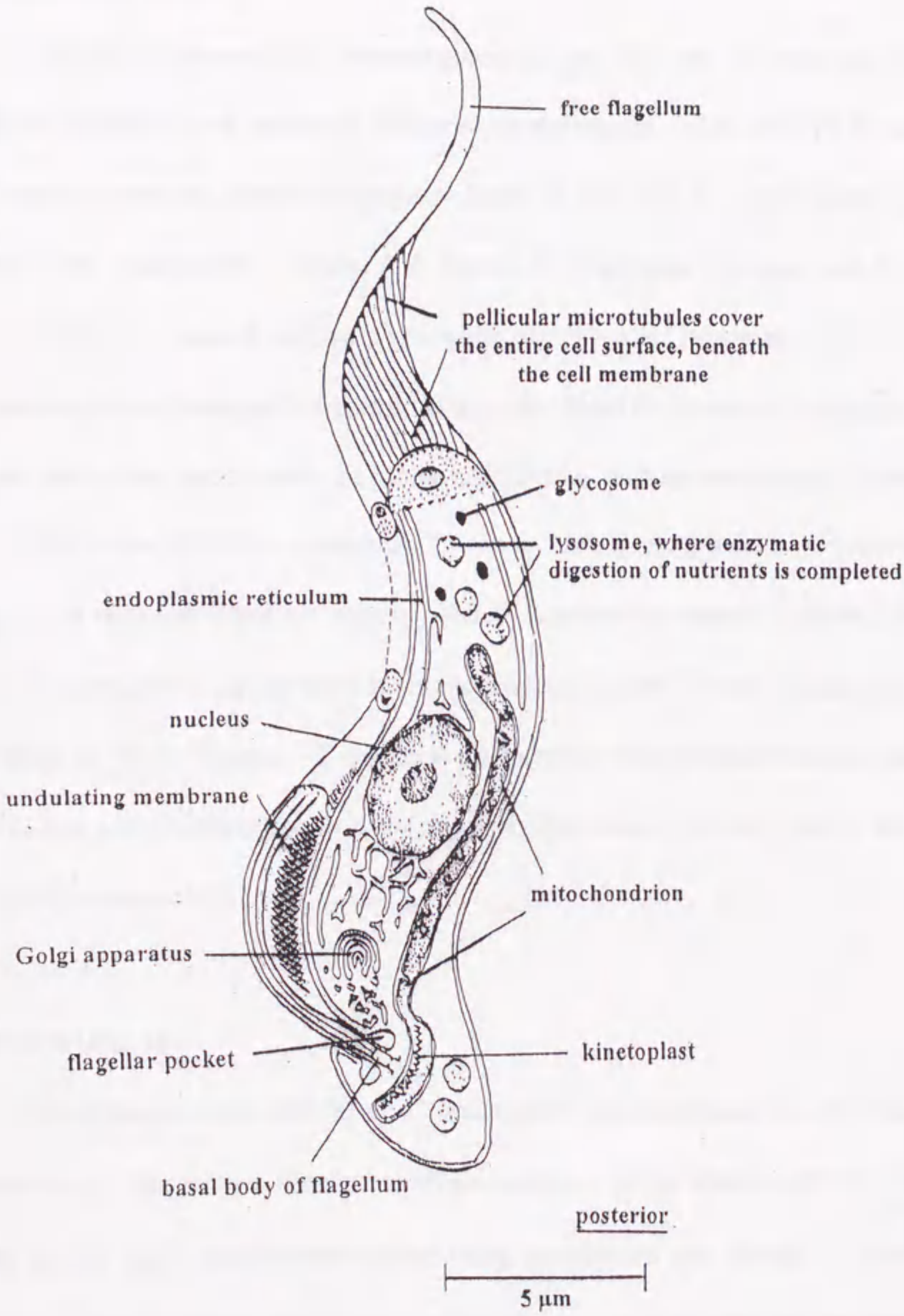


Figure 1. Schematic diagram of a bloodstream form trypanosome

The diagram illustrates the major organelles [adapted from Vickerman (1962)] (152).

Pathogens and vectors

African trypanosomosis (tsetse-transmitted) in man and animals are caused by various subspecies and species of salivarian trypanosomes. The African trypanosomes that cause acute and chronic sleeping sickness in man are *T. b. gambiense* and *T. b. rhodesiense*, respectively. While, *T. b. brucei*, *T. congolense*, *T. simiae* and *T. vivax* are the pathogens of animal African trypanosomosis (so-called Nagana). All the African trypanosomes are biologically transmitted by tsetse flies (*Glossina* spp.), although *T. vivax* is also transmitted mechanically by blood-sucking flies such as tabanids and *Stomoxys*.

While the NTTAT is caused by *T. evansi*, and *T. equiperdum*. *T. evansi* causes "Surra" in wild and domestic animals, and *T. equiperdum* causes "Dourine" in horses. Both *T. evansi* and *T. equiperdum* are monomorphic "slender" forms in mammalian hosts, and have no "insect" stages. *T. evansi* is mechanically transmitted by blood-sucking flies (Tabanidae and *Stomoxys* spp.) or vampire bats (*Desmodus rotundus*), and *T. equiperdum* is sexually transmitted.

Life cycle (Fig. 2)

Bloodstream forms (BSFs) of *T. brucei* show pleomorphism but the other tsetse-transmitted trypanosomes usually are monomorphic. In the pleomorphic *T. brucei*, the long slender BSFs predominate during rising parasitemia and change into the stumpy forms through the intermediate forms in host animals. It is thought that the stumpy BSFs are infectious to tsetse flies (27). On the other hand, in the monomorphic trypanosomes such as *T. congolense*, *T. simiae* and *T. vivax*, BSFs are "slender" forms and not difference in morphology during the infection in animals. The long slender and intermediate BSFs multiply actively by binary fission. While, stumpy forms do not multiply in the host

animals.

When BSFs were ingested along with blood meals by a tsetse fly, they undergo morphological transformations and change to "insect forms", lose the infectivity to animals (152). Firstly, they transform to the procyclic forms which have a highly branched mitochondrion. The kinetoplast is located halfway between the posterior end and the nucleus, instead of in a posterior end as in the BSFs. Secondly, the procyclic forms transform to the epimastigotes which have also a well developed mitochondrion, but of which the kinetoplast is located anterior position of the nucleus. The procyclic forms and the epimastigotes also actively multiply by binary fission in their insect vectors. Finally, the epimastigotes transform to the metacyclic forms which again become infectious to animals, although they are non-dividing forms. When the metacyclic forms are inoculated by an infected tsetse fly into a host animal, they become BSFs within 6 h and start to divide by binary fission.

Both *T. evansi* and *T. equiperdum* are monomorphic "slender" forms in mammalian hosts, and have no "insect" stages.

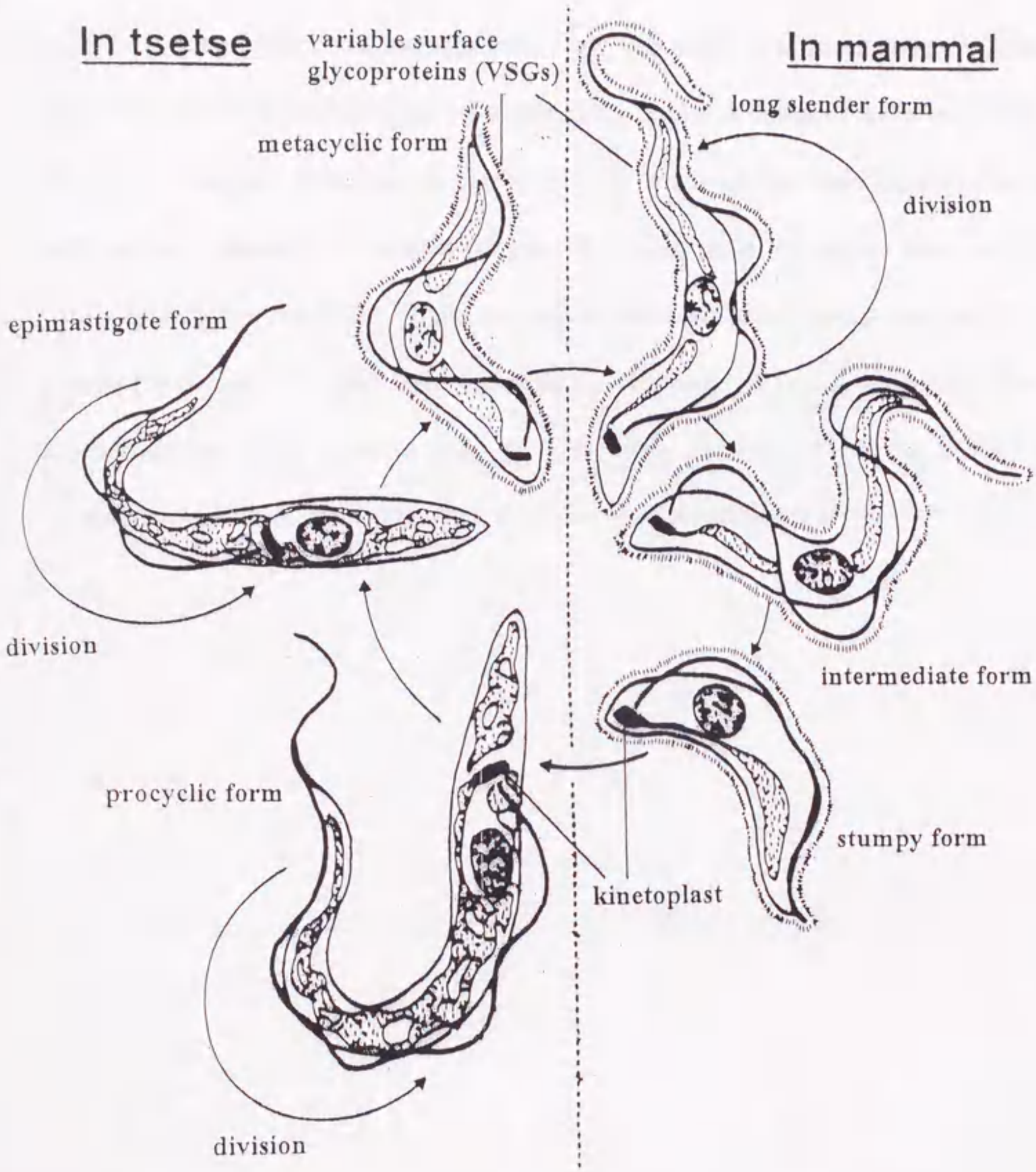


Figure 2. The life cycle of pleomorphic *T. brucei*

[adapted from Hirumi and Hirumi (1994)] (49)

Distribution

The tsetse-transmitted trypanosomoses are prevalent in a vast region of Africa (approximately 10 million km^2) and its distribution overlaps with that of the tsetse fly (Fig. 3) (161). Thirty-six countries are involved in the tsetse-infested areas (so-called tsetse belt), and approximately 50 million peoples, 50 million cattle, 30 million sheep and 40 million goats are at risk (45). While, the non-tsetse transmitted trypanosomes are widely prevalent out side of tsetse belt including in Northern Africa, Asia, Middle East, Kazakhstan and South America (Fig. 3). Therefore, the area affected by NTTAT is approximately 3 times greater than that of African trypanosomoses (161).

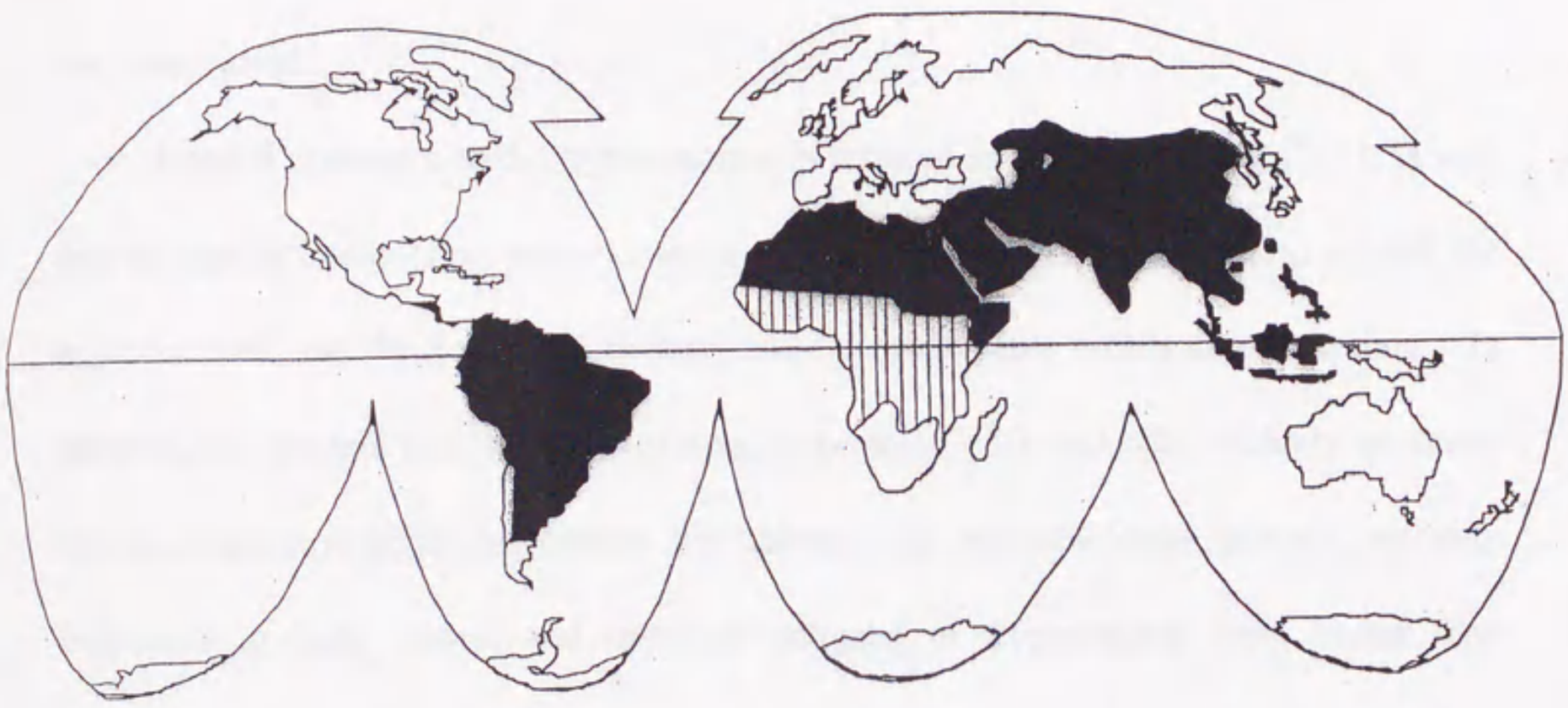


Figure 3. Geographic distribution of tsetse-transmitted trypanosomosis (vertical stripes) and NTTAT (solid area)

Symptoms

The clinical signs of the animal trypanosomoses are parasitemia, intermittent fever, anemia, weight loss, progressive weakness and reduced productivity, and abortion and infertility in breeding animals. Although host susceptibility and virulence of parasite may vary, many affected hosts die of anemia, heart failure and/or secondary infections unless they are treated.

Natural resistance to the trypanosomes is referred as "trypanotolerance". It is well known that N'Dama (*Bos taurus*), which is indigenous to Africa, is able to control the infection well, but the Zebu (*Bos indicus*) and European cattle breeds are susceptible. In general, it is thought that variable surface glycoprotein (VSG) -specific antibody responses are an important factor for control the disease. In trypanotolerant animals, antibody responses to both variant and invariant antigens of trypanosome were higher than susceptible ones (3, 25, 59, 141). In addition to the VSG-specific antibodies, it was reported that both mouse and human tumor necrosis factor (TNF) inhibits the development of *T. brucei in vivo* (66) and *in vitro* (62). This trypanostatic activity was due to the direct interaction between the TNF and the parasite through the lectin-like activity of the TNF (62). The trypanocidal activity of a serum protein in trypanotolerant Cape buffalo (*Syncerus caffer*) also reported (112), and the protein was identified as xanthine oxidase (86). Although mechanisms which determine host resistance to trypanosomes are not fully understood, elucidation of the mechanisms may suggest new strategies for control of the trypanosomes.

Antigenic variation

Salivarian trypanosomes elude very effectively the host immune responses through antigenic variation by which trypanosomes continually change the antigens that constitute their surface coat (154). The first demonstration that the antigenic variation occurs in the course of trypanosome infection was made by Ritz (1916) (119). He showed that antigenic variation could occur in an infection initiated with a single trypanosome and distinguished 22 different antigenic types (155). While, Seed (1964) (128) reported that the loss of variable antigen in the procyclic forms of *T. brucei*. Furthermore, Vickerman (1969) (153) demonstrated that bloodstream and metacyclic forms of *T. brucei* had a 12 nm thick surface coat outside the plasma membrane, but the coat was missing from all other developmental stages in tsetse fly. In consideration of all observations described above, Vickerman (1969) (153) proposed that the coat contained the variable antigen, and finally Vickerman and Luckins (1969) (156) proved this hypothesis by using ferritin-conjugated variable antigen types (VATs) specific antibodies. These antibodies did not bind to trypanosomes deprived of the coat by pronase digestion.

The coat was purified and further characterized by Cross (1975) (24) as a matrix of identical glycoprotein molecules that differed only in their amino acid sequences. At present, the surface coat of trypanosomes are called VSGs and VAT refers to a trypanosome expressing a particular VSG. It is speculated that the VSG repertoires are more than 1,000 in each trypanosome (10). Therefore, the VSG is not suitable for a vaccine in spite of its high immunogenicity. During the infection, host immune system generates antibodies that are directed against particular VSGs expressed on the invading parasites. However, a few trypanosomes spontaneously switch its particular VSGs to different VSGs, and a frequency of VSG switch is estimated at 10^{-2} per cell per generation

(143). This allows the survival of the trypanosomes expressing the different VSG. Since the antigenic variation is a continuous event, finally the host immune system succumbs to the trypanosomes.

The factor(s) which induces the antigenic variation is still not known, but it is speculated spontaneous event (110). The antigenic variation is not initiated by an antibody response to particular VSG, but may be enhanced by such response which will select for low numbers of new variant trypanosomes. The observations that the antigenic variation occurs *in vitro* may support this speculation (31). The genetic mechanisms involved in the antigenic variation are well reviewed by Pays and Nolan (1998) (110). They discussed two different processes; the VSG switches arise either by the alternative use of different VSG expression sites or by DNA recombination events which change the VSG present in the active expression site.

Like the BSFs of trypanosomes, the metacyclic forms of the parasite also possesses a VSG. It was reported that the metacyclic VSG repertoire was limited to at most 27 (144). Scientists hoped that a multivalent metacyclic VSG vaccine might be developed. However, vaccination with the metacyclic VATs is also problematic because of its heterogeneity and instability (11).

I-2: *In vitro* cultivation of salivarian trypanosome BSFs

Until 1977 no method was available to grow the BSFs in *in vitro* culture. Inavailability of *in vitro* systems that support the growth of animal infective BSFs and metacyclic forms in culture had constrained the rapid progress of basic studies of trypanosomoses and the development of control measures against the diseases. This problem has overcome by the development of long-term culture of the BSFs of *T. brucei* by Hirumi et al. (1977) (46). All previous attempts to cultivate the BSFs resulted either in the death of the trypanosomes or in their transformation to non-animal infective insect forms. In the original methods, BSFs of African trypanosomes were propagated *in vitro* in the presence of mammalian feeder layer cells. At present, axenic cultivation of African trypanosome BSFs, namely *T. brucei*, *T. congolense* and *T. vivax*, are established (49). However, the serum requirement in the axenic culture systems is an obstacle to certain specific studies, which require completely defined culture system such as serum protein-free culture system, due to the undefined nature of serum. Recently, the development of a serum-free medium for the cultivation of *T. brucei* and *T. evansi* BSFs was achieved by Hirumi et al. (1997) (50). The serum-free culture would be an ultimate *in vitro* system for studying growth-promoting factors, drug-sensitivity test, mode of trypanocidal activity, gene-selection/cloning, cell division cycle and gene expression which have been difficult earlier due to the serum supplementation in the culture medium.

I-3: Vaccine development

Like many parasitic diseases, there are no effective vaccines for trypanosomoses (78). The antigenic variation of bloodstream trypomastigotes is a major problem to develop the vaccine, therefore a number of attempts have been made to discover invariant surface proteins as vaccine candidates. Several kinds of invariant surface proteins have been reported (105) and 2 of these proteins, namely ISG 65 and ISG 75, were examined whether they are effective as vaccines (163). The ISG 65 and the ISG 75, however, were not protective due to the inaccessibility of antibodies and its small amount of expression on the cell surface (100- to 10,000-fold less than the VSG) (105, 163). Despite the difficulties described above, some results have been obtained in experimental attempts of vaccination with flagellar pocket preparations both in mice and in cattle (74, 100). Therefore, it seems that the flagellar pocket may contain useful antigens as vaccine candidates, although their reports have not yet been confirmed by other investigators.

I-4: Control of trypanosomoses

At present, effective control measures of trypanosomoses are based on two approaches, namely, control of the vector populations and the use of trypanocidal drugs for prophylaxis and/or for treatment of the infection.

Vector control

Until the development of synthetic organochlorine insecticides such as DDT and dieldrin, vegetation clearance was the major method for the vector control. Since the insecticides were developed, the large-scale insecticide spraying was carried out. In West Africa, where over 200,000 km^2 of land were cleared of tsetse fly by the insecticide spraying (9). Thus, the large-scale insecticide spraying was very effective method for the vector control. However, the method has become widely unacceptable in terms of the protection of environments. The sterile insect technique was one of the alternative method for the vector control. However, this method was not only expensive but also the sterile males of some species have been shown to be efficient vectors of pathogenic *Trypanosoma* species and would potentially increase the risk of trypanosomosis (75, 76). At present, tsetse traps and screens are the most effective method for the vector control. These methods have the advantage of inexpensive, easy to use, and sustainable for the clean environment. Moreover, the improvements of the design and color of traps have made them more effective.

Chemotherapy

A number of trypanocidal drugs have been found to be effective in treatment and prophylaxis of the trypanosomoses. In case of African trypanosomoses in man, suramin and pentamidine are commonly used for a treatment of an early stage of the disease. Suramin is effective on both *T. b. rhodesiense* and *T. b. gambiense*, but pentamidine is not recommended for *T. b. rhodesiense* (27). In severe case of the disease where the trypanosomes appear in the CSF at the late stage of the infection, Mel B is the choice for the reasons of its ability to penetrate into CSF. However, the Mel B, which is an organic arsenical, must be used with utmost care because of fatal side effects.

In animal trypanosomoses, quinapyramine dimethosulfate, homidium bromide B vet C, pyrithidium bromide, isometamidium chloride, diminazene aceturate and suramin are used for treatment (132). In endemic areas, quinapyramine dimethosulfate, pyrithidium bromide and isometamidium chlolide are also used for chemoprophylaxis (132). Although an appropriate use of these trypanocidal drugs is very effective to control the disease, the frequent use for a long period in endemic areas causes the emergence of drug resistant parasites. Moreover, there is poor prospect of new drugs being developed because of the small size of the market.

I-5: Diagnosis

Parasite detection

At present, several diagnostic techniques are available for the disease. The most primitive but reliable technique is direct detection of trypanosomes by microscopic examination of blood and/or CSF. Wet and stained preparations are commonly used for this purpose. This technique is applicable in the field but not suitable for screening large number of materials. Moreover, the sensitivity of this technique is low because of the fluctuating nature of the parasitemia. Trypanosome concentration techniques, such as microhematocrit centrifugation (160, 162) and miniature anion-exchange centrifugation technique (MAEC) (64), are more sensitive than the direct microscopic examination of blood smears. The microhematocrit centrifugation technique can detect the presence of trypanosomes in the blood of experimentally infected cattle 6-10 d earlier than the microscopic examination of the blood smear (77). At the same time, the technique can also assess the status of anemia. However, its sensitivity is at least 4 times less than an antigen detection enzyme linked immunosorbent assay (ELISA) (70). The MAEC technique was developed by Lumsden et al. (1979) (64) and was based on a modification of the technique described by Lanham and Godfrey (1970) (57) for separation of trypanosomes from infected blood by using DEAE-cellulose column. It was reported that the sensitivity of the MAEC technique is less than that of the microhematocrit centrifugation (108). Sub-inoculation of materials, such as blood and CSF, into susceptible animals is a useful technique for detection and isolation of parasites. Experimental rodents are commonly used for this purpose, but a virulence of trypanosome to the experimental rodents highly varies among isolates. For example, since *T. b. gambiense* usually shows a low virulence to experimental rodents, semi-lethally irradiated

or chemically immunosuppressed mice have been used for detection of the parasites. However, the technique is expensive and is time-consuming to develop the infection in the recipient animals.

Serodiagnosis

Serodiagnosis includes indirect immunofluorescence antibody test, complement fixation test, ELISA and direct agglutination test (a card agglutination test for trypanosomosis: CATT). The antigenic variation is one of the major problems for serodiagnosis of salivarian trypanosomes, therefore common antigens are used for detection of antibodies. However, two major problems still remain (90). Firstly, these tests detect antibodies and can not, therefore, distinguish an active infection from one that has been cured. Secondly, except for the CATT in which commonly occurring VATs are used for detection of the specific antibodies (67), the other techniques do not utilize common antigens of defined specificity or purity. Therefore, these techniques are difficult to standardize and tend to give false-positive reactions (151, 157).

Antigen detection ELISA

An antigen detection ELISA was first reported by Rae and Luckins (1984) (111). They demonstrated that trypanosomal antigens were able to be detected in sera of rabbits and goats experimentally infected with *T. evansi* or *T. congolense* by using a homologous antiserum against each parasite. Furthermore, the trypanosomal antigens were also detected in sera of goats infected with *T. brucei* and *T. vivax* by using the antiserum against *T. evansi*. In the study by Rae and Luckins (1984) (111), the possible use of the antigen detection ELISA was clearly demonstrated, but a problem was its lack of species

specificity. Further improvement of the antigen detection ELISA was made by using trypanosome species specific monoclonal antibodies (61, 93). The species specific antigen detection ELISA appeared to be more sensitive than the parasitological methods, such as microscopic examination of the infected blood and microhematocrit centrifugation (91, 94). Moreover, the antigen detection ELISA was able to distinguish an active infection from one that has been cured, and usefulness of which was proved by several studies (38, 55, 92).

Polymerase chain reaction (PCR)

A PCR-based diagnostic technique is one of the most sensitive and specific technique of all diagnostic techniques. An application of the PCR to the diagnosis of the African trypanosomosis was initially reported by Moser et al. (1989) (80). They demonstrated that 10% of the DNA in a single parasite of *T. congolense* or *T. brucei* spp. was enough to detect these parasite. Evaluation of the PCR technique in comparison to parasitological techniques and antigen detection ELISA was made by Desquesnes (1997) (29), Ijaz et al. (1998) (52) and Masake et al. (1997) (69). In most cases, sensitivity of the PCR technique was higher than parasitological techniques and antigen detection ELISA. However, there were the PCR negative samples which were parasitologically positive (29, 69). This problem might be due to variation in the processing of samples or presence of *Taq* polymerase inhibitors (69). Therefore, improvement of the PCR techniques including sample preparation and DNA extraction is necessary to develop more reliable and simple PCR technique.

II: Surra

II-1: Problems of *T. evansi* infection (Surra)

Surra is a NTTAT caused by *T. evansi* infection, and is distributed in almost all tropical and subtropical regions of the world where domestic animals are very important as source of food, means of transportation and draft power (Fig. 3). In 1995 and 1996, *T. evansi* infection was reported from Bhutan, India, Indonesia, Iran, Laos, Mongolia, Myanmar, Nepal, Pakistan, Philippines and Thailand (106), and there are some evidences that the disease is more widely distributed even in some countries which have not reported the occurrence of the disease before (44, 88, 96, 124, 142). Although the disease causes serious problem in such many countries, *T. evansi* infection has been scientifically neglected for a long period of time in comparison with tsetse-transmitted trypanosomoses.

Surra in buffalo and cattle is usually chronic or asymptomatic, resulting in production losses and reduced draft power. While, the disease in horses is quite acute and is often fatal shortly after infection (161). *T. evansi* infects wild animals as well as domestic animals and it is possible that certain wild animals, such as wild deer, act as reservoirs of the disease (63). In addition to blood sucking flies, *T. evansi* is transmitted by vampire bats especially in South and Central America. The relationship between vampire bats and *T. evansi* is unique because the bat serves as both a vector and a host (or a reservoir).

II-2: Molecular characteristics of *T. evansi* and diagnosis of Surra

T. evansi is not only morphologically but also biochemically similar to *T. brucei*. In general, it is thought that *T. evansi* "recently" evolved from *T. brucei*, and spread from Africa to other areas of the world. Theoretically, a tsetse tansmissibility test will be the best way to distinguish *T. evansi* from *T. brucei*, but the experiment may fail even with *T. brucei*. Therefore, attempts have been made in order to distinguish the two trypanosome species. Analyses of isoenzyme electrophoresis patterns are a useful tool for characterization of a variety of protozoan parasite, such as *Trypanosoma*, *Leishmania*, *Theileria* and *Toxoplasma* (1, 8, 39, 87). However, Gibson et al.(1980) (36) demonstrated that *T. evansi* was almost indistinguishable from *T. brucei*, especially from West African tsetse-transmitted trypanosome, by means of the isoenzyme analyses. Similarities of isoenzyme electrophoresis patterns between *T. evansi* and West African *T. brucei* were also demonstrated by Stevens et al. (1992) (136) and Gibson et al. (1983) (37). Therefore, the analyses of isoenzyme electrophoresis patterns are also not sufficient enough to distinguish *T. evansi* from West African *T. brucei*.

The sequence analyses of the ribosomal RNA genes (rRNAs) are a useful tool for molecular phylogenic analysis among related species. According to the results from the sequence analyses of rRNA, *T. evansi* was not so different as to be classified as an independent species from *T. brucei* (145). Furthermore, Artama et al. (1992) (2) demonstrated that the 177 bp nuclear DNA repeat was identical in the two species, and the PCR amplification patterns of procyclic acidic repetitive protein (PARP) genes, which encoded a major surface glycoprotein of the procyclic forms of *T. brucei* (83, 115), were the same. These results may suggest a striking resemblance between *T. evansi* and *T. brucei* at a genomic DNA level.

The well characterized difference between *T. evansi* and *T. brucei* was found in the kinetoplast DNA (kDNA) (16). The kDNA is a mitochondrial DNA of protozoa belonging to the order Kinetoplastida, and is organized into a network containing thousands of topologically interlocked DNA circles (16). There are two kinds of circular DNA in a typical kDNA network, namely minicircle DNA and maxicircle DNA. The maxicircle DNA is 20–40 kbp circular DNA molecules which are functionally analogous to conventional mitochondrial DNA (137), and the minicircle DNA is 1–2.5 kbp circular DNA molecules encoding small guide RNA which involves post-transcriptional editing of maxicircle DNA transcripts (12, 13, 148). *T. brucei* possesses a typical kDNA which consists of 50–100 copies of homogeneous maxicircles and thousands of copies of heterogeneous minicircles (137), but *T. evansi* has no maxicircle DNA (16) and has thousands of the minicircle DNA with (nearly) the same sequence (104, 133). Nevertheless, all *T. evansi* minicircle DNA sequences reported could be classified into two groups (type A and B) (15, 133). Masiga and Gibson (1990) (71) demonstrated that the "*T. evansi* specific" minicircle DNA fragments were useful tools for the distinction of kinetoplastic *T. evansi* from *T. brucei*. Likewise, *T. evansi* specific PCR primer was identified using *T. evansi* specific minicircle DNA sequence, and it was proposed that such "*T. evansi* specific PCR" was an important diagnostic tool in areas where both *T. evansi* and *T. brucei* were prevalent (2). However, it was found the existence of naturally akinetoplastic *T. evansi* strains in South America and China (65, 71, 104). Therefore, kDNA-based diagnosis and identification of *T. evansi* may overlook infections caused by such akinetoplastic *T. evansi*. Moreover, since it is generally thought that *T. evansi* is "recently" evolved from *T. brucei*, it will be possible that certain strains of *T. brucei* may have the *T. evansi* specific minicircle DNA sequence in their heterogeneous minicircle

DNA. If so, the "*T. evansi* minicircle DNA specific PCR" not only overlooks akinetoplastic *T. evansi* infection but also misdiagnoses *T. brucei* infections for those of *T. evansi*. Therefore, it is necessary to evaluate the specificity of the "*T. evansi* minicircle DNA specific PCR" by using various strains of *T. brucei*, including of low virulent strains of *T. b. gambiense*, in spite of inavailability of *in vivo* and *in vitro* systems that are able to produce sufficient number of such low virulent strains of trypanosomes for the studies.

III: Objective of the study

Salivarian trypanosomosis is caused by various species of trypanosomes such as *T. evansi* and *T. brucei*, and causes vast economical losses. Since certain species and isolates of trypanosomes show low virulence to experimental rodents, it is difficult to isolate and propagate such parasites for further analyses of biological, immunological and molecular biological aspects. In this study, *in vivo* and *in vitro* systems that support the growth of low virulent strains of trypanosomes were explored (Chapter 1), and the "*T. evansi* minicircle DNA specific PCR" was evaluated by using total and/or purified kDNA from various strains of trypanosomes produced in such systems. Further characterizations of the PARP gene in *T. evansi* and *T. brucei* regarding its nucleic acid sequences and gene expression were performed, and also the potential use of the PARP primers as a diagnostic tools for *T. evansi* and *T. brucei* was explored (Chapter 2). Host immune responses against *T. b. gambiense* IL3253 infection, which shows extremely low virulence to mice, were examined (Chapter 3). Especially, the role of interleukin 4 was studied in relation to host defense during IL3253 infection in mice.

CHAPTER 1:

Establishment of *in vivo* and *in vitro* culture methods for low virulent salivarian trypanosome bloodstream forms (BSFs)

1-1: Introduction

Trypanosoma brucei gambiense, the pathogen of chronic human trypanosomosis, is characterized by its low virulence to rodents, sequences of ribosomal RNA genes (rRNAs), isoenzyme patterns, restriction fragment length polymorphisms (RFLPs) in variable surface glycoprotein (VSG) genes and restricted VSG repertoire (18, 40, 43, 73, 107). A low virulent *T. b. gambiense* has made it difficult to analyze biological and immunological properties of the parasites because of the difficulty in preparing sufficient amounts of parasites. Irradiated mice have been used for isolation, propagation and cloning of salivarian trypanosomes. But certain variable antigen types (VATs) of trypanosomes do not grow well in irradiated mice (26) and radiation facilities are not always available. Immunosuppressed mice have been available to clone and propagate trypanosomes without the requirement of specific facilities (131). However, effects of immunosuppressants on trypanosomes were not well defined.

Severe combined immunodeficient (SCID) mice were originally reported by Bosma et al. (1983) (17). SCID mice have no functional T- and B-cells (17, 30) and proved to be highly susceptible hosts for many parasitic diseases. SCID mice become a useful model system for investigations of many aspects of parasites (89, 135). These studies encouraged the author to investigate the SCID mouse as a susceptible host for *T. b. gambiense*, a trypanosome species with a generally low virulence for rodents.

In vitro culture systems also have been useful for propagating various species of

trypanosomes (6, 32, 46, 49, 50, 164). However, to initiate primary cultures of salivarian trypanosomes, it is necessary to prepare millions of long slender BSFs from infected blood of a susceptible host. Therefore certain isolates, specially "low virulent" trypanosomes, have been faced with difficulties in initiating their primary cultures because of lack of susceptible experimental animals.

The objectives of this study are as follows. 1) To establish effective *in vivo* and *in vitro* culture systems for production of BSFs of *T. b. gambiense*, which shows a very low virulence in normal immunocompetent (BALB/c) mice, in sufficient amounts for various investigations of cell biological, immunological, biochemical and molecular biological aspects. 2) To examine *in vivo* growth characteristics and small chromosomes of various strains of trypanosomes.

1-2: Materials and methods

1-2a: Experimental animals

BALB/c/A-+/+ (BALB/c) and C.B-17/Icr-scid/scid (SCID) mice were obtained from Japan CLEA Inc., Japan. Mice were housed under conventional or specific pathogen free conditions and used for experiments at 5 to 6 weeks of age.

1-2b: Parasites

Trypanosomes used in entire study are listed in Table 2. Except for data of Tansui and Welcome strains, all information was obtained from the International Livestock Research Institute (ILRI), Nairobi, Kenya.

1-2c: Parasite infection

The frozen parasites were rapidly thawed at 37°C. Live trypanosomes observed by phase-contrast microscopy were diluted at 1 : 9 in phosphate-buffered saline (pH 7.8) containing 1% glucose (PSG) and then intraperitoneally injected into mice. When the parasitemia in the tail blood was higher than 100 BSFs/×400 microscopic field, infected blood was collected by cardial puncture. Parasite concentration was adjusted to 1×10^4 BSFs/ml in PSG, and then each mouse in experimental groups was inoculated with 5×10^3 BSFs.

Table 2. History of *Trypanosoma* strains tested

Species	Strain	Place	Year
T. brucei brucei	GUTat3.1	Uganda	1966
T. b. brucei	221	ND	ND
T. b. gambiense	IL1922	Ivory Coast	1952
T. b. gambiense	IL3248	Nigeria	1969
T. b. gambiense	IL3250	Nigeria	1969
T. b. gambiense	IL3253	South Sudan	1982
T. b. gambiense	IL3254	South Sudan	1982
T. b. gambiense	IL3301	Nigeria	1969
T. b. gambiense	IL3707	Nigeria	1968
T. b. gambiense	Welcome	ND	ND
T. b. rhodesiense	IL1501	Kenya	1980
T. b. rhodesiense	IL2343*	Ivory Coast	1978
T. evansi	IL1695	Kenya	1978
T. evansi	IL1934	South America	1971
T. evansi	IL3354	Mali	1988
T. evansi	IL3382	Mali	1988
T. evansi	IL3960	Kenya	1980
T. evansi	IL3962	Sudan	1976
T. evansi	Tansui	Taiwan	ND
T. congolense	IL3000	Kenya/Tanzania border	1966

Place: place of isolation, Year: year of isolation, ND: no data

*IL2343 was originally isolated from a 'gambiense' sleeping sickness patient but was later classified as *T. b. rhodesiense* (43).

Except for data of Tansui and Welcome strains, all information was obtained from ILRI's Biological Service Unit.

1-2d: Estimation of parasitemia

Three different techniques were employed for the estimation of parasitemia. Basic method to estimate the parasitemia was to observe tail blood smear by phase-contrast microscopy at×400 magnification. When no parasites were observed in the tail blood, the buffy coat method was used. Briefly, infected blood was collected into a heparinized hematocrit tube. The blood was centrifuged at 15,000*g* for 5 min. The buffy coat was collected onto a slide glass and observed by phase-contrast microscopy. When the parasitemia was higher than 100 BSFs/×400 microscopic field, then the parasite concentration in the blood was counted by a hemocytometer. To convert the data obtained form the tail blood smear and buffy coat observation to the actual concentration of the parasites in the blood, a conversion table was made (data not shown). Briefly, a known number of parasites was serially diluted in mouse blood, and then the numbers of parasites in blood smears and buffy coats were determined. The conversion table was made on both actual concentrations of the parasites and the results of the tail blood and buffy coat counts.

1-2e: Pulsed field gel electrophoresis (PFGE)

BSFs were purified from infected blood by means of DE52 column chromatography (57). The BSFs were embedded in 1% low melting agarose at a concentration of 6×10^8 BSFs/ml. The sample-embedded agarose blocks were incubated in sodium dodecyl sulfate (SDS) lysis buffer containing 1 mg/ml proteinase K (lysis buffer) with a gentle agitation at 55°C. The incubation was continued for 3 d and the lysis buffer was changed every 12 h. Then, the agarose blocks were washed 3 times by TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). PFGE was performed using a CHEF-DRIII system (Bio-Rad

Laboratories, U.S.A.). Slices from the agarose blocks were sealed into the wells of 1% agarose gel containing 0.5× Tris-borate-EDTA (TBE) buffer (45 mM Tris-borate, 0.5 mM EDTA). The PFGE was carried out at 6 V/cm for 21 h. Field angle was 120°, and switch time was gradually increased from 60 s to 120 s during the electrophoresis. Chamber buffer (0.5× TBE) was recirculated at 14°C.

1-2f: Sera and plasmas

Fetal bovine serum (FBS) was purchased from ICN Biomedicals Japan Co. Ltd., Japan. Adult bovine serum (ABS), adult bovine plasma (ABP), adult horse serum(HoS), adult horse plasma (HoP), human serum (HuS) and human plasma (HuP) were obtained from healthy donors.

1-2g: *In vitro* cultivation of *T. brucei* BSFs by feeder cell layer system

BSFs of *T. brucei* were cultured by using the procedure of Hirumi et al. (1977) (46). Briefly, BSFs of *T. brucei* were obtained from a mouse that was showing a ascending parasitemia. The parasites were separated from the blood cells by a low speed centrifugation at 100*g* for 7 min. Then the concentration of BSFs was adjusted to 0.5-1.0 $\times 10^6$ BSFs/ml in RPMI-1640 medium supplemented with 20% serum or plasma, 0.75 mM L-cysteine hydrochloride, 0.10 mM bathocuproine disulfonic acid disodium salt, 1.00 mM hypoxanthine and 2.00 mM L-glutamine. The suspension of the BSFs was transferred to culture flasks (5 ml/flask), in which embryonic fibroblast-like cells of *Microtus montanus* were cultured confluently, and the flasks were kept in a CO_2 incubator (5% CO_2 in air) at 37°C. General growth patterns of BSFs were observed by phase-contrast microscopy every day and the cultures were maintained by changing medium and making subcultures

appropriately.

1-2h: Axenic cultivation of *T. brucei* BSFs

Axenic cultivation of *T. brucei* BSFs was carried out by using HMI-9 medium which was designed for the cultivation of BSFs of *T. brucei* in a feeder cell-free system (47). *T. brucei* BSFs were isolated from infected mouse blood at a ascending parasitemia by a low speed centrifugation at 100*g* for 7 min. BSF-containing supernatant was transferred to T-25 culture flasks (5 ml/flask) which were kept for 2-3 h in a CO_2 incubator (5% CO_2 in air) at 37˚C to settle remaining blood cells on the bottom of the flasks. Culture supernatants were collected from the flasks by gentle pipetting and adjust concentration of BSFs to 4.0-8.0$\times 10^5$ BSFs/ml by adding an appropriate amount of HMI-9 medium. Five ml of the adjusted BSFs suspensions were transferred to new T-25 flasks and the flasks were incubated in the CO_2 incubator. Numbers of trypanosomes in cultures were determined by hemocytometer. To maintain the cultures with best condition, an appropriate volume of the fresh medium was added and subcultures were made.

1-2i: Calculation of a population doubling time (PDT)

PDT was calculated by using the equation as follows (103).

$PDT = \{(T-T_0)\log 2\}/(\log N/N_0)$

N_0 is an initial concentration of cells at any selected time (T_0).

N is a final concentration of cells at time (T).

1-2j: Statistical analyses

Statistical determinations of the difference between means of experimental groups were made using an unpaired, 2-tailed Student's *t* test.

1-3: Results

1-3a: Growth characteristics of various strains of trypanosomes in BALB/c mice

Seven strains of *T. b. gambiense*, 2 strains of *T. b. rhodesiense*, and 6 strains of *T. evansi* were examined for their infectivity, survival rate, the highest parasitemia and the lowest packed cell volume (PCV) in BALB/c mice (Table 3). Five out of 7 strains of *T. b. gambiense* (IL3248, IL3250, IL3301, IL3253 and IL3254) and 3 out of 6 strains of *T. evansi* (IL1695, IL3354 and IL3382) developed chronic infection in the BALB/c mice with sporadic parasitemia (Fig. 4), although 2 mice infected with *T. evansi* IL1695 died of high parasitemia. The lowest PCVs in these chronically infected groups were ranged from 32.0% to 46.5% during the infection. While, *T. b. gambiense* IL1922 and IL3707, *T. b. rhodesiense* IL2343 and IL1501, and 3 strains of *T. evansi* (IL1934, IL3960 and IL3962) caused fatal infection in the BALB/c mice with high parasitemia (>1×10^8 BSFs/ml) (Fig. 5). The lowest PCVs in the acutely infected groups were ranged from 18.0% to 46.8%.

Table 3. Growth characteristics of trypanosomes in BALB/c mice

Strain	SR (%)	HP (BSFs/ml)	LPCV (%)
T. b. gambiense IL1922	0	$(5.6\pm0.5)\times10^8$	46.8±0.5
T. b. gambiense IL3248	100	ND	44.6±1.7
T. b. gambiense IL3250	100	$(1.7\pm1.3)\times10^6$	40.4±4.2
T. b. gambiense IL3253	100	$(1.9\pm2.2)\times10^6$	46.5±1.5
T. b. gambiense IL3254	100	$(5.1\pm2.6)\times10^5$	38.6±2.5
T. b. gambiense IL3301	100	$(1.0\pm0.8)\times10^5$	34.5±4.4
T. b. gambiense IL3707	0	$(1.1\pm0.3)\times10^9$	39.8±6.6
T. b. rhodesiense IL1501	0	$(1.7\pm0.6)\times10^9$	29.4±2.1
T. b. rhodesiense IL2343	0	$(1.4\pm0.4)\times10^9$	32.5±3.3
T. evansi IL1695	60	4.5×10^8	32.0
T. evansi IL1934	0	1.5×10^9	28.0
T. evansi IL3354	100	1.8×10^6	46.0
T. evansi IL3382	100	4.5×10^7	45.0
T. evansi IL3960	0	1.8×10^9	23.0
T. evansi IL3962	0	1.5×10^9	18.0

The results of *T. b. gambiense* and *T. b. rhodesiense* are expressed as means±SD for the 5 mice in each group.

The data of *T. evansi* are mean value of the 5 mice in each group.

SR: survival rate at 60 d after infection, HP: highest parasitemia,

LPCV: lowest PCV, ND: not detected

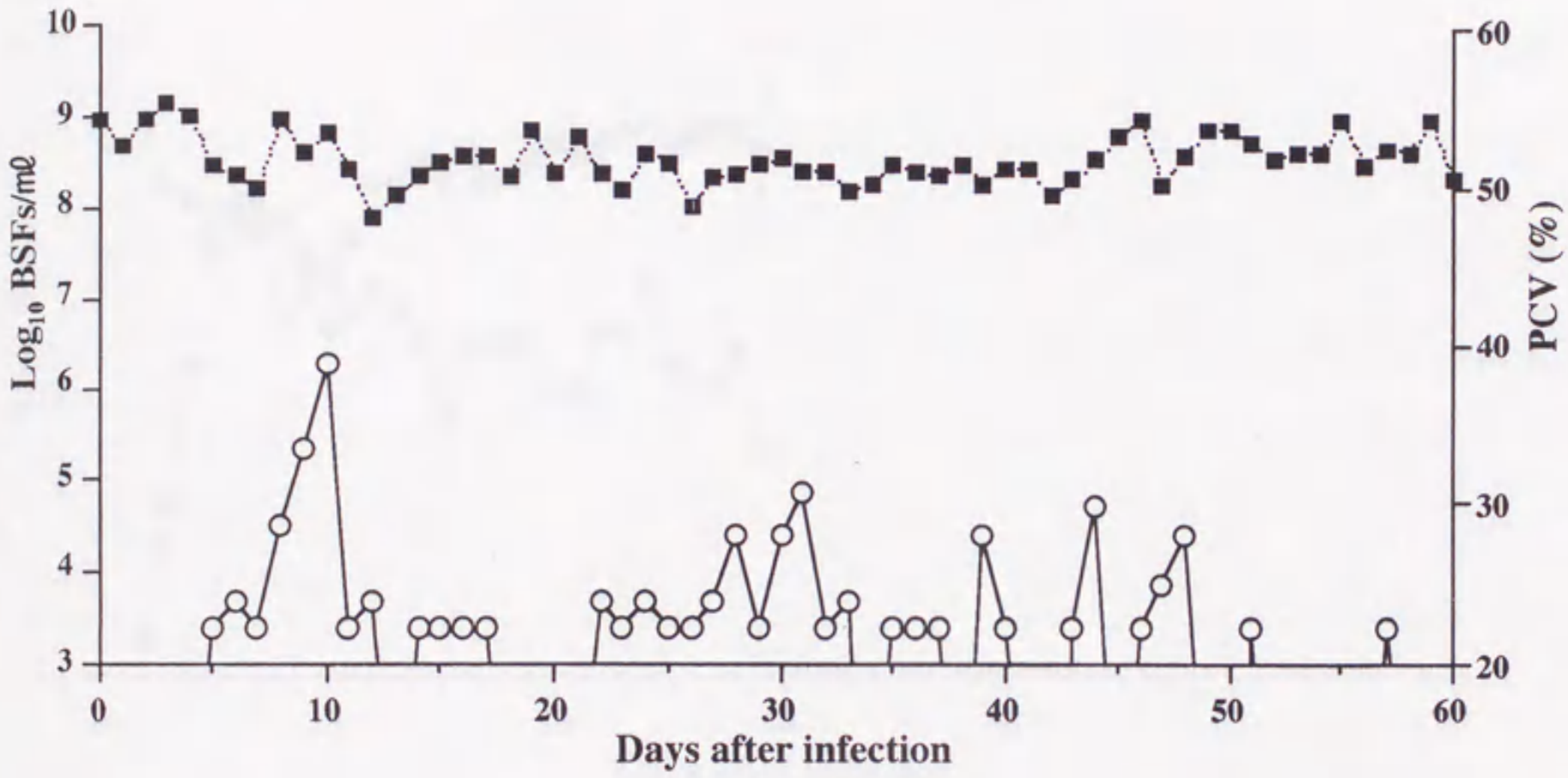


Figure 4. Typical growth pattern of a low virulent trypanosome in a BALB/c mouse

Mice were infected intraperitoneally with 5×10^3 BSFs of *T. b. gambiense* IL3253.

Parasitemia (open circle) and packed cell volume (solid square) are representative value of 5 mice.

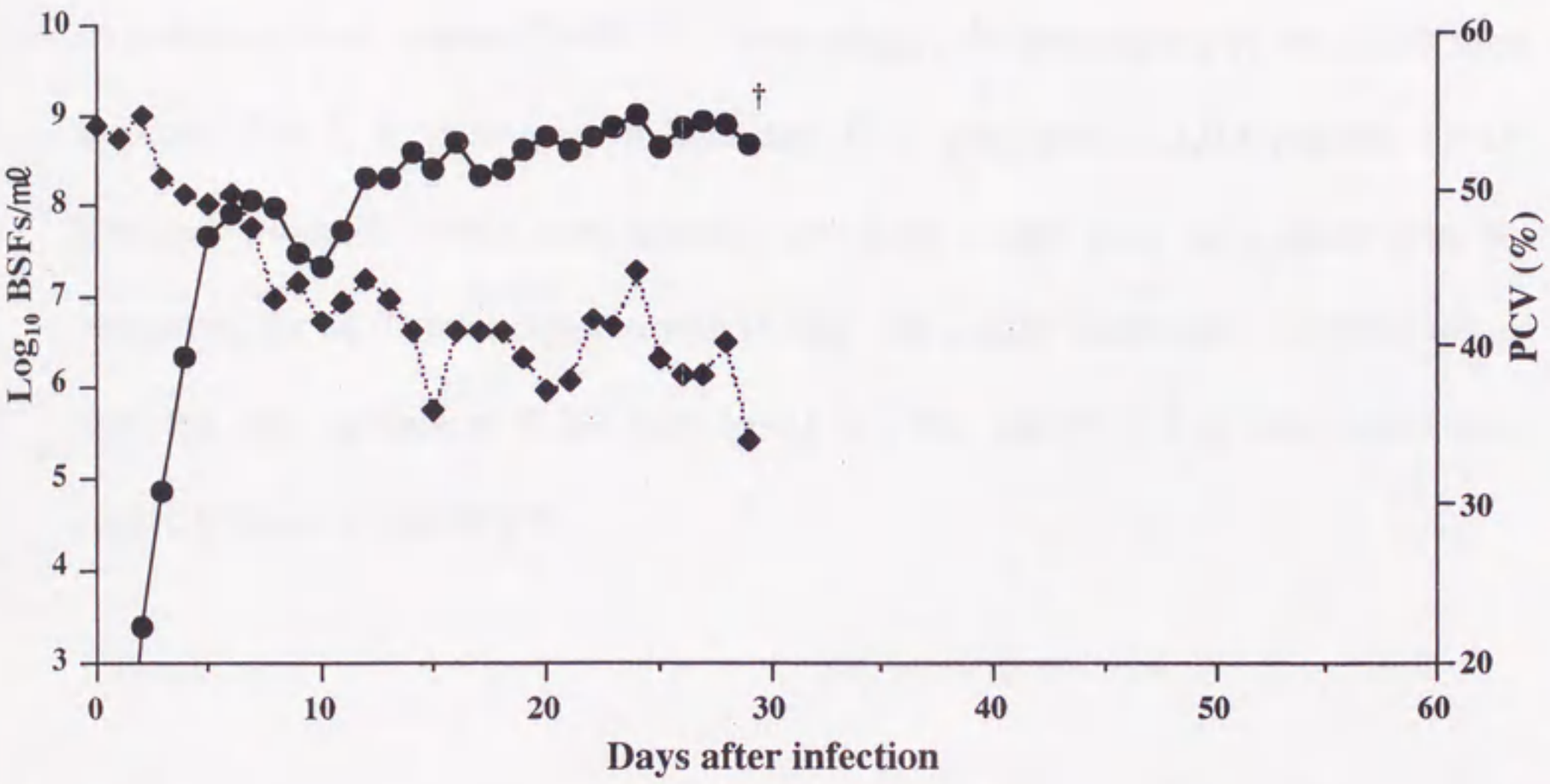


Figure 5. Typical growth pattern of a high virulent trypanosome in a BALB/c mouse

Mice were infected intraperitoneally with 5×10^3 BSFs of *T. b. rhodesiense* IL2343.

Parasitemia (solid circle) and packed cell volume (solid diamond) are representative value of 5 mice.

† indicates death of the mouse.

1-3b: Growth characteristics of *Trypanosoma* strains in SCID mice

Although the results of *T. evansi* strains were not shown in Table 4, SCID mice showed extremely high susceptibility to all *Trypanosoma* strains tested, including low virulent strains which develop chronic infection in BALB/c mice. All SCID mice developed high parasitemia (>1×10^8 BSFs/ml) and died within 47 d regardless of the trypanosome strain tested (Table 4). Interestingly, the parasitemia of the SCID mice infected with *T. b. gambiense* IL3253 and *T. b. gambiense* IL3254 reached 1×10^8 BSFs/ml within 2 weeks after infection and kept a high level until death (Fig. 6). Moreover, the SCID mice infected with IL3253 and IL3254 significantly survived longer than the other groups of SCID mice ($p<0.05$). The lowest PCV of each group were ranged from 29.3 % to 48.0 %.

Table 4. Growth characteristics of *T. b. gambiense* and *T. b. rhodesiense* in SCID mice

Strain	PP (d)	SP (d)	HP (BSFs/ml)	LPCV (%)
T. b. gambiense IL1922	1.0±0.0	4.0±0.0	$(6.1\pm2.1)\times10^{8}$	41.4±3.1
T. b. gambiense IL3248	3.2±0.5	12.8±1.1	$(8.8\pm1.0)\times10^{8}$	31.0±3.5
T. b. gambiense IL3250	3.0±0.0	12.2±0.5	$(1.9\pm0.4)\times10^{9}$	38.4±4.2
T. b. gambiense IL3253	8.0±0.7	31.2±13.1	$(2.1\pm0.3)\times10^{9}$	29.3±6.2
T. b. gambiense IL3254	1.2±0.5	41.6±4.2	$(9.1\pm1.6)\times10^{8}$	35.6±2.6
T. b. gambiense IL3301	1.3±0.5	8.5±0.6	$(1.2\pm0.2)\times10^{9}$	40.0±2.9
T. b. gambiense IL3707	2.0±0.7	7.0±0.0	$(1.4\pm0.2)\times10^{9}$	48.0±2.9
T. b. rhodesiense IL1501	3.2±0.5	9.8±1.9	$(1.2\pm0.2)\times10^{9}$	32.2±3.3
T. b. rhodesiense IL2343	3.5±0.5	10.0±0.0	$(1.3\pm0.1)\times10^{9}$	43.0±2.3

The results are expressed as means±SD for the five mice in each group.

PP: prepatent period, SP: survival period, HP: highest parasitemia, LPCV: lowest PCV

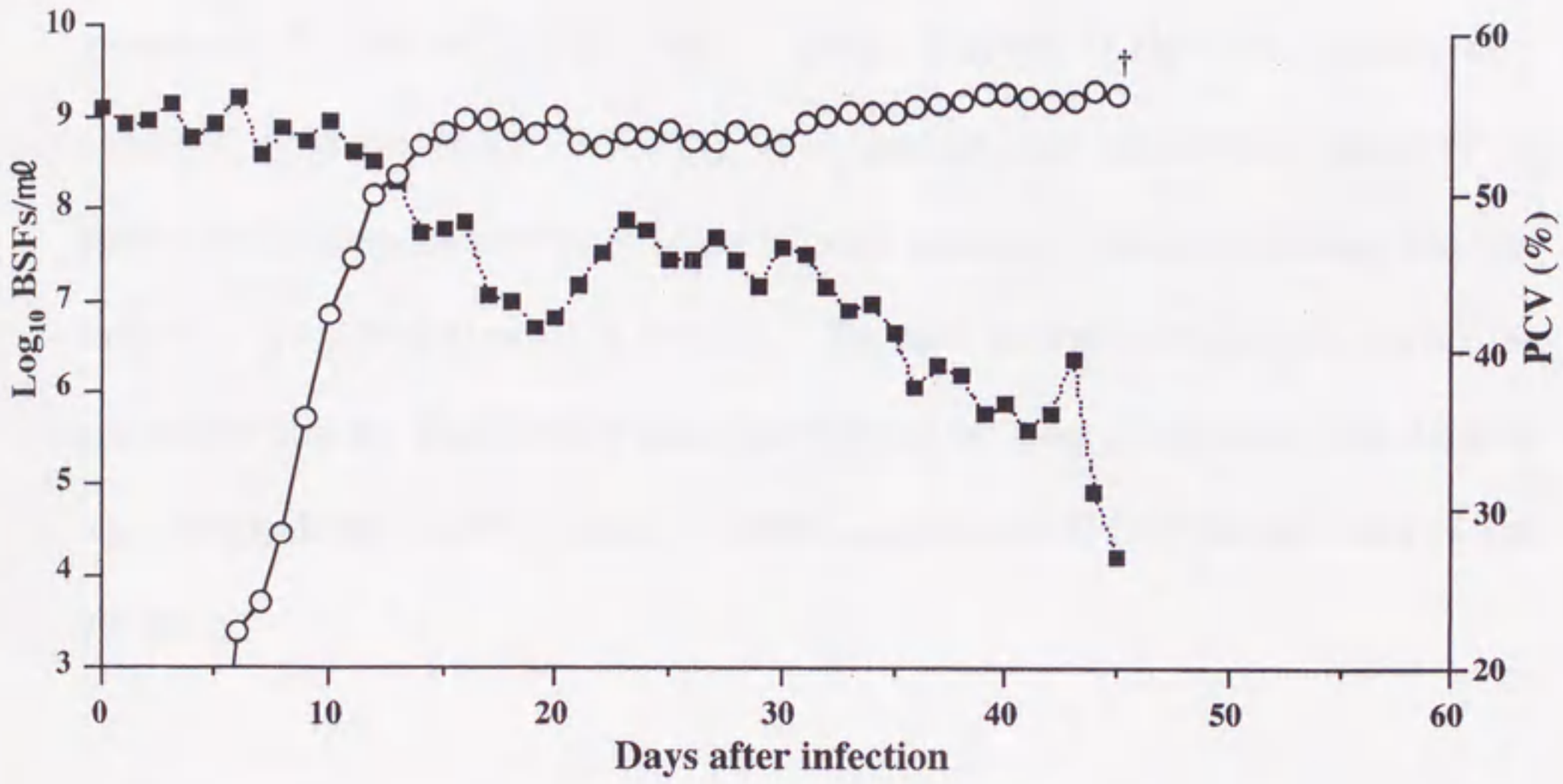


Figure 6. Typical growth pattern of *T. b. gambiense* IL3253 BSFs and *T. b. gambiense* IL3254 BSFs in SCID mice

SCID mice were infected intraperitoneally with 5×10^3 BSFs of *T. b. gambiense* IL3253 or *T. b. gambiense* IL3254.

Parasitemia (open circle) and packed cell volume (solid square) are representative value of 10 mice.

† indicates death of the mouse.

1-3c: PFGE analysis of various strains of trypanosomes

Three classes of chromosome had been identified from salivarian trypanosomes, that are the mini-chromosomes (< 150 kb), the intermediate size chromosomes (150 kb - 1,000 kb) and the large chromosomes (> 1,000 kb) (35, 65, 138, 139). The mini- and intermediate size chromosomes of 14 strains of trypanosomes including low virulent *T. b. gambiense* (IL3250 and IL3301) and *T. evansi* (IL1695, IL3354 and IL3382) were examined by means of the PFGE (Fig. 7). Although, each strain had a unique PFGE pattern, the relative intensity of ethidium bromide staining of mini-chromosomes was low in the *T. b. gambiense* (lanes 2, 3, 4 and 5). The band patterns varied among not only the species but also the strains which were isolated from the same geographical area (Lanes 2 and 7: Ivory Coast, Lanes 3, 4 and 5: Nigeria, Lanes 9 and 13: Kenya, and Lanes 11 and 12: Mali).

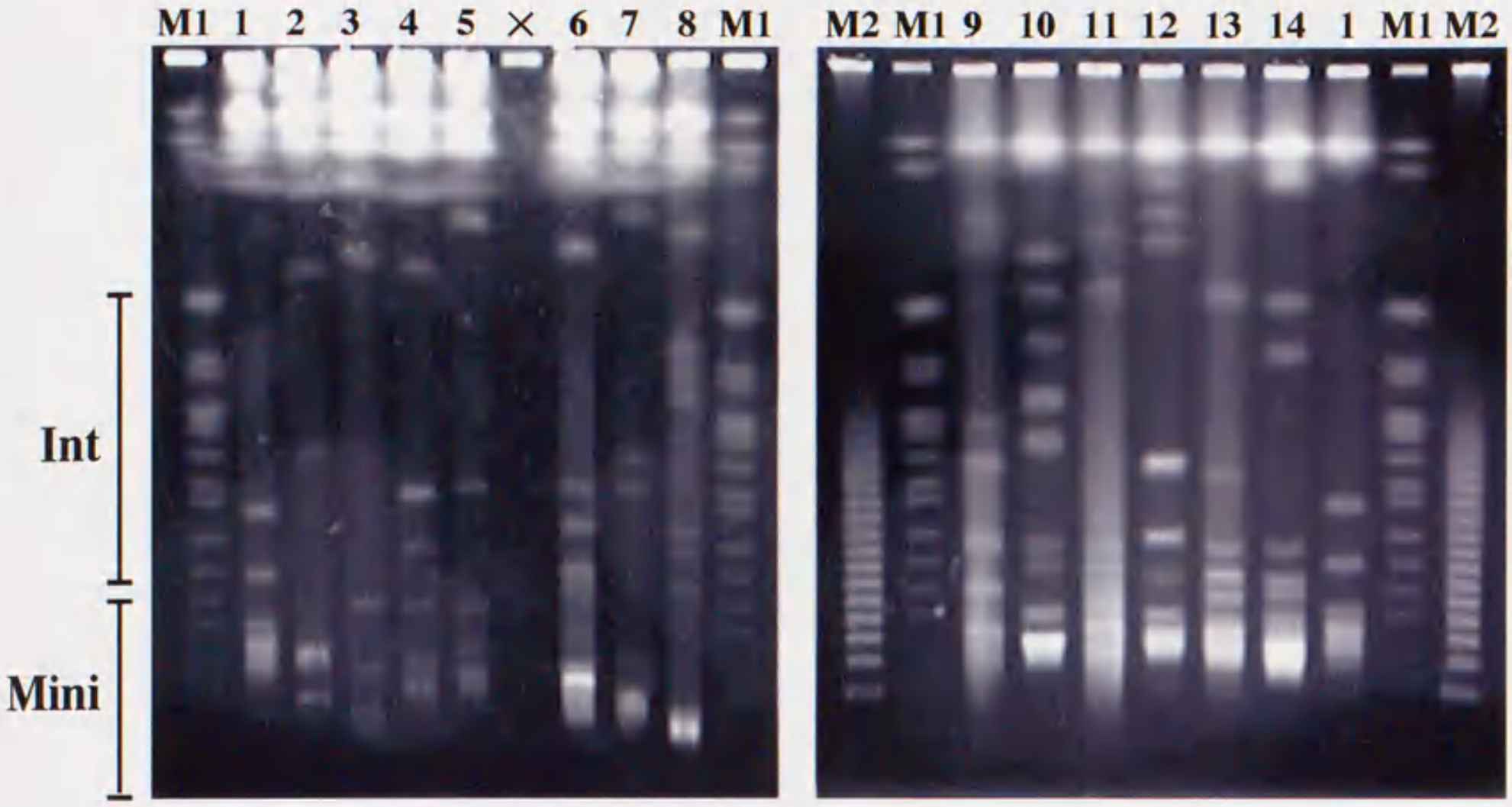


Figure 7. Size-fractionation of the chromosomes (48.5 kb-2,200 kb) of various strains of *T. evansi* and *T. brucei*

The strains examined are as follows: (1) *T. evansi* Tansui, (2) *T. b. gambiense* IL1922, (3) *T. b. gambiense* IL3707, (4) *T. b. gambiense* IL3250, (5) *T. b. gambiense* IL3301, (6) *T. b. rhodesiense* IL1501, (7) *T. b. rhodesiense* IL2343, (8) *T. congolense* IL3000, (9) *T. evansi* IL1695, (10) *T. evansi* IL1934, (11) *T. evansi* IL3354, (12) *T. evansi* IL3382, (13) *T. evansi* IL3960, and (14) *T. evansi* IL3962.

Lanes M1 and M2 contain *Saccharomyces cervisiae* YNN295 chromosome (Bio-Rad Laboratories, U.S.A.) and λ ladder (Bio-Rad Laboratories, U.S.A.), respectively.

Sample preparation was a failure in lane ×.

Mini: mini-chromosomes, Int: intermediate size chromosomes

1-3d: *In vitro* cultivation of BSFs of a low virulent strain by feeder cell layer system

Millions of long slender BSFs are needed for initiation of *in vitro* cultivation of animal infective salivarian trypanosomes. Therefore, an application of *in vitro* culture systems has been biased toward high virulent strains of *Trypanosoma* which propagate well in small experimental animals. To date, successful *in vitro* cultivation of animal infective trypanosomes of a low virulent strain has never been reported. Since SCID mice were highly susceptible to variable *Trypanosoma* strains tested (Table 4), sufficient number of long slender BSFs could be prepared by using SCID mouse system regardless of virulence of the parasites.

BSFs of *T. b. gambiense* IL3253, that is a typical low virulent strain, were obtained from infected SCID mouse blood. Three kinds of basal media and 7 kinds of supplements were examined for their ability to support the growth of the BSFs (Table 5). As a result, the best growth was obtained in RPMI-1640 supplemented with HuP (Fig. 8a), moderate growth in the media supplemented with FBS, HoS or HuS (Fig. 8b), and no growth in the media with others.

Table 5. *In vitro* cultivation of BSFs of a low virulent strain (*T. b. gambiense* IL3253) with feeder cell layer

Basal medium	Supplement		Growth
RPMI-1640*	FB	S	+
	AB	S	−
	AB	P	−
	Ho	S	+
	Ho	P	−
	Hu	S	+
	Hu	P	++
IMDM Hybri-Max®	FB	S	−
S-Clone SF-B	FB	S	−

*: Hirumi et al. (1977) (46), FB: Fetal bovine,

AB: Adult bovine, Ho: Horse, Hu: Human, S: Serum,

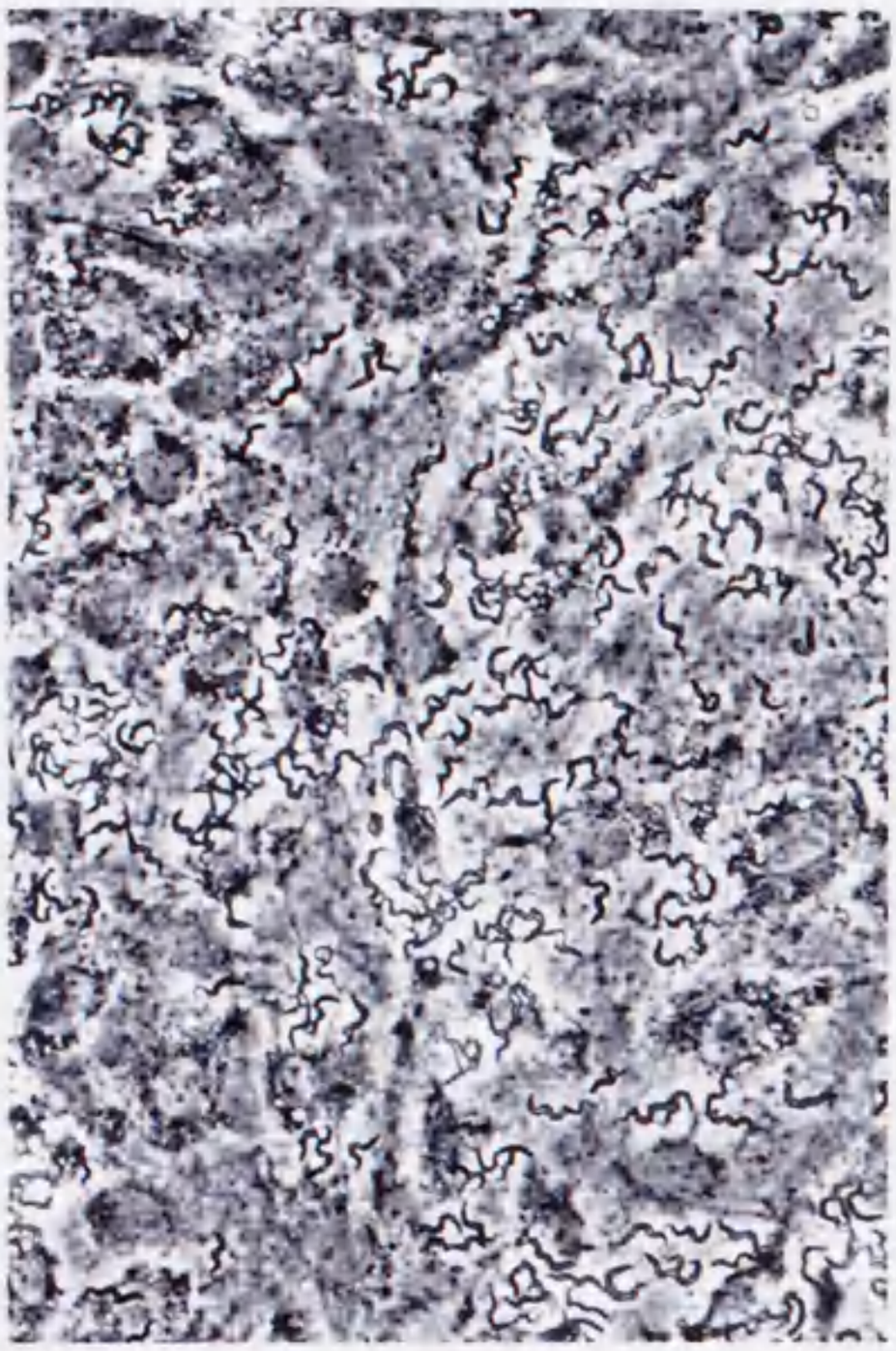
P: Plasma,

IMDM: Iscove's modified Dulbecco's MEM,

++: Support best growth, +: Support growth,

−: No growth

(a)



(b)

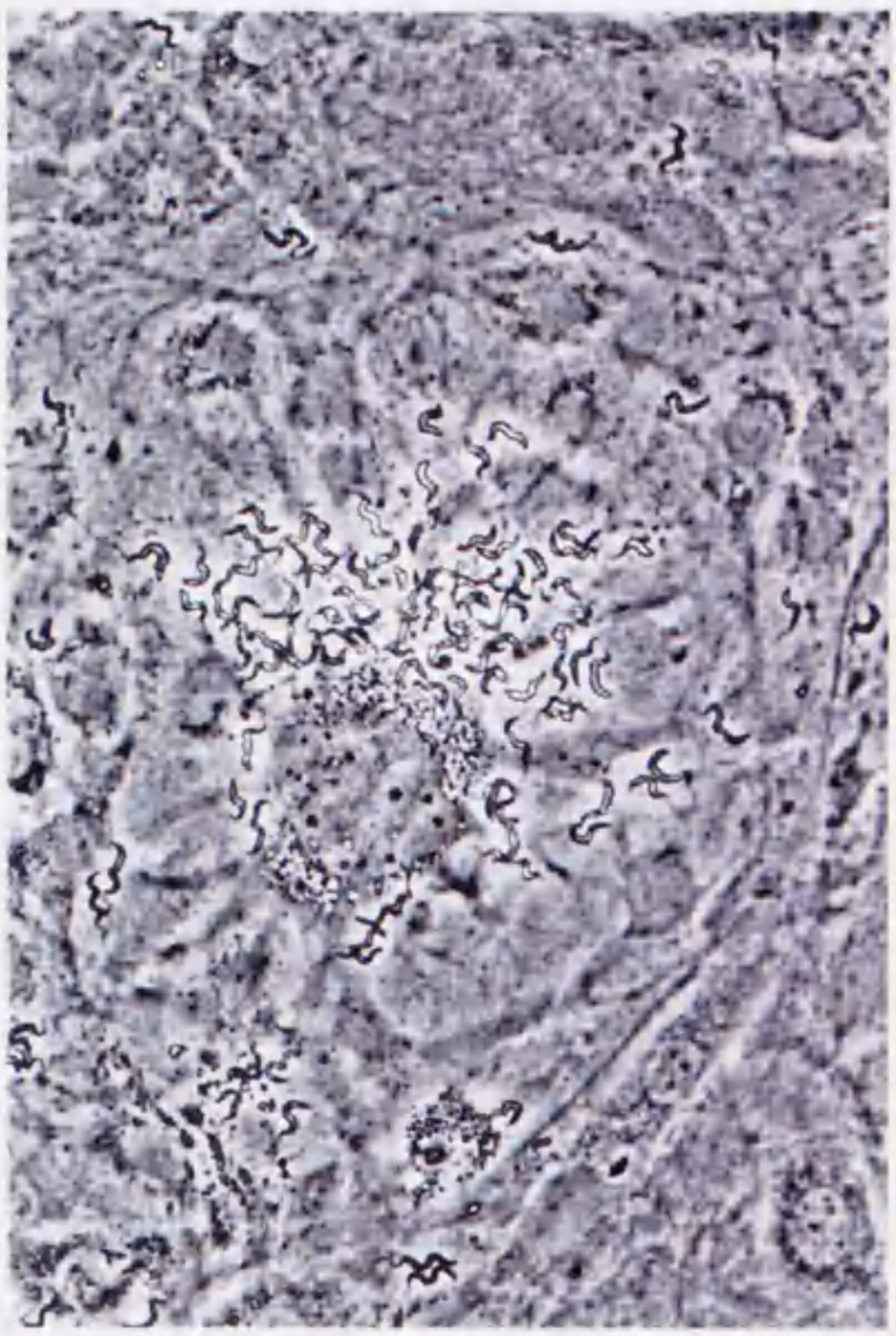


Figure 8. The growth of *T. b. gambiense* IL3253 BSFs in the presence of feeder cell layer in the medium supplemented with HuP (a) and with HoS (b)

1-3e: Axenic cultivation of BSFs of the low virulent strain

T. b. rhodesiense IL2343 is a high virulent strain and is well grown in an axenic culture system as described earlier (49).

Firstly, to replace the basal Iscove's modified Dulbecco's MEM (IMDM) (Flow Lab., U.K.) which was used in the original system but has become commercially unavailable, currently available four media, namely IMDM (Sigma, U.S.A.), IMDM Hybri-Max® (Sigma, U.S.A.), IMDM (Gibco BRL, U.S.A.) and S-Clone SF-B (Sanko Junyaku, Japan), were tested for their ability to support the growth of *T. b. rhodesiense* IL2343 BSFs and *T. b. gambiense* IL3253 BSFs. Since IMDM used in the original axenic culture system was supplemented with bovine serum albumin, L-α-phosphatidylcholine and bovine holo-transferrin, 2 out of the 4 media were supplemented with the same concentrations of the supplements (Table 6). Although all the media supported the growth of "high virulent" IL2343 BSFs, none of them supported "low virulent" IL3253 BSFs. The shortest population doubling time (SPDT) of the IL2343 was shorter in S-Clone SF-B (6.8 h) than that in IMDM Hybri-Max® (7.9 h), and the highest population density (HPD) was 3.1×10^6 BSFs/ml and 2.9×10^6 BSFs/ml, respectively (Fig. 9 and Table 9). Growth characteristics of the IL2343 in the other 2 media were nearly the same as in the IMDM Hybri-Max® (Data not shown).

Secondly, to establish an axenic culture system which supports the growth of "low virulent" IL3253 BSFs, combinations of 2 basal media and 3 supplements were tested for their ability to support the growth of *T. b. gambiense* IL3253 BSFs (Table 8). The growth of the IL3253 BSFs was examined in the IMDM Hybri-Max® supplemented with HuP (IMDM-HuP), HuS or HoS (IMDM-HoS). The best growth was obtained in the medium supplemented with HuP, and moderate growth in the other media (Table 8). The SPDTs

of the parasites were 8.7 and 13.4 h in the IMDM-HuP and IMDM-HoS, respectively (Table 9). The HPD was 2 times higher in the IMDM-HuP (1.7×10^6 BSFs/ml) than that in IMDM-HoS (5.4×10^5 BSFs/ml), although HPDs of the IL3253 BSFs were obtained from 3 d-culture in both media (Fig. 10 and Table 9). Moreover, the S-Clone SF-B, which supported the growth of "high virulent" IL2343 BSFs, supplemented with HuS or HuP were examined for their ability to support the growth of the IL3253 BSFs. As a result, only the medium supplemented with HuP supported a moderate growth of the parasites (Table 8). The number of the parasites decreased during initial 24 h and increased gradually the following 72 h (Fig. 11). The SPDT and HPD of the parasites in the medium were 13.0 h and 6.1×10^5 BSFs/ml, respectively (Table 9).

Table 6. Culture media tested*

Basal medium	Supplements				
	BSF-SF	L-Glu (mM)	BSA (μg/ml)	PC (μg/ml)	TF (μg/ml)
IMDM Hybri-Max®	+	8	400	100	1
SIGMA IMDM	+	8	400	100	1
Gibco IMDM	+	2	–	–	–
S-Clone SF-B	+	2	–	–	–

*: All media were supplemented with 20% (v/v) fetal bovine serum.

IMDM: Iscove's modified Dulbecco's MEM,

BSF-SF: Bloodstream form supporting factors (Table 7),

L-Glu: L-Glutamine, BSA: Bovine serum albumin,

PC: L-α-Phosphatidylcholine, TF: Bovine holo-transferrin

Table. 7. Final concentration (conc.) of BSF-SF in medium (47)

Components	Final conc. (mM)
Bathocuproine disulfonic acid disodium salt	0.10
Pyruvic acid sodium salt	1.00
Thymidine	0.16
2-β-Mercaptoethanol	0.14
Hypoxanthine	1.00
L-Cysteine hydrochloride	1.50

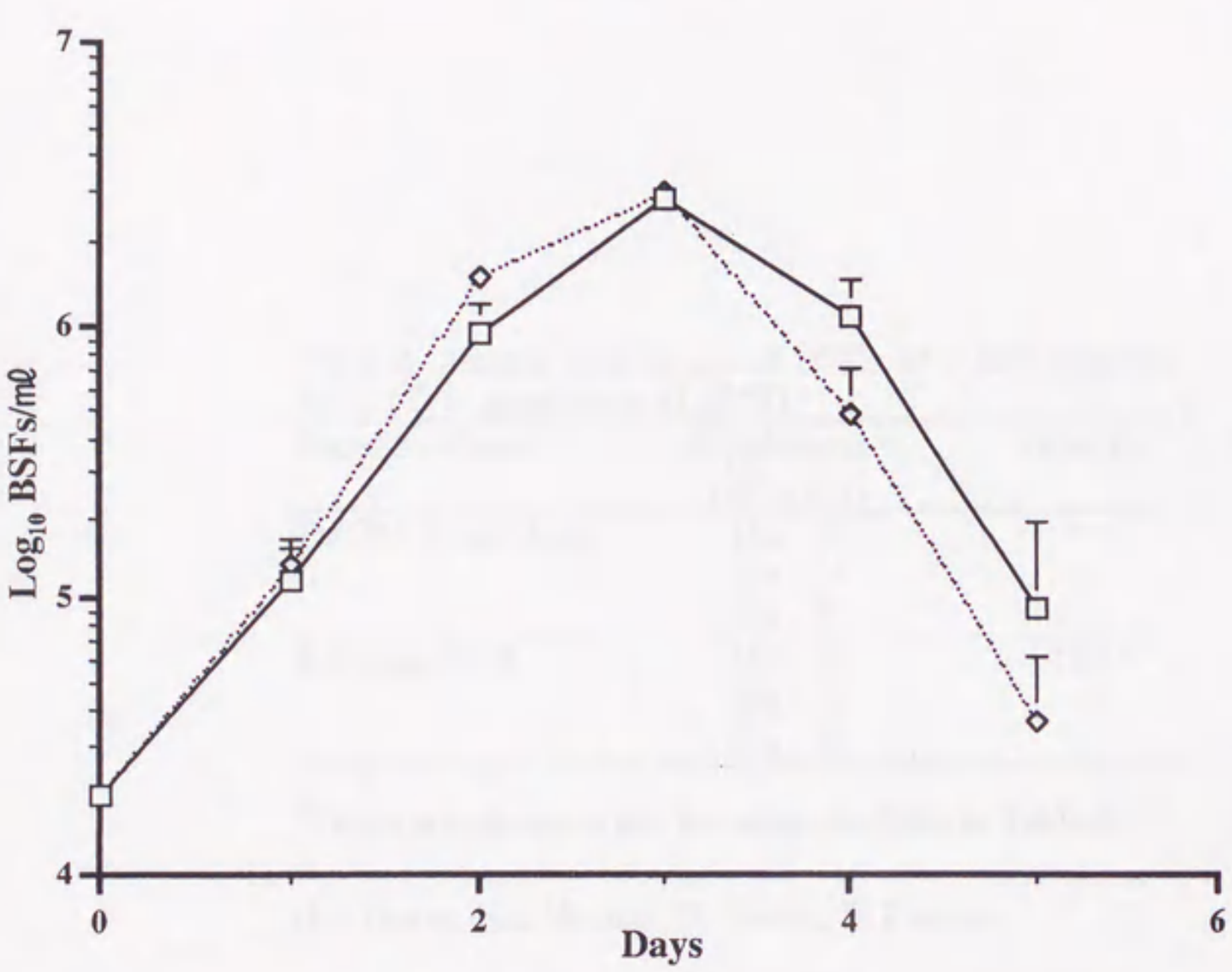


Figure 9. Growth curves of *T. b. rhodesiense* IL2343 in IMDM Hybri-Max® (open square) and S-Clone SF-B (open diamond)

The data are derived from 9 cultures, and expressed as means±SD.

Table 8. Axenic cultivation of BSFs of a low virulent strain (*T. b. gambiense* IL3253)

Basal medium	Supplement* (20% v/v)		Growth
IMDM Hybri-Max®	Ho	S	+
	Hu	S	+
	Hu	P	++
S-Clone SF-B	Ho	S	ND
	Hu	S	−
	Hu	P	+

*Other supplements are the same as those in Table 6.

Ho: Horse, Hu: Human, S: Serum, P: Plasma,

ND: No data,

IMDM: Iscove's modified Dulbecco's MEM,

++: Support best growth, +: Support growth,

−: No growth

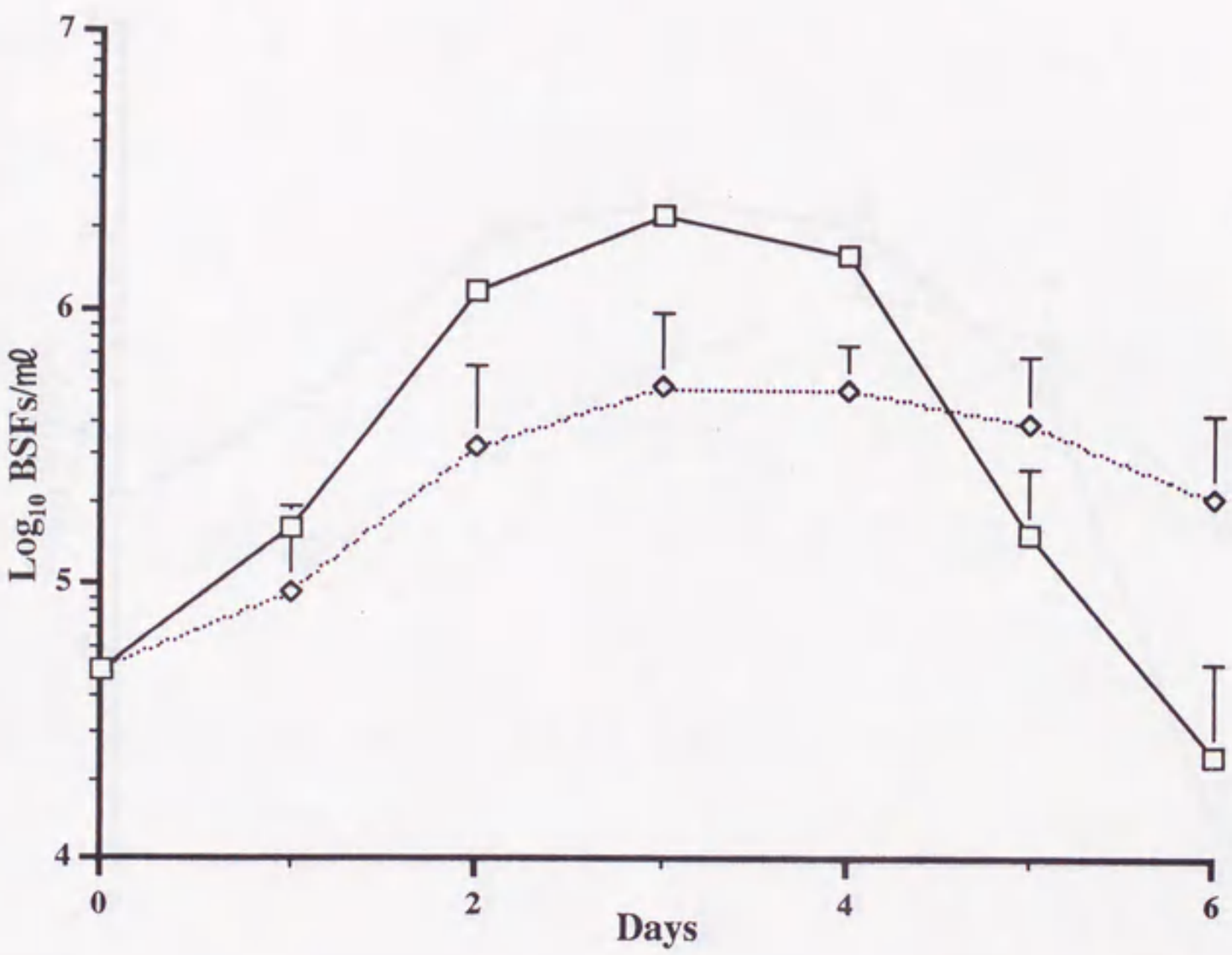


Figure 10. Growth curves of *T. b. gambiense* IL3253 in IMDM Hybri-Max® supplemented with 20 % HuP (open square) and with 20% HoS (open diamond) The data are derived from 9 cultures, and expressed as means±SD.

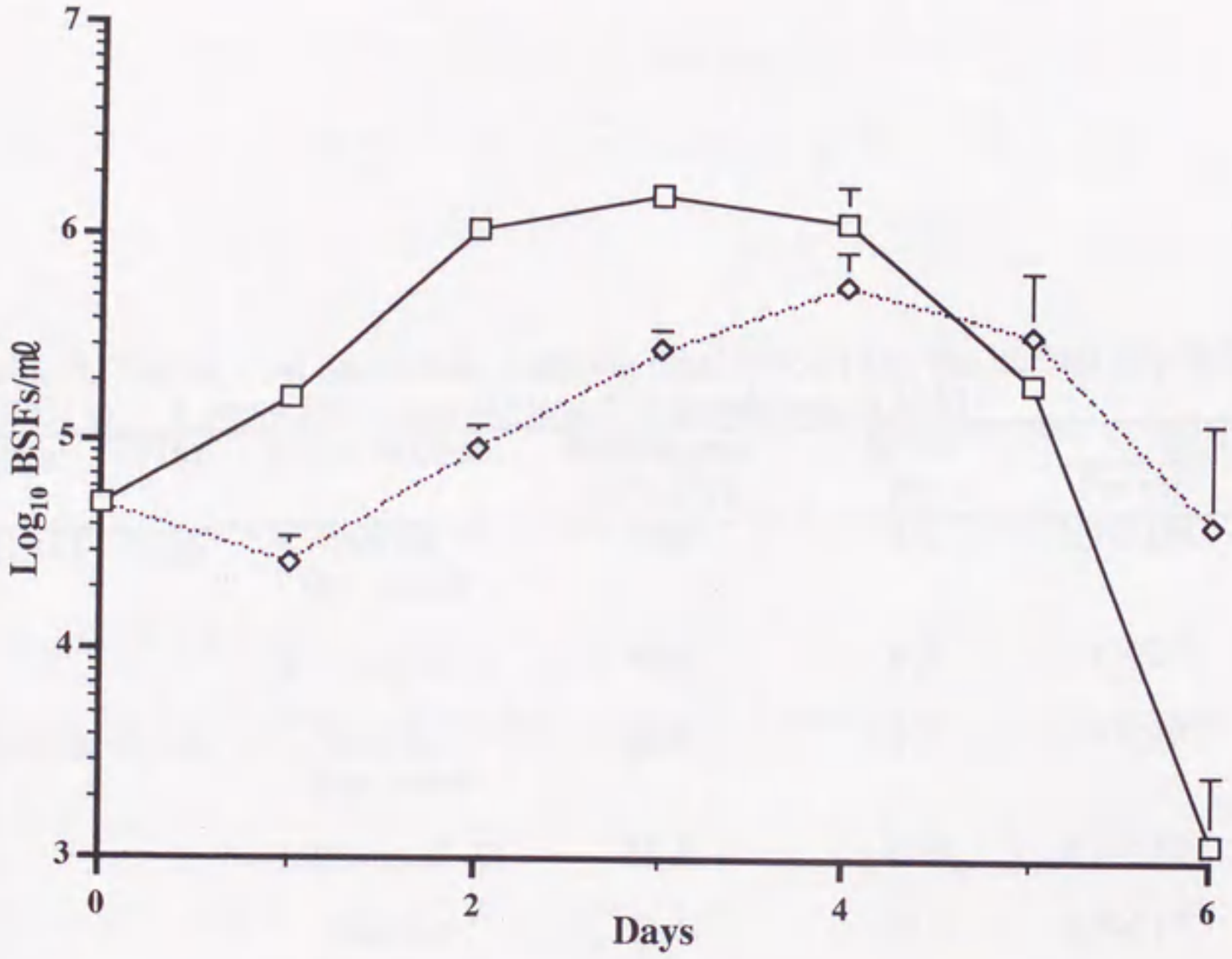


Figure 11. Growth curves of *T. b. gambiense* IL3253 in IMDM Hybri-Max® supplemented with 20 % HuP (open square) and S-Clone SF-B supplemented with 20% HuP (open diamond)

The data are derived from 9 cultures, and expressed as means±SD.

Table 9. The shortest population doubling time (SPDT) and the highest population density (HPD) in *T. b. rhodesiense* IL2343 and *T. b. gambiense* IL3253

Strain	VIM	Basal medium	Supplement (20% v/v)	SPDT (h)	HPD	
					Per ml	On day
IL2343	High	IMDM Hybri-Max®	FBS	7.9	2.9×10^6	3
		S-Clone SF-B	FBS	6.8	3.1×10^6	3
IL3253	Low	IMDM Hybri-Max®	HuP	8.7	1.7×10^6	3
		S-Clone SF-B	HuP	13.0	6.1×10^5	4
		IMDM Hybri-Max®	HoS	13.4	5.4×10^5	3

VIM: Virulence in mice, IMDM: Iscove's modified Dulbecco's MEM,

FBS: Fetal bovine, serum, HuP: Human plasma, HoS: Horse serum

1-4: Discussion

1-4a: Growth characteristics of trypanosomes in BALB/c and SCID mice

Various species and strains of salivarian trypanosomes were examined for their infectivity, survival rate, the highest parasitemia and the lowest PCV in BALB/c and SCID mice. *T. b. gambiense* (IL3248, IL3250, IL3253, IL3254 and IL3301) and *T. evansi* (IL3354 and IL3382) caused chronic infection in BALB/c mice with sporadic parasitemia (Fig. 4). Interestingly, *T. b. gambiense* IL1922 (isolated in Ivory Coast on 1952) and IL3707 (isolated in Nigeria on 1968) showed a high virulence to the BALB/c mice (Table 3). There is strong evidence that Type II *T. b. gambiense* (or non-gambiense) occurs in West Africa including Ivory Coast and Nigeria, which differs from Type I (or true-gambiense) in its greater virulence to rodents, repetitive DNA sequences of ribosomal DNA, isoenzyme electrophoresis patterns, PCR of VSG genes and RFLPs (18, 40, 43, 73, 107). For example, *T. b. rhodesiense* IL2343 originating from TH144/78E[020] strain was isolated as *T. b. gambiense* from a patient in Ivory Coast (33), but further characterizations of IL2343 concluded that the isolate should be referred to as *T. b. rhodesiense* or Type II *T. b. gambiense* (43, 73, 107, 116). Thus, *T. b. gambiense* IL1922 and IL3707, both characterized by high parasitemia ($>5\times10^8$ BSFs/ml) in BALB/c mice, may require further characterization to be classified as "true-gambiense". On the other hand, parasitemia in SCID mice at the terminal stage of infection was higher than 1 $\times10^8$ BSFs/ml regardless of the trypanosome strain tested (Table 4). These results demonstrate that SCID mice are highly susceptible hosts for all trypanosome strains tested including "low virulent" strains and can be useful for propagating large amounts of such parasites. The SCID mice infected with *T. b. gambiense* IL3253 and *T. b. gambiense* IL3254, originally isolated from patients in South Sudan in 1982, survived significantly

longer than the other groups of SCID mice. This persistent parasitemia in the SCID mice may depend on the virulence of the parasite and/or protective effects by non-specific immune responses (7). The levels of the lowest PCV ranged from 34.5 % to 46.5 % among chronically infected BALB/c mice (Table 3). These results indicate that the chronically infected mice showed a slight anemia. While, the lowest PCV varied in levels from 18.0 % (sever anemia) to 48.0 % (normal) among fatally infected groups of BALB/c and SCID mice (Tables 3 and 4).

1-4b: PFGE analysis of mini- and intermediate size chromosomes of trypanosomes

Chromosomes of *T. brucei* can be divided into 3 size classes, the mini-chromosomes (< 150 kb) and 2 larger groups of 150 kb – 1,000 kb (intermediate size chromosomes) and > 1,000 kb (large size chromosomes) (35, 65, 138, 139). There are approximately 100 mini-chromosomes in the single parasite (146). The mini-chromosomes are considered to be reservoirs of non-expressed VSG genes (147). It was reported that the number of mini-chromosomes and the size of the VSG gene repertoire of *T. b. gambiense* were smaller than those of *T. b. brucei* (28). As a result of PFGE analysis, 4 strains of *T. b. gambiense* (IL1922, IL3707, IL3250 and IL3301) were distinguished from the other species of *Trypanosoma* (*T. b. rhodesiense*, *T. evansi* and *T. congolense*) by their faint bands of mini-chromosomes (Fig. 7). These findings extend the previous studies described above, and may also suggest that the number of mini-chromosomes and the VSG gene repertoire of *T. b. gambiense* were smaller than the other 3 species of trypanosomes. Since all the *T. b. gambiense* strains tested, including high virulent strains (IL1922 and IL3707), appeared to have the small number of mini-chromosomes, the size of VSG repertoire may have no relationship with the virulence of the parasite.

1-4c: *In vitro* cultivation of BSFs of a low virulent strain (*T. b. gambiense* IL3253)

The first *in vitro* cultivation of *T. brucei* BSFs was reported by Hirumi et al. (1977) (46). In this system, BSFs of *T. b. brucei* 427 strain were successfully cultivated by using a modified RPMI-1640 medium supplemented with FBS and bovine fibroblast-like cells.

To establish in vitro cultivation of a low virulent strain, *T. b. gambiense* IL3253, firstly the modified RPMI-1640 supplemented with FBS and 2 other basal media were examined for their ability to support the growth of the IL3253 BSFs (Table 5). As a result, the modified RPMI-1640 was only support growth of the IL3253 BSFs, although the growth of the BSFs was inferior to that of a high virulent *T. b. rhodesiense* IL2343 (data not shown). Therefore, it was concluded that the modified RPMI-1640 was suitable medium for the IL3253 BSFs in the feeder cell layer system.

Secondly, various serum (or plasma) supplements were examined in order to obtain the best growth of the IL3253 BSFs in the feeder cell layer system. Since human is a natural host of the *T. b. gambiense*, the author inferred that the IL3253 BSFs would show the best growth in the medium supplemented with HuP or HuS. In the feeder cell layer system, BSFs grow in intercellular spaces of the feeder cells without cytopathic effects (46). RPMI-1640 supplemented with HuP supported the growth of the IL3253 BSFs and the BSFs appeared in almost every part of the intercellular spaces of the feeder cells (Fig. 8a). While, the growth supporting effects of FBS, HoS and HuS were inferior to that of HuP and intercellular accumulations of the BSFs were sporadically observed (Fig. 8b). Therefore, the RPMI-1640 containing 20% HuP, 0.75 mM L-cysteine hydrochloride, 0.10 mM bathocuproine disulfonic acid disodium salt, 1.00 mM hypoxanthine and 2.00 mM L-glutamine is the most suitable medium for the *in vitro* cultivation of IL3253 BSFs in the feeder cell layer system.

Axenic cultivation of *T. brucei* BSFs was reported by Hirumi and Hirumi (1989) (47). In this system, 2 strains of *T. brucei* (GUTat3.1 and IL2343), which were highly virulent to BALB/c mice, were continuously cultured without feeder cell layers by using HMI-9 medium. The HMI-9 medium was a modified IMDM supplemented with 20% FBS, BSF supporting factors and L-glutamine. The basal IMDM (Flow Lab., U.K.), which contained bovine serum albumin, L-α-phosphatidylcholine and bovine holo-transferrin, was used in the Hirumi's system but has become commercially unavailable. Therefore, 4 kinds of basal media were tested for their suitability to the axenic cultivation of *T. brucei* BSFs by using *T. b. rhodesiense* IL2343, of which the axenic cultivation was already established by Hirumi and Hirumi (1989) (47), and *T. b. gambiense* IL3253. Although the FBS-containing medium supported the growth of IL3253 BSFs in the feeder cell layer system, all the media tested only supported the growth of IL2343 BSFs in the axenic culture system. Therefore, HuP, HuS and HoS were examined for their growth-promoting effects on the IL3253 BSFs. As the author expected, HMI-9 medium supplemented with HuP was the most suitable medium for the axenic cultivation of the IL3253 BSFs. The HMI-9 medium containing HuS or HoS also supported the moderate growth of the BSFs. According to the PDT, S-Clone SF-B, which is a serum-free medium for mouse hybridoma cells, was suitable basal medium for a high virulent IL2343 BSFs but not for a low virulent IL3253 BSFs (Table 9).

Interestingly, although both the IL2343 and IL3253 were isolated form human, IL2343 BSFs did not grow in the medium supplemented with HuP or HuS and was rapidly lysed. The cytolytic activities of normal HuP and HuS are due to haptoglobin-related proteins, named trypanosome lytic factor (41) and the host ranges of *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense* depend on the sensitivity of the parasite to lysis by the

factor (118). It was reported that the resistance to normal HuP and HuS is an unstable phenotype in *T. b. rhodesiense* (72, 117). Thus the IL2343 is *T. b. rhodesiense* which is infectious to human, and may lose resistance to HuS and HuP during syringe passages in experimental animals.

1-5: Summary

T. b. gambiense is divided into Type I (or true-gambiense) and Type II (or non-gambiense) by its virulence to rodents, repetitive DNA sequences of ribosomal DNA, isoenzyme electrophoresis patterns, PCR of VSG genes and RFLPs (18, 40, 43, 73, 107). Type I *T. b. gambiense* is characterized by its low virulence to rodents. Therefore, it is difficult to prepare sufficient amounts of the parasites in order to analyze biological features of the parasites. Thus, in this chapter, *in vivo* and *in vitro* culture methods which support the growth of BSFs of such "low virulent" trypanosomes were developed.

Firstly, the susceptibility of SCID mice to various strains of trypanosomes was examined in terms of their infectivity, course of parasitemia, PCV and survival rate (or period) in comparison with those of BALB/c mice. All strains of trypanosomes tested, including a low virulent *T. b. gambiense*, caused high parasitemia (> 1×10^8 BSFs/ml) in SCID mice, the survival periods ranged from 5 to 47 d. Therefore, it is concluded that SCID mouse is a useful tool for preparation of a large number of BSF regardless of its virulence for mice.

Although many live parasites (> 1×10^7 BSFs) have to be prepared, PFGE is one of the useful tools for epidemiological study of trypanosomes (65). Secondly, mini-chromosomes of the various strains of trypanosomes were examined by means of the PFGE. Surprisingly, all strains tested had a unique electrophoresis pattern and there were no relationships among the PFGE patterns, species and virulence of the parasite, and the place of isolation.

Thirdly, *in vitro* cultivation of a low virulent strain, *T. b. gambiense* IL3253, was established by using long slender BSFs of the IL3253 obtained from infected SCID mice that were showing a ascending parasitemia. In the feeder cell layer system, the growth of

IL3253 BSFs was fully supported by using RPMI-1640 medium supplemented with 20% HuP, 0.75 mM L-cysteine hydrochloride, 0.10 mM bathocuproine disulfonic acid disodium salt, 1.00 mM hypoxanthine and 2.00 mM L-glutamine, and embryonic fibroblast-like cells of *Microtus montanus*. Moreover, an axenic culture system for the IL3253 BSFs was established using HMI-9 medium, originally reported by Hirumi and Hirumi (1989) (47), supplemented with 20% HuP was the best medium for the axenic cultivation of the IL3253 BSFs. In this system, the SPDT of the BSFs was 8.7 h and the HPD was 1.7×10^6 BSFs/ml.

In conclusion, both *in vivo* and *in vitro* systems which support the growth of the low virulent strain (IL3253) BSFs were established. The results clearly indicate that the SCID mouse is highly susceptible for "low virulence" *Trypanosoma* strains. This makes SCID mouse as a useful tool for detection of the parasites from animals suspected of having trypanosomes, isolation of the parasites, and the propagation of large amounts of such parasites. Furthermore, the results demonstrate that the *in vitro* culture systems, specially the axenic culture system, would be useful tool for studying growth-promoting factors, drug-sensitivity test, mode of trypanocidal activity, gene-selection/cloning and cell division cycle of a low virulent trypanosome which has been difficult to examine earlier.

CHAPTER 2:

Kinetoplast DNA and procyclic acidic repetitive protein A-α (PARP A-α) gene of *Trypanosoma evansi* and *T. brucei*

2-1: Introduction

T. evansi is a causative agent of Surra which is a wasting disease of domestic animals in South America, Northern Africa, Middle East and Asia. The parasite is transmitted mechanically by biting flies, such as Tabanidae and *Stomoxys*, and vampire, and does not have any developmental stages in its insect vectors, and its bloodstream forms (BSFs) are morphologically indistinguishable from those of *T. brucei.* Close relationship between *T. evansi* and *T. brucei* was further documented by Gibson et al. (1983) (37), Masiga and Gibson (1990) (71) and Stevens et al. (1992) (136), suggesting that *T. evansi* has been derived from *T. brucei* .

On the other hand, the difference between *T. evansi* and *T. brucei*, which lies in kinetoplast DNA (kDNA), has been also well documented. The kDNA of *T. brucei* consists of 50-100 homogeneous maxicircles and 5,000-10,000 heterogeneous minicircles (16). The maxicircle DNA resembles a conventional mitochondrial DNA in which it encodes ribosomal RNA and a small number of proteins. While, the minicircle DNA encodes guide RNA which plays an important role for post-transcriptional editing of maxicircle DNA transcripts (12, 13, 148). In contrast, *T. evansi* lacks maxicircle DNA (16), and its minicircle DNAs are highly homogeneous and possess the same or nearly the same DNA sequences (104, 133). It was further reported that several features of the sequence of minicircle DNAs were specific for *T. evansi*, and that such sequences would be of useful in distinguishing *T. evansi* from *T. brucei* by means of DNA hybridization

technique and polymerase chain reaction (PCR) (2, 71). However, it is necessary to consider wide prevalence of akinetoplastic strains of *T. evansi* when such a *T. evansi* specific minicircle DNA sequence is used as a diagnostic tool. Moreover, if origin(s) of *T. evansi* was *T. brucei* spp., it would be possible that certain strains of *T. brucei* might have the *T. evansi* specific minicircle DNA sequence in their heterogeneous minicircle DNA. In this study, the author examined such a possibility in various strains of *T. brucei*, including *T. b. brucei*, *T. b. gambiense* and *T. b. rhodesiense*.

PARP is a major surface glycoprotein of procyclic and epimastigote form of *T. brucei* (83, 115). Although the function of the PARP is not fully understood, it may play a role in the protection of the parasite against proteases present in the midgut of tsetse vectors (*Glossina* spp.) (121). The PARP expression in BSFs is down-regulated relative to procyclic forms at several levels (51). However, a relatively high level of transcription occurs from the PARP promoter in the BSFs (109, 149, 150). In consideration with life cycle of *T. evansi*, the PARP seems to be unnecessary in *T. evansi*. However, interestingly, the PARP A-α gene was found by PCR-based analyses of *T. evansi* (2). In this study, potential use of the PARP primers as a diagnostic tool for *T. evansi* was explored, specially its application outside of the tsetse belt where both of the kinetoplastic and akinetoplastic strains of *T. evansi* are known to be widely prevalent but not *T. brucei*. Moreover, further characterization of the PARP A-α gene of *T. evansi* was made regarding its gene expression in *T. evansi*.

2-2: Materials and methods

2-2a: Trypanosomes

Histories of the strains of *T. evansi* and those of *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense* and *T. congolense* are listed in Table 2. The BSFs of trypanosomes were propagated in normal, semi-lethally irradiated (600 rad) or sever combined immunodeficient (SCID) mice (Chapter 1), and separated from the blood cells by means of DE-52 cellulose column chromatography (57). The procyclic forms of *T. b. rhodesiense* IL2343 were produced *in vitro* following the procedure by Hirumi and Hirumi (1991) (48).

2-2b: Extraction of DNA

kDNA was extracted from the trypanosomes as described by Waitumbi and Young (1994) (158) with minor modifications. Briefly, isolated trypanosomes ($>1\times10^7$) were incubated in lysing buffer (1% SDS, 100 μg/ml proteinase K, 0.2 M NaCl, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0) at 50˚C for 24 h. The genomic DNA was sheared by repeatedly passing the lysate through a 27G needle, and kDNA was pelleted by centrifugation at 12,000*g* at 20˚C for 30 min. kDNA networks were washed 3 times in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The purified kDNA networks were stored in TE buffer at 4˚C up to use. Genomic DNA was extracted as described elsewhere (54).

2-2c: DNA cloning and sequencing

The kDNA networks of *T. evansi* was digested with *Taq* I and the fragment was separated by means of high performance liquid chromatography (TOSO, Japan). The fragment ends were converted to blunt by using Klenow fragment. Then the fragments

were ligated into the unique *Sma* I site of pUC 19 plasmids (54). The cloned fragments of kDNA were sequenced by means of dye primer cycle sequencing method (The Perkin-Elmer Co., U.S.A.). The PCR products from genomic DNA of trypanosomes (described below) were sequenced directly.

2-2d: PCR

PCR amplification of minicircle DNA and the PARP A-α gene was performed as described before (2). For the PCR amplification of minicircle DNA, the PARP A-α gene and PARP core promoter, the following oligonucleotide primer sets were used. Minicircle primers: 5'-CAA CGA CAA AGA GTC AGT-3' and 5'-ACG TGT TTT GTG TAT GGT-3', PARP A-α gene primers: 5'-CAC AAT GGC ACC TCG TTC CC-3' and 5'-TTA GAA TGC GGC AAC GAG A-3', PARP core promoter primers: 5'-GCA GTC AGC CTT TGT TGT CA-3' and 5'-TTA GAA TGC GGC AAC GAG A-3'. The amplifications were conducted for 30 cycles in a DNA Thermal Cycler (The Perkin-Elmer Co., U.S.A.) in which each cycle carried out by successive incubations of 94˚C for 30 s, 60˚C for 30 s, and 72˚C for 30 s.

2-2e: Reverse transcription PCR (RT-PCR)

Total RNA was extracted by an acid guanidium thiocyanate-phenol-chloroform method (21). To delete contamination of genomic DNA, 10 μg of the total RNA was treated with 200 units of deoxyribonuclease I (Takara Shuzo Co. Ltd., Japan). Oligo-dT primed cDNA was synthesized by using kit (Pharmacia Biotech Ltd., U.S.A.). PCR amplifications of cDNA were conducted in a 50 μl reaction mixture containing 1 μl cDNA reaction mixture, 0.5 μM each of primers, 2.5 units of *Taq* DNA polymerase (The Perkin-

Elmer Co., U.S.A.), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, and 200 μM each of dATP, dGTP, dCTP and dTTP. The oligonucleotide primers and the temperature conditions for the amplification were the same as described above.

2-3: Results

2-3a: Cloning and sequencing of minicircle DNA

Firstly, the determination of the complete sequences of minicircle DNA of all the *T. evansi* strains was made, except for akinetoplastic *T. evansi* IL1934. All minicircle DNA sequences were aligned from the GGGGTTGGTGTA sequence which is the putative replication origin of minicircle DNA (Fig. 12) (14, 97). Minicircle DNA sequence was highly conserved among *T. evansi* strains which isolated in different countries or places. Attempts to isolate the kDNA networks of the akinetoplastic *T. evansi* IL1934 were made several times but were vain as expected. Sequence homology was calculated among newly determined 6 minicircle DNA sequences and 4 previously reported complete minicircle DNA sequences of *T. evansi* (Table 10) (104). The homology was more than 96% among all the samples tested.

Secondly, possible existence of the "*T. evansi* specific" minicircle DNA in the kDNA networks of various strains of *T. brucei* was examined. Total DNA and/or kDNA were prepared from 7 strains of *T. evansi* (IL1695, IL1934, IL3354, IL3382, IL3960, IL3962 and Tansui), 2 strains of *T. b. brucei* (GUTat3.1 and 221), 8 strains of *T. b. gambiense* (IL1922, IL3248, IL3250, IL3253, IL3254, IL3301, IL3707 and Welcome), 2 strains of *T. b. rhodesiense* (IL1501 and IL2343) and *T. congolense* IL3000. Minicircle DNA was then amplified by means of "*T. evansi* minicircle DNA specific PCR" (2). Positive reaction was obtained from total DNA and/or purified kDNA of 6 strains of *T. evansi* (IL1695, IL3354, IL3382, IL3960, IL3962 and Tansui), *T. b. brucei* GUTat3.1, *T. b. gambiense* Welcome and 2 strains of *T. b. rhodesiense* (IL1501 and IL2343). Nucleotide sequence of these PCR products was determined and the sequence homology was calculated among them (Table 11). The homology was more than 93% among *T. evansi*

strains, *T. b. gambiense* Welcome and *T. b. rhodesiense* IL1501, while, the homology was less than 62% among *T. evansi* strains, *T. b. brucei* GUTat3.1 and *T. b. rhodesiense* IL2343.

```
IL1695 GGGGTTGGTG TAATACACAC ACGGTTTTTC TCAGGGTTTT GAGAAAATTC GCAGTTTTCC TGGGGTTCTC AGTACAGTTA ATTTGGATTT  90
IL3354 ---------- ---------- ---------- ---------- ----C----- ---------- ---------- ---------- ----------  90
IL3382 ---------- ---------- ---T------ ---------- ----C----- ---------- ---------- ---------- ----------  90
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------  90
IL3962 ---------- ---------- ---------- ---------- ----C----- ---------- --A------- ---------- ----------  90
Tansui ---------- ---------- ---------- ---------- ---GC----- A--------- ---------- ---G------ ----------  90

IL1695 AATTGATTTC CTATAGAGAA AAATAGAATA ATAGATAAGT AATCATGAAT ATAGATATAT ATAATTGTAC ATATACCAAC AAACAGAATA 180
IL3354 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 180
IL3382 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 180
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 180
IL3962 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 180
Tansui ---------- ---G------ ---------- ---------- ---------- ---------- ---------- ---------- ---------- 180

IL1695 ACTAATGCAC AGTGATGATA ATAGTTAATT AATTATATAT AAAGTTGTAA TCTATCTATT ATTATATTTA GTTGAGTGAC GTGAGAATAA 270
IL3354 ---------- ---------G ---------- ---------- ---------- ---------- ---------- --------G- ---------- 270
IL3382 ---------- ---------G ---------- ---------- ---------- ---------- ---------- --------G- ---------- 270
IL3960 ------A--- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 270
IL3962 ---------- ---------G ---------- ---------- ---------- ---------- ---------- ---------G- ---------- 270
Tansui ---------- ---------G ---------- ---------- ---------- ---------- ---------- A--------- -----G---- 270

IL1695 GGTGATATTT CAATCCTGAA CAAAAGAAAT GGTGTAATAG ATAGAAGATA ATGAGAAGTT AATTATAAAT ATATCATACA AAATAACAAT 360
IL3354 ---------- ---------- ---------- -A-------- ---------- --A------- ---------- ---------- ---------- 360
IL3382 ---------- ---------- ---------- -A-------- ---------- ---------- ---------- ---------- ---------- 360
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 360
IL3962 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 360
Tansui ---------* -------A-- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 359

IL1695 GATCACAGAT AAGAGTGAAT ATAGATAGAG AATTTAATTA TTATTATTGT GTATATTGAA TTACATATTT ATTATTTTAT TTTAGTATAT 450
IL3354 ---------- ---------- ---------- ---------- ---------- -A-------- ---------- ---------- ---------- 450
IL3382 ---------- ---------- ---------- ---------- ---------- -A-------- ---------- ---------- ---------- 450
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 450
IL3962 ---------- ---------- ---------- ---------- ---------- -A-------- ---------- ---------- ---------- 450
Tansui ---------- ---------- ---------- ---------- ---------- ---------- ---------- --*------- ---------- 448

IL1695 AGGATGCAGA AATTAGCAGT ATAAAATAAG GATAAAAGAG TTTATAGGTG AAGTTGAAGT GAATCAGCGT CTTTTGAGGG AAGTAAAGTA 540
IL3354 ----C----- --A------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 540
IL3382 ----C----- --A------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 540
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 540
IL3962 ----C----- --A------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 540
Tansui ---------- --*--A---- ---------- ---------- ---------- ---------- -------T-- ---------- ---------- 537

IL1695 ATATAATAGA TAGAAACATA ATAATAATTT AATTTGATAG TATATACATA TCAACAACGA CAAAGAGTCA GTGAAATTAG AGATAAAGTT 630
IL3354 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 630
IL3382 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 630
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 630
IL3962 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 630
Tansui ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---G------ ---------- 627

IL1695 ATTGTAGTTA TATAATTAAA TTAATGT*AT CTA**TTATT TATTTGTTTT ATACGAGGAG AGGGAATAAG AGGGAAAATT CATTGGAGAT 717
IL3354 ---------- ---------- ----G--*-- ---**----- ---------- ---------- ---------- ---------- ---------- 717
IL3382 ---------- ---------- -------*-- ---**----- ---------- ---------- ---------- ---------- ---------- 717
IL3960 ---------- ---------- -------*-- ---**----- ---------- ---------- ---------- ---------- ---------- 717
IL3962 ---------- ---------- -------C-- --CAT-C--- ---------- ---------- ---------- ---------- ---------- 720
Tansui ---------- ---------- -------*-- ---**----- ---------- ---------- -----*---- ---------- ---------- 713

IL1695 ACTAGGGTGA GAGAGTTAAT AGAGTAATTG TAGTCGGGAG TATGGAGTAG TTATAATTAT ATTGGCGAAA AGGAAAGAGC TAAAAAGTCG 807
IL3354 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 807
IL3382 ---------- ---------- ---------- ---------- ----A----- ---------- ---------- --A------- ---------A 807
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 807
IL3962 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 810
Tansui ---------- ---------- ---------- ----T---G- ---------- ---------- -----T---- --------G-G ---------T- 803

IL1695 TGTAGTAGAA TAGAGGTTGA TAGGAATAAG GGATGGAATT TGTAGAAGTA GTTGGTAAAA ATCTATAGAA ATCGTTAAAA TTGGCTAAAA 897
IL3354 ---------- ---------- ---------- A--------- ---------- ---------- ---------- ---------- ---------- 897
IL3382 ---------- ---------- ---------- A--------- ---------- ---------- ---------- ---------- ---------- 897
IL3960 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 897
IL3962 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- 900
Tansui ---------- ---------- ---------- T-G------- ---------- ---------- ---------- ---------- ---------- 893

IL1695 ATCGGGCTAA AAAAACGGAA AATCTTATGG GCGTGCAAAT TTCACCATAC ACAAAACACG TGCTATTTTC *GGGGGTTTT TTAGGTCCGA 986
IL3354 ---------G- ---------- -----C---- --------G-- ---------- ---------- ---------- *---------- ---------- 986
IL3382 ---------G- ---------- -----C---- ---------- ---------- ---------- ---------- *---------- ---------- 986
IL3960 ---------G- ---------- ---------- ---------- ---------- ---------- ---------- *---------- ---------- 986
IL3962 ---------G- ---------- -----C---- --------G-- ---------- ---------- ---------- *---------- ---------- 989
Tansui ---------G- ---------- ---------- ---------- ---------- ---------- ---------- C---------- ---------- 983

IL1695 GGTACTTCGA GA  998
IL3354 ---------- --  998
IL3382 ---------- --  998
IL3960 ---------- --  998
IL3962 ---------- -- 1001
Tansui ---------- A-  995
```

Figure 12. Alignments of the entire sequences of the minicircle DNA from *T. evansi* strains IL1695, IL3354, IL3382, IL3960, IL3962 and Tansui

Nucleotides identical to those in IL1695 sequence are indicated by dashes (-).

Deletions are marked by asterisks (*).

Table 10. Pairwise analysis of sequence homology (%) among the minicircle DNAs from *T. evansi* strains

	IL1695	IL3354	IL3382	IL3960	IL3962	Tansui	MA1*	MA2*	ET*	SH*
IL1695		98	98	99	98	97	97	97	98	97
IL3354			99	98	99	96	96	96	98	96
IL3382				98	98	96	96	96	98	97
IL3960					98	97	97	97	98	97
IL3962						96	96	96	97	96
Tansui							97	96	97	97
MA1*								99	98	98
MA2*									98	98
ET*										97

*Ou et al. 1991 (104).

Table 11. Pairwise analysis of sequence homology (%) among the minicircle DNA fragments from *T. evansi* and *T. brucei*

	GUTat 3.1	Welcome	IL1501	IL2343
IL1695	61	99	94	24
IL3354	61	98	95	24
IL3382	61	98	93	24
IL3960	61	100	95	24
IL3962	61	99	95	24
Tansui	62	97	94	24
GUTat 3.1		37	37	12
Welcome			95	24
IL1501				24

2-3b: Sequence analyses of the PARP A-α gene

The PARP A-α gene was amplified by PCR from genomic DNA of 5 strains of *T. evansi* (IL1695, IL1934, IL3354, IL3382 and IL3960), *T. b. brucei* 221, *T. b. gambiense* Welcome and *T. b. rhodesiense* IL2343. The determinations of the DNA sequence of all the PCR products were then made in order to compare the PARP A-α gene of *T. evansi* with that of *T. brucei*. Since PCR was targeted to the open reading frame of the PARP A-α gene, nucleotide sequence had been translated to amino acid sequence. Then, the amino acid sequences of the 5 strains of *T. evansi*, *T. b. brucei* 221, *T. b. gambiense* Welcome and *T. b. rhodesiense* IL2343 were aligned (Fig. 13). Except for the number of GPEET repeat, the amino acid sequence was nearly identical among *T. evansi* and *T. brucei* strains tested.

A PARP promoter is one of the well known promoter in *T. brucei* (19, 130). Oligonucleotide primer pair was designed to amplify from the PARP core promoter region to the end of the open reading frame of the PARP A-α gene. The PCR products were cloned and sequenced. The PARP core promoter sequences of *T. evansi* IL1695 and *T. b. rhodesiense* IL2343 were the same (Fig. 14).

```
427[1)]     MAPRS LYLLA ILLFS ANLFA GVGFA AAADE SASNV IVKGG KGKER EDGPE  50
221         ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50
Welcome     ----- ----- ----- ----- ----- -T--- ----- ----- ----- -----  50
IL2343      ----- ----- V---- ----- ----- ----- ----- ----- ----- -----  50
IL1695      ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50
IL1934      ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50
IL3354      ----- ----- V---- ----- ----- ----- ----- ----- ----- -----  50
IL3382      ----- ----- V---- ----- ----- ----- ----- ----- ----- -----  50
IL3960      ----- ----- ----- ----- ----- ----- ----- ----- ----- -----  50

427[1)]     EPEET GPEET GPEET GPEET GPEET GPEET GPEET EPEPE PGAAT LKSVA 100
221         ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- 100
Welcome     ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
IL2343      ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
IL1695      ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
IL1934      ----- ----- ----- ----- ----- ***** ***** ----- ----- -----  90
IL3354      ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- 100
IL3382      ----- ----- ----- ----- ----- ----- ----- ----- ----- ----- 100
IL3960      ----- ----- ----- ----- ----- ----- ***** ----- ----- -----  95
```

Figure 13. Alignments of the predictive amino acid sequences of the PARP A-α (M_1-A_{100}) from *T. evansi* (IL1695, IL1934, IL3354, IL3382 and IL3960) and *T. brucei* (427, 221, Welcome and IL2343) strains

Amino acids identical to those in *T. brucei* 427 sequence are indicated by dashes (-).

Deletions are marked by asterisks (*).

[1)]Mowatt et al. (1989) (84)

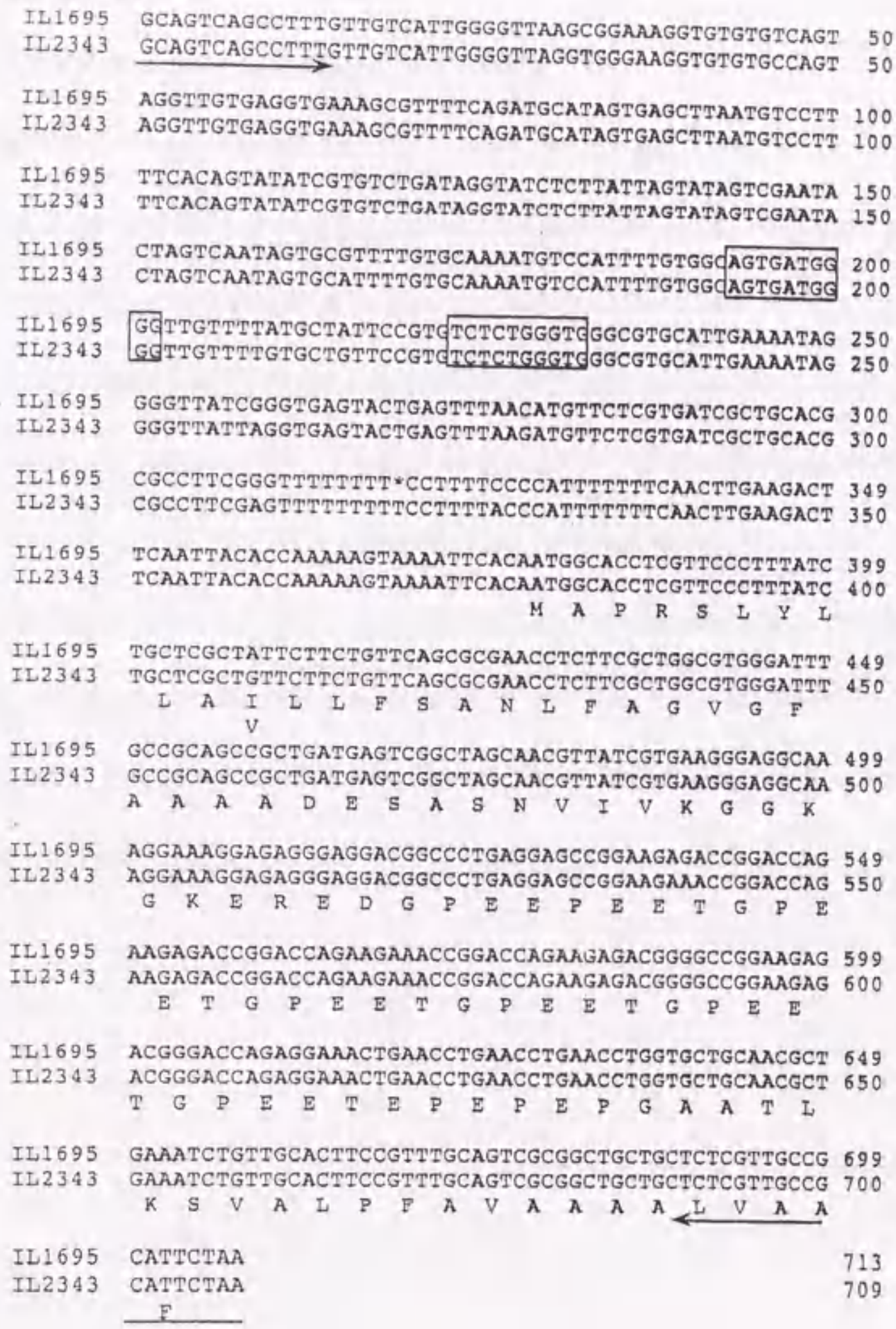


Figure 14. Comparison of the nucleotide sequences of the PARP core promoter (boxed sequences) from *T. evansi* IL1695 and *T. b. rhodesiense* IL2343

Arrows show the sequences of sense and anti-sense primers for PCR amplification.

Deletion is marked by asterisks (*).

The predictive amino acid sequences shown under the nucleotide sequences are the entire sequence of the PARP A-α gene.

2-3c: The PARP A-α gene expression in *T. evansi*

Total RNA was obtained from procyclic forms and BSFs of *T. b. rhodesiense* IL2343, and *T. evansi* IL1695. The oligo-dT primed cDNA was employed as a template for PCR. Regions of the PARP A-α gene primers and the PARP core promoter primers are illustrated in Figure 15. Since one of the PARP core promoter primer region was out of the transcription initiation site, this primer pair helped to discriminate between positive reaction caused by genomic DNA contamination and real positive reaction. The results are shown in Figure 16. RT-PCR by using the PARP A-α gene primer was positive in all the cDNA samples, and the same amplification patterns were observed in the genomic DNA samples. Expectedly, there were no amplification in the cDNA samples with the PARP core promoter primer.

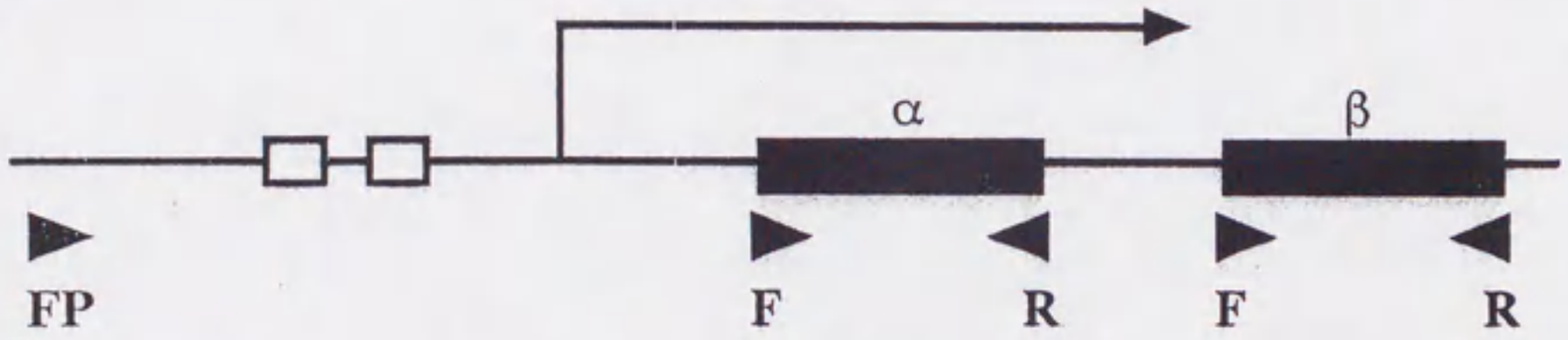


Figure 15. Map of the PARP A gene showing the positions of the binding sites (arrow head) for the PARP A-α gene primers (F and R) and the PARP core promoter primers (FP and R)

Transcription is schematically represented by arrowed line.

Closed boxes indicate open reading frames of the PARP A-α (α) and the PARP A-β (β).

Open boxes indicate the PARP core promoter region.

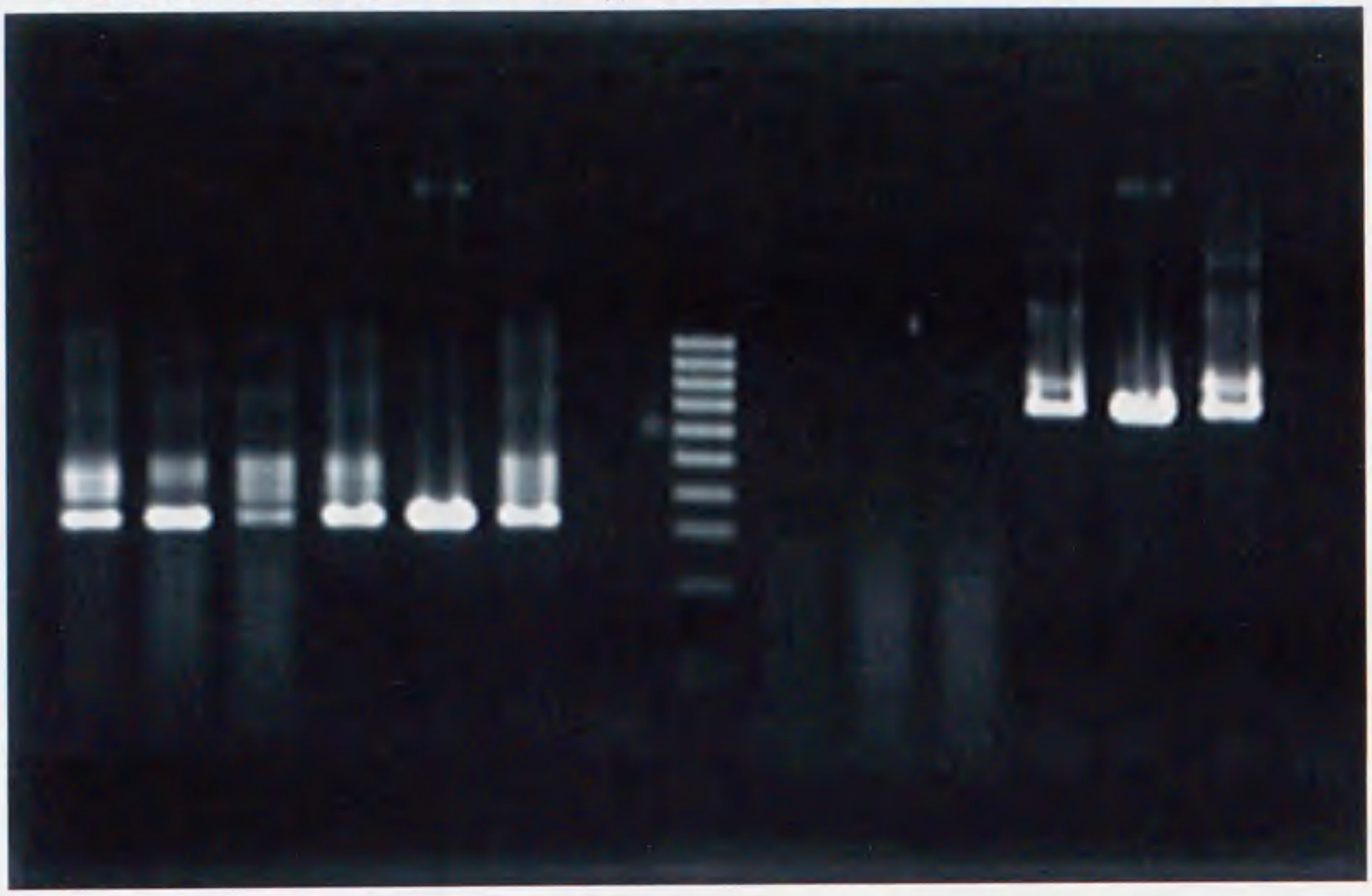


Figure 16. RT-PCR amplification patterns of primer pairs specific for the PARP A-α gene (PARP) and PARP core promoter (Promoter) of *T. brucei*, and template cDNAs of *T. b. rhodesiense* IL2343 procyclic forms (TbP), IL2343 BSFs (TbB) and *T. evansi* IL1695 (Te)

Lane TbG, P and TeG are the IL2343 genomic DNA, the PARP A-α gene cloned into pUC19 plasmid and *T. evansi* IL1695 genomic DNA respectively.

Lane (-) is negative control without template DNA, and lane M is 100 bp ladder.

2-3d: Evaluation of the specificity of the PARP A-α gene primers

To assess a potential use of the PARP A-α gene primer as a diagnostic tool for *T. evansi* and *T. brucei* infections, PCR was performed by using various species of protozoan parasites including *Trypanosoma*, *Babesia*, *Neospora* and *Toxoplasma*. Genomic DNA was extracted form 7 strains of *T. evansi* (IL1695, IL1934, IL3354, IL3382, IL3960, IL3962 and Tansui), 10 strains of *T. brucei* (IL1922, IL3248, IL3250, IL3253, IL3254, IL3301, IL3707, Welcome, IL1501 and GUTat3.1), *T. congolense* IL3000, *Babesia equi* USDA strain, and *B. caballi* USDA strain, *Neospora caninum* NC-1 strain and *Toxoplasma gondii* RH strain, and used as templates for the PCR. As a result, the same amplification patterns were obtained from all *T. brucei* and *T. evansi* strains tested, while the PCR was negative in the other species tested (Fig. 17).

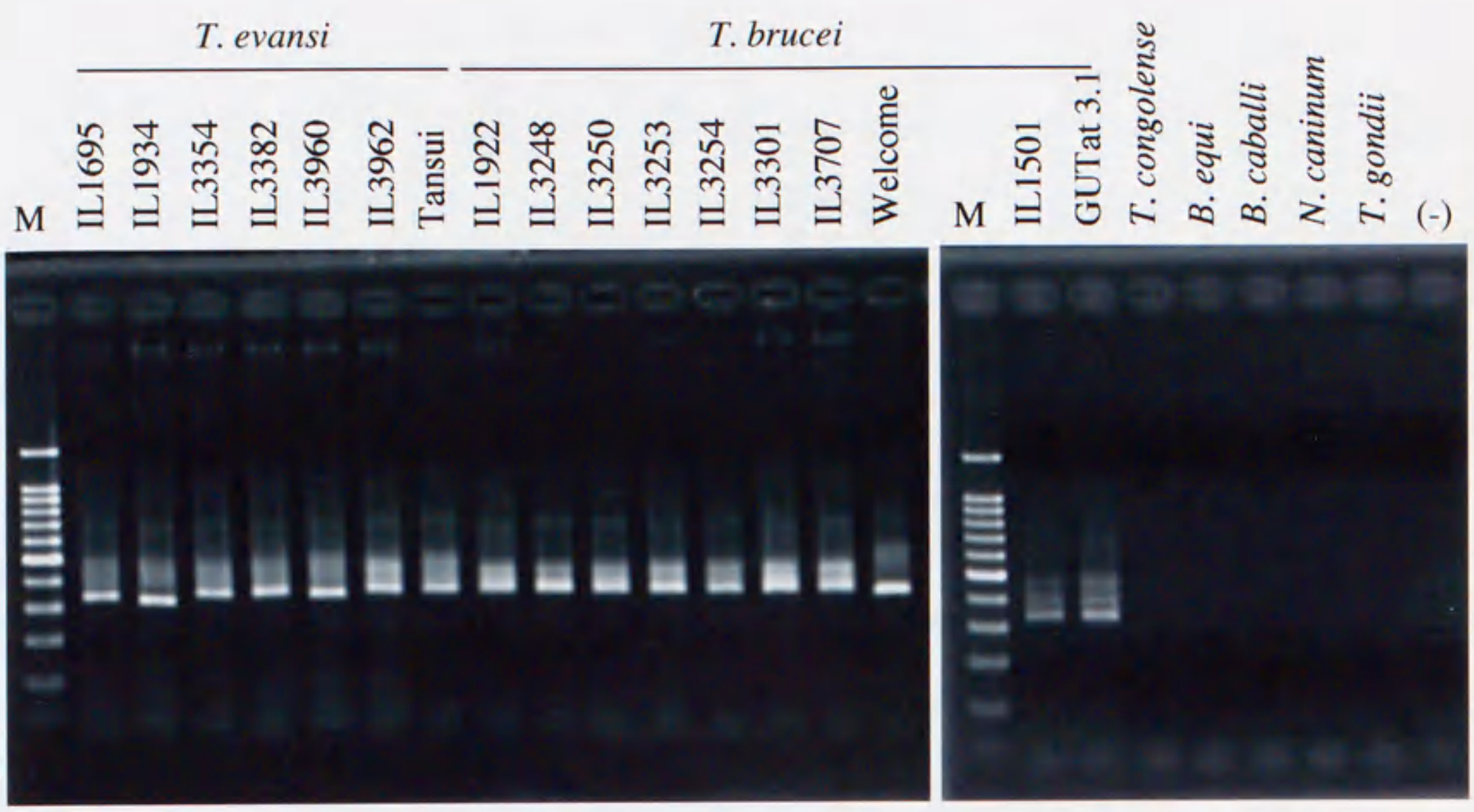


Figure 17. PCR amplification patterns of primer pairs specific for the PARP A-α gene among several species of protozoan parasites

Lane M is 100 bp ladder, and lane (-) is negative control without template DNA.

2-4: Discussion

In the previous studies, the kDNA minicircle of *T. evansi* was characterized by restriction enzyme digestion patterns, and partial and total sequencings (15, 104, 133). According to the analyses of restriction enzyme digestion patterns of kDNA minicircles (15, 133), *T. evansi* kDNA minicircles have been classified into 4 closely related types (A1, A2, A3 and A4) and one different type (B1). In this study, the author has determined total sequences of kDNA minicircles of 6 *T. evansi* strains isolated from several countries or places of Africa, and Taiwan (Fig. 12). It was reported that the kDNA minicircle of *T. evansi* IL1695 belonged to the type A1 minicircle (15). The results of the total sequence obtained in this study showed that the five strains belonged to the type A1 or A2 minicircle (A1: IL1695, IL3382, IL3960 and IL3962, A2: IL3354), and to one new related type (Tansui). Total sequences of kDNA minicircle of *T. evansi* strains (NJ and SH: China, MA: The Philippines, ET: Ethiopia) were also reported by Ou et al. (1991) (104). The author calculated the sequence homology among 10 *T. evansi* strains including 4 previously reported sequences (Table. 10). The results clearly indicated that the kDNA minicircle of *T. evansi* is highly conserved among the strains isolated from several countries or places of Africa and Asia.

It has been suggested that *T. evansi* was "recently" evolved from *T. brucei*. Moreover, it was reported that putative guide RNA sequences were coded in minicircle DNA of *T. evansi* (34). Therefore, the author inferred that *T. evansi* type kDNA minicircle might exist in several strains of *T. brucei*. To elucidate this speculation, total DNA and/or purified kDNA networks from various strains of *T. brucei* and a strain of *T. congolense* were examined by means of "*T. evansi* minicircle specific PCR" (2). The result revealed that *T. b. gambiense* Welcome strain and *T. b. rhodesiense* IL1501 also

possess *T. evansi* type minicircle DNA (Table 11). This suggests that although the minicircle DNA of *T. evansi* might be not functional, it has a potential for a source of guide RNA which might be unnecessary in *T. evansi*, and that the two *T. brucei* strains seem to be closely related to *T. evansi* in terms of minicircle DNA.

Comparative study of the PARP A-α gene sequences among 4 strains of *T. brucei* and 5 strains of *T. evansi* revealed that the PARP A-α gene of *T. evansi* is highly conserved. A predictive amino acid sequence of the PARP A-α of *T. evansi* and *T. brucei* was nearly the same (Fig. 13). The author also examined nucleotide sequences of a PARP core promoter region of *T. evansi* IL1695. Interestingly, the PARP core promoter sequence of *T. evansi* IL1695 and *T. b. rhodesiense* IL2343 was the identical (Fig. 14). The PARP expression in BSFs is down-regulated relative to procyclic forms. However, it has been demonstrated that the PARP promoter is active in BSFs (109, 149, 150). Therefore, the author hypothesized that the PARP A-α gene was expressed in *T. evansi*. The RT-PCR amplification patterns clearly indicated that the PARP A-α gene expresses in the BSF not only of *T. brucei* but also of *T. evansi* (Fig. 16). The several bands in each lane are caused by different PARP genes. The PARP expression has been shown to be up-regulated by differentiation triggering factors such as low temperature and mild acid stress (109, 122). Since *T. evansi* survives for a few days in its insect vector (161), the PARP expression may be up-regulated during this period, although due to the absence of maxicircle DNA and the loss of minicircle DNA heterogeneity, *T. evansi* may not be able to differentiate into the insect form.

In conclusion, this study also strongly indicated that *T. evansi* was "recently" evolved from *T. brucei* on the bases of the finding of the *T. brucei* strains which have "*T. evansi* specific" minicircle DNA sequences in their kDNA networks, and a further evidence

that the PARP A-α gene is not only conserved but also expressed in *T. evansi*, in addition to the previous findings (37, 71, 136). Until now, it is not clear if the impairment of kDNA is the only reason why *T. evansi* does not have developmental stages in its insect vector. Comparative study of responses of *T. brucei* and *T. evansi* to differentiation triggering factors may clarify the molecular mechanisms that underlie the differentiation during the life cycle of *T. brucei*.

Although "*T. evansi* specific" minicircle DNA primer is a useful tool for diagnosis of *T. evansi* (2), it may overlook akinetoplastic strains in where such strains, such as *T. evansi* IL1934, are prevalent. The finding of the "*T. evansi* type" minicircle DNA sequence in 2 strains of *T. brucei* also suggests need of precaution in diagnosing *T. evansi* infection particularly in areas where a critical distinction of *T. evansi* and *T. brucei* infections is required.

Although the PCR detection using the PARP A-α gene primers is also unable to distinct *T. evansi* infection from that of *T. brucei*, it detects the infection with akinetoplastic strains of *T. evansi*. Therefore, the PARP A-α gene primers may be a useful tool for the diagnosis of *T. evansi* in the tsetse-free regions, and may be also used inside of the tsetse belt if the distinction between *T. evansi* and *T. brucei* infections are not so critical.

2-5: Summary

T. evansi is morphologically indistinguishable from *T. brucei*. Close relationship between *T. evansi* and *T. brucei* was further documented by Gibson et al. (1983) (37), Masiga and Gibson (1990) (71) and Stevens et al. (1992) (136), suggesting that *T. evansi* is derived from *T. brucei*. However, *T. evansi* has been distinguished from *T. brucei* by their lack of maxicircle DNA, minicircle DNA homogeneity and lack of developmental stages in its insect vectors (137). Although several features of the minicircle DNA sequence have been reported to be specific for *T. evansi* (2, 71), the author reports here that two strains of *T. brucei* (*T. b. gambiense* Welcome and *T. b. rhodesiense* IL1501) also possess "the *T. evansi* specific" minicircle DNA sequence.

Furthermore, Artama et al. (1992) (2) reported the similarity of PCR amplification patterns of the PARP A-α gene, which encodes a major surface protein of procyclic forms of *T. brucei* (83, 115), between *T. evansi* and *T. brucei*. In this study, the author confirmed the existence of the similarity and suggested the use of the PARP A-α gene primer as a diagnostic tool for *T. evansi* infection. Although the PCR detection using this primer could not still distinguish the *T. evansi* infection from the *T. brucei* infection, it might be specially useful outside of the tsetse belt where both the kinetoplastic and akinetoplastic strains of *T. evansi* are widely prevalent but not *T. brucei*.

Moreover, the PARP A-α gene of *T. evansi* was sequenced, and the expression of the PARP A-α gene in *T. evansi* was, for the first time, demonstrated by means of RT-PCR, although *T. evansi* is lacking the procyclic stage in its life cycle.

CHAPTER 3:

Interleukin 4 (IL-4) is a crucial cytokine in controlling *Trypanosoma brucei gambiense* infection in mice

3-1: Introduction

T. b. gambiense, which is an obligate extracellular parasite, causes chronic sleeping sickness in man. Although inbred mouse is useful tool for immunological studies on African sleeping sickness in man, its application is limited to the parasites which are infectious to mice. It is well known that type 1 *T. b. gambiense* (or true-gambiense) is characterized in its extremely low virulence to rodents (18, 40, 43, 73, 107). Therefore, host immune responses against such parasites are hardly examined by mouse model system. Since the efficient propagation systems for low virulent trypanosomes were established in Chapter 1, it became possible for studies of such parasites in immunological, molecular biological and biochemical aspects. In this chapter, host immune responses against low virulent strain of *T. b. gambiense* were examined utilizing the propagation systems described above.

In general, antibody-dependent immune responses are important to control salivarian trypanosomes. Both T-cell-independent and T-cell-dependent B-cell responses to the variable surface glycoprotein (VSG) molecule occur during infection with *T. brucei*, and in the presence of T-cells, the VSG specific B-cell responses are greatly enhanced (114). This finding suggests that T helper cells play important role(s) in controlling *T. brucei* infection. In this chapter, *in vivo* $CD4^+$ T-cell depletion was performed to examine the importance of the $CD4^+$ T-cell in controlling *T. b. gambiense* IL3253 infection in mice.

Naive T helper cells recognize major histocompatibility complex-binding antigenic

peptide and differentiate into functionally distinct T helper cell subsets. T helper 1-type (Th1) cells and T helper 2-type (Th2) cells are characterized by their cytokine production pattern (81, 82). Briefly, the Th1 cell is characterized by its production of interleukin (IL) 2, interferon γ (IFN-γ) and lymphotoxin, and promotes cell-mediated immune responses whereas the Th2 cell distinctively produces IL-4, IL-5, IL-6 and IL-10, and promotes humoral immune responses. Recently, the importance of the Th1/Th2 balance has been demonstrated in experimental leishmaniosis and a variety of autoimmune diseases (42, 60, 98, 129). VSG-responsive T-cells in infected mice are $CD4^+$ T-cells, express the T-cell receptor αβ and produce Th1 cytokines. These cells are detectable in the peritoneal T-cell populations of infected mice. These VSG-specific T-cells do not proliferate in response to VSG, but produce Th1 cytokines (125). In this chapter, the author demonstrates that IL-4, which is a Th2-type cytokine, was detected in serum obtained from *T. b. gambiense*-infected mice and was important in control of the infection.

3-2: Materials and methods

3-2a: Mice

BALB/c/A-+/+ (BALB/c), BALB/c/A-nu/nu (nude) and severe combined immunodeficient (SCID) mice were obtained from Japan CLEA Inc. (Tokyo, Japan). Mice were housed in specific pathogen-free conditions and used for experiments at 5 to 6 weeks of age.

3-2b: Parasites

Bloodstream forms (BSFs) of *T. b. gambiense* IL3253, which cause chronic infection in mice, were used. Parasites were propagated in SCID mice as an inoculum (Chapter 1).

3-2c: Parasite infection

Parasite infection was performed by the same procedure as described in Chapter 1 (1-2c).

3-2d: Estimation of parasitemia

Parasitemia was estimated by the same method as described in Chapter 1 (1-2d).

3-2e: Monoclonal antibodies

The GK1.5 and the 53-6.72 monoclonal antibodies (mAbs) were used for depletion of $CD4^+$ and $CD8^+$ T-cells, respectively (58, 159). Prior to performing the T-cell subset depletion, effect of the GK1.5 and the 53-6.72 mAbs were assessed by means of flow cytometry. Mice were inoculated intraperitoneally (i.p.) with 0.5 mg of the GK1.5 mAb, the 53-6.72 mAb or normal rat-IgG one day before the flow cytometric analysis. The

spleen cell suspension was then prepared from each mouse and expression of CD4 and CD8 was examined by using flow cytometry (EPICS® XL, BECKMAN COULTER, U.S.A.) after staining with fluorescein isothiocyanate (FITC)-conjugated mAbs (FITC-conjugated rat anti-mouse CD4 and FITC-conjugated rat anti-mouse CD8α, Pharmingen, U.S.A.).

In the experiments, 5 BALB/c mice were inoculated i.p. with 0.5 mg of the GK1.5, the 53-6.72 mAb or non-related rat IgG a day before infection. The injection was repeated every 5 d after infection.

The 11B11 mAb and the R4-6A2 mAb, which block biological activity of IL-4 and IFN-γ, respectively (99, 134), were used for *in vivo* blocking of the cytokines. Each BALB/c mouse (five mice were used) received 0.5 mg of the 11B11, the R4-6A2, or the rat-IgG by the same procedure as the T-cell subset depletion.

All the mAbs were prepared from ascites of the hybridoma inoculated nude mice by means of ammonium sulfate precipitation.

3-2f: Enzyme linked immunosorbent assay (ELISA)

To measure the concentration of IL-4 and IFN-γ in the sera obtained from infected mice, ELISA kit (Mouse ELISA IL-4, Mouse ELISA IFNγ, ENDOGEN, Inc., U.S.A) were used. Serum was collected from each infected mouse every other day after infection and was stored in liquid nitrogen until used. A standard curve was used to determine the amount of the cytokines in the sera. The standard curve was generated by plotting the absorbance obtained for each of the cytokine concentrations on the vertical axis versus the corresponding cytokine concentration on the horizontal axis. Sensitivity of the ELISA kits was <5 pg/ml for IL-4 and <15 pg/ml for IFN-γ.

3-2g: Statistical analysis

Statistical determinations of the difference between means of experimental groups were made using an unpaired, 2-tailed Student's *t* test.

3-3: Results

3-3a: Susceptibility to IL3253 in congenitally immunodeficient mice

BALB/c, nude and SCID mice were infected i.p. with 5×10^3 BSFs of IL3253. Parasitemia of each mouse was determined every day. Susceptibility to IL3253 was clearly different among BALB/c, nude and SCID mice (Fig. 18). The BALB/c mice developed a chronic infection with sporadic parasitemia. The nude and the SCID mice showed medium and high susceptibility to IL3253 infection, respectively. Although parasitemia was persistent and undulated between 1.0×10^4 and 1.8×10^6 BSFs/ml in the nude mice, the infection was not fatal. Moreover, the parasitemia in the nude mice was significantly higher than that in the BALB/c mice on days 13-20, 23 and 25-30 post-infection (PI) ($P<0.05$). The SCID mice developed high parasitemia from day 10 PI onward (SCID vs nude and BALB/c; $P<0.05$) and died within 50 d.

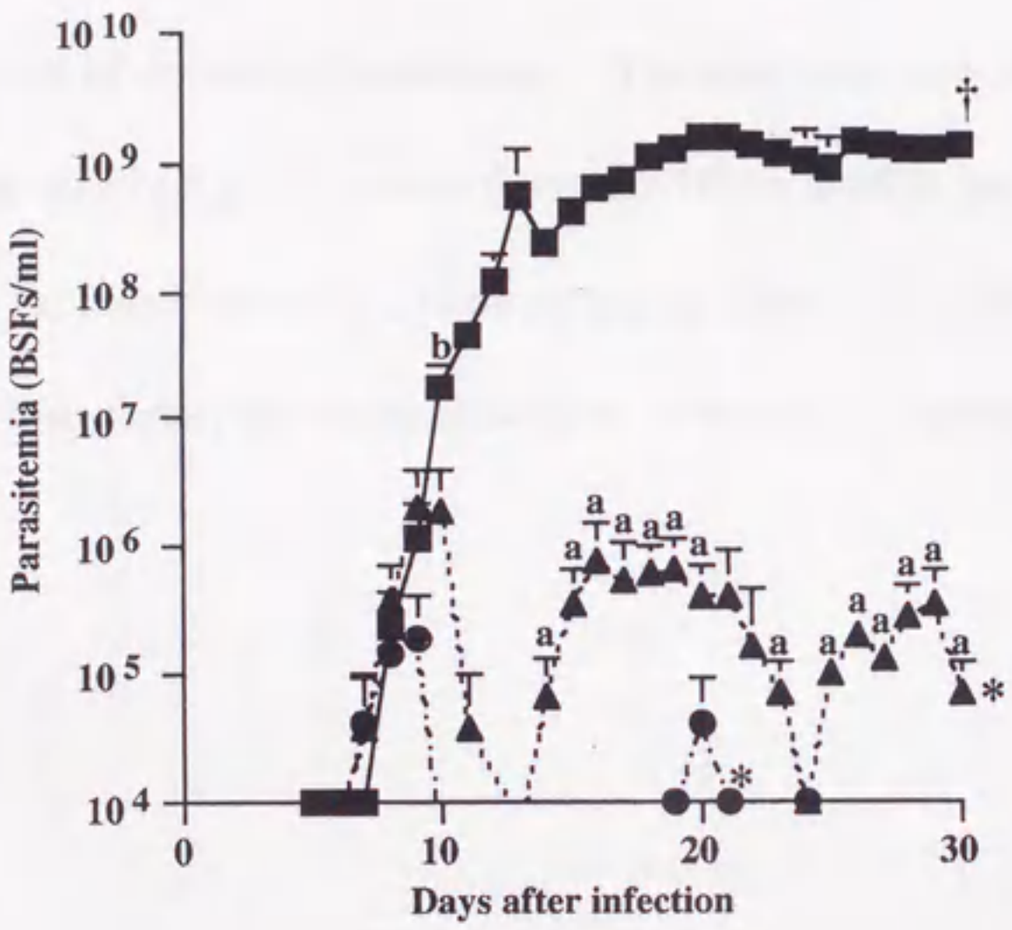


Figure 18. Mean parasitemia levels in BALB/c, nude and SCID mice infected with *T. b. gambiense* IL3253

The solid circle represents BALB/c mice and the solid triangle depicts nude mice.

The solid square indicates SCID mice. †: All mice died within 50 d.

*: All mice survived more than 60 d.

The values represent the means ± SD of 5 mice per group.

a: The parasitemia in the nude mice was significantly higher than that of the BALB/c mice ($P<0.05$).

The parasitemia in the SCID mice was significantly higher than that of BALB/c and nude mice on day 10 PI (**b**) onward ($P<0.05$).

3-3b: Kinetics of IL-4 and IFN-γ production in IL3253 infected mice

To analyze the kinetics of IL-4 and IFN-γ production during IL3253 infection in BALB/c mice, the mice were infected with 5×10^3 of IL3253 BSFs. Every other day 2 mice were killed for serum collection by cardiac puncture under anesthesia. Serum IL-4 levels in infected mice increased from day 10 PI. The time of increased IL-4 was consistent with that of decreased parasitemia. The maximum concentration of IL-4 was 250 pg/ml on day 12 PI (Fig. 19a), while the serum IFN-γ level increased on days 8-10 PI, and then rapidly decreased on day 12 PI onward (Fig. 19b). The time of the IFN-γ surge occurred earlier than that of the increase in IL-4. Results were similar for 3 independent experiments.

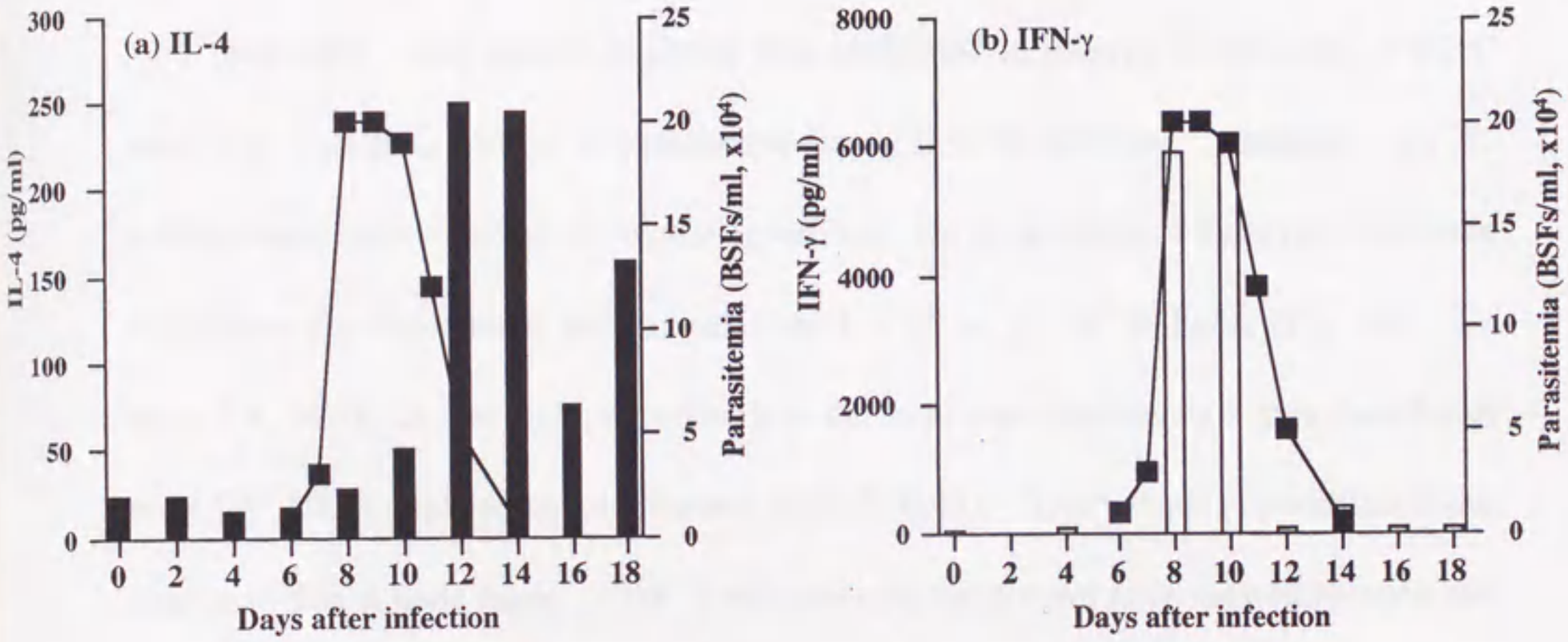


Figure 19. IL-4 and IFN-γ responses in *T. b. gambiense* IL3253 infected BALB/c mice

The solid bar (a) represents IL-4 responses and the open bar (b) depicts IFN-γ responses.

The results shown are from a single representative experiment of three independent experiments.

The solid line (a and b) indicates mean parasitemia from the two mice which were sacrificed for serum collection.

3-3c: IL3253 infection and cytokine production in T-cell subset-depleted BALB/c mice

The GK1.5 and the 53-6.72 mAbs were used for depletion of $CD4^+$ cells and $CD8^+$ cells, respectively. Firstly, the efficacy of the mAbs was examined by means of flow cytometry. Depletion of T cell subsets was observed in the spleen cell populations which were obtained from the mice treated with corresponding mAbs (Table 12).

Secondly, T-cell subset depletion was performed to analyze involvement of $CD4^+$ and $CD8^+$ T-cells in control of parasitemia during IL3253 infection. Although $CD4^+$ T-cell-depleted mice survived during the experiment, the parasitemia of these mice persisted throughout the experiment and ranged from 1×10^4 to 1×10^6 BSFs/ml (Fig. 20). On days 7, 8, 16-18, 21 and 25 PI, parasitemia in the mice was significantly higher than that of the $CD8^+$ T-cell-depleted and the control mice ($P<0.05$). This pattern of parasitemia was similar to that of nude mice. $CD8^+$ T-cell-depleted and control mice showed sporadic and low parasitemia and there were no significant differences between their levels of parasitemia (Fig. 20).

Serum was collected form each T-cell subset-depleted mouse every other day. The sera were pooled and IL-4 and IFN-γ levels during IL3253 infection were determined. IL-4 was not detected in $CD4^+$ T-cell-depleted mice. However, IL-4 was detected in both control and $CD8^+$ T-cell-depleted mice from day 10 and day 8 PI, respectively. The highest concentration of IL-4 was 36 pg/ml in the control and 70 pg/ml in the $CD8^+$ T-cell-depleted group (Fig. 21a). Serum IFN-γ levels decreased in the $CD4^+$ T-cell-depleted group. The highest IFN-γ level was 460 pg/ml on day 10 PI. In the $CD8^+$ T-cell-depleted mice, the highest IFN-γ level was 2,600 pg/ml on day 10 PI. This level was not significantly different from that of the control mice (Fig. 21b).

Table 12. Evaluation of an effect of the anti-mouse CD4 mAb or anti-mouse CD8 mAb treatment on spleen cell population by using flow cytometry

	Treatment of mice		
	GK1.5	53.6-72	rat IgG
$CD4^+$ cell (%)	0.3±0.2	61.3±3.1	55.9±0.3
$CD8^+$ cell (%)	32.7±16.9	0.3±0.5	14.8±4.9

The data represent mean±SD of triplicate samples.

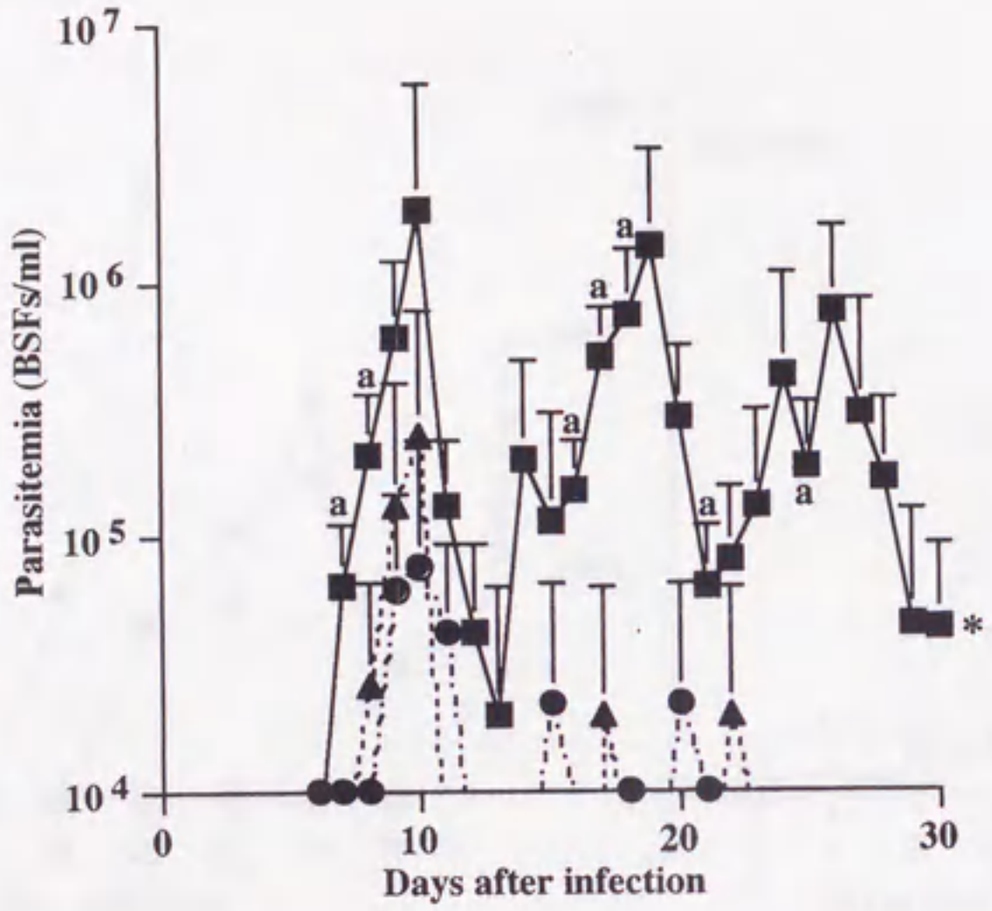


Figure 20. Changes of parasitemia in T-cell subset depleted BALB/c mice infected with *T. b. gambiense* IL3253

The solid square represents $CD4^+$ T-cell depleted and the solid triangle depicts $CD8^+$ T-cell depleted mice.

The solid circle indicates control mice treated with non-related rat IgG.

*: All the mice were healthy and the experiment was terminated on day 30 PI.

a: The parasitemia in the $CD4^+$ T-cell depleted mice was significantly higher than that of the $CD8^+$ T-cell depleted and the control mice ($P<0.05$).

The values represent the means ± SD of 5 mice per group.

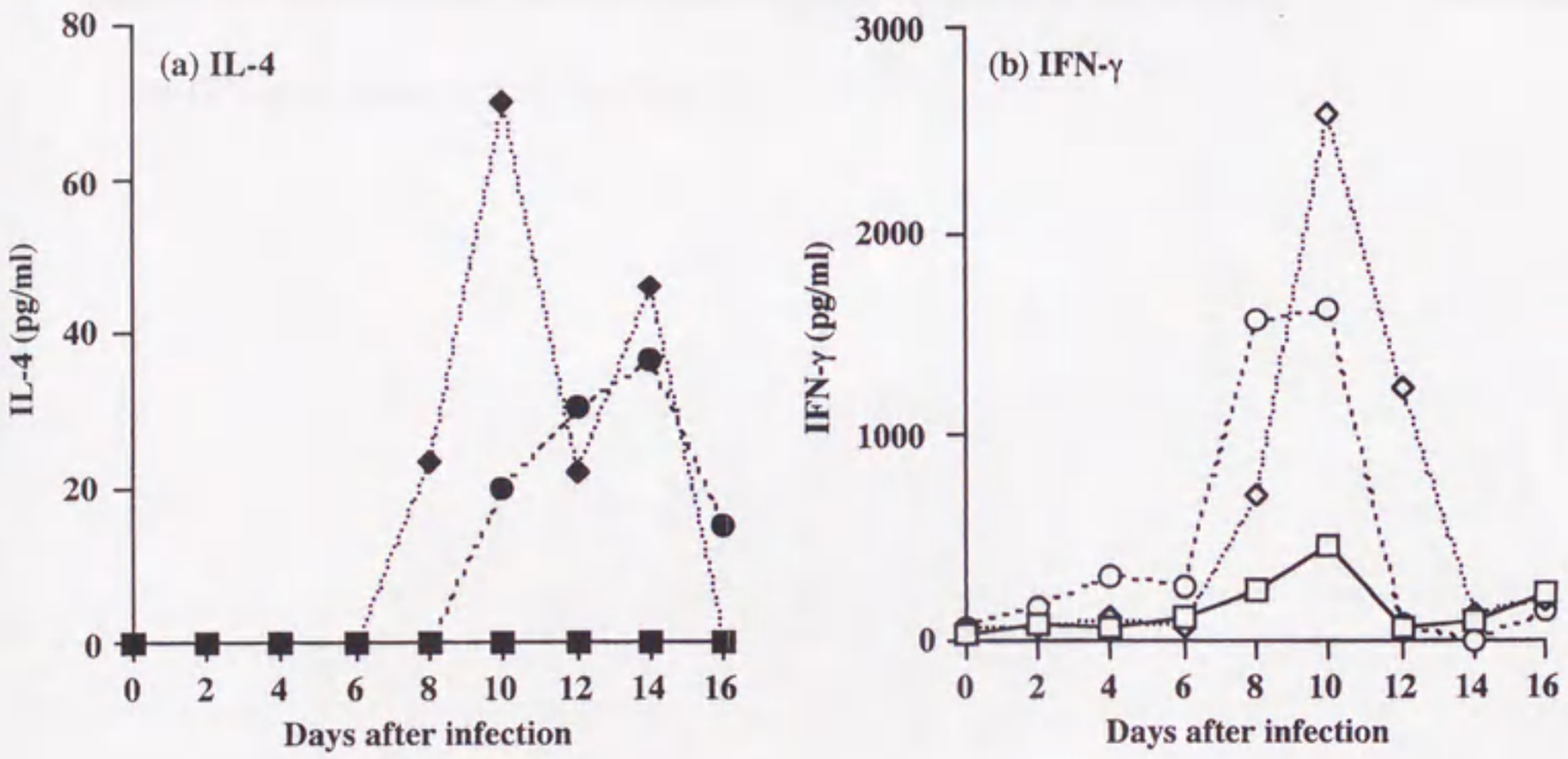


Figure 21. IL-4 and IFN-γ responses in T-cell subset depleted mice infected with *T. b. gambiense* IL3253

The square represents $CD4^+$ T-cell depleted and the diamond depicts $CD8^+$ T-cell depleted mice.

The circle indicates control mice treated with non-related rat IgG.

The results are expressed as the means for duplicate values in mixed sera from five mice.

3-3d: *In vivo* blocking of IL-4 and IFN-γ in IL3253 infected mice

The contribution of IL-4 and IFN-γ in controlling parasitemia was examined by means of mAb blocking of these cytokines *in vivo*. The parasitemia in anti-IL-4 mAb treated mice relapsed significantly longer than that of the control mice (anti-IL-4 vs control; $P<0.01$), although all the mice survived. There was little difference between the anti-IFN-γ mAb-treated and the control group in terms of the relapses of the parasitemia (anti-IFN-γ vs control; $P>0.1$) (Fig. 22).

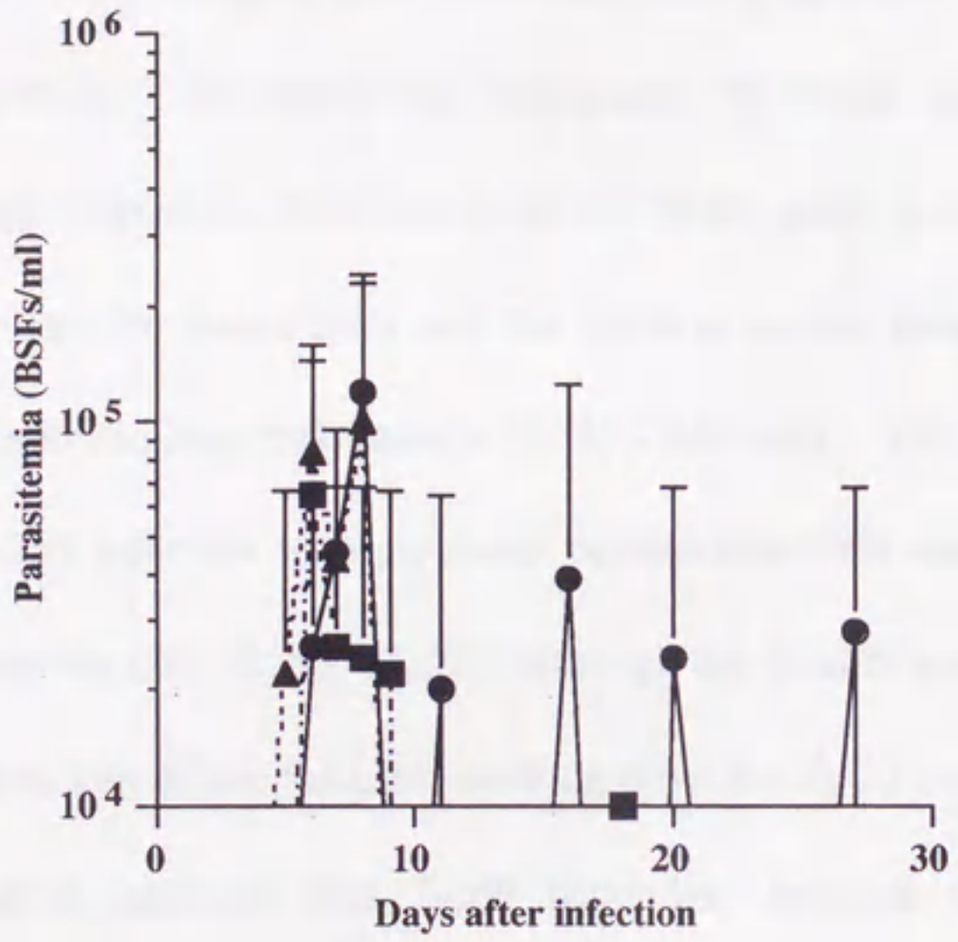


Figure 22. Changes of parasitemia in anti-IL-4 or anti-IFN-γ mAb treated mice infected with *T. b. gambiense* IL3253

The circle represents anti-IL-4 mAb treated and the triangle depicts anti-IFN-γ mAb treated mice.

The square indicate control mice treated with non-related rat IgG.

The values represent the means ± SD of 5 mice per group.

3-4: Discussion

The objective of this chapter was to examine the role of IL-4 in relation to host defense during *T. b. gambiense* infection in mice. It has been reported that athymic nude mice can respond to VSG and sometimes control infections of *T. brucei* (20, 22, 56, 68, 79). The previous studies suggest that T-cell independent immune responses can control infections of *T. brucei*. To assess the importance of T-cell dependent and T-cell independent immune responses, BALB/c, nude and SCID mice were infected with *T. b. gambiense* IL3253 and the parasitemia and the survival period were observed for 30 d. The BALB/c mice showed high resistance to IL3253 infection. The nude mice were also able to control IL3253 infection with persistent parasitemia. This result is consistent with those of previous studies (20, 22, 56, 68, 79), although the level of parasitemia of the nude mice was higher than that of the BALB/c mice on days 13-20, 23 and 25-30 PI ($P<0.05$). Therefore, the author conclude that T-cell dependent immune responses effectively contribute to the control of IL3253 infection. The SCID mice, which have no functional T- and B-cells, showed high susceptibility to IL3253 infection (Chapter 1). From this finding, B-cells appear to have a role in protection against IL3253 infection. Interestingly, the SCID mice infected with IL3253 survived more than 30 d with high parasitemia (Fig. 18). This result may suggest an involvement of natural immunity and/or an absence of immunopathological effects in the SCID mouse during the IL3253 infection. Several aspects of natural immunity in the SCID mouse were well reviewed by Bancroft et al., (1991) (7).

T cells can be divided into two distinct populations. The $CD4^+$ T-cell mainly regulates immune responses as a helper cell, and the $CD8^+$ T-cell is predominantly a cytotoxic cell. To examine the roles of $CD4^+$ and $CD8^+$ T-cells in IL3253 infection in the

BALB/c mouse, parasitemia and the cytokine production pattern of the mice depleted of $CD4^+$ or $CD8^+$ T-cell were observed. The mean parasitemia in $CD4^+$ T-cell-depleted mice was clearly higher than that of the control and the $CD8^+$ T-cell-depleted mice on days 7, 8, 16-18, 21 and 25 PI ($P<0.05$). The pattern of parasitemia in the $CD4^+$ T-cell-depleted mice was similar to that of nude mice. This indicates that $CD4^+$ T-cell mediated immune responses are important for control of IL3253 infection.

It has been reported that trypanosome-derived lymphocyte-triggering factor (TLTF) induces $CD8^+$ T-cells to IFN-γ secretion and the IFN-γ derived from $CD8^+$ T-cell, promotes parasite growth (101, 102). Therefore, depletion of $CD8^+$ cells in infected rats abrogated IFN-γ production, suppressed parasite growth and increased the survival time of the animals (5). According to the results (Fig. 20), there were no differences in the extent of parasitemia between the $CD8^+$ depleted mice and the control mice. This may be explained by a species-restricted recognition of IFN-γ (101), since *T. b. gambiense*, which was used in the studies, is distinct form *T. b. brucei* by its infectivity to humans.

Recently, the importance of the cytokine production pattern in several diseases has been reported in terms of the Th1/Th2 balance (113, 127, 140). According to the results (Fig. 19), both IL-4 and IFN-γ were detected from IL3253 infected mice. Serum concentrations of these cytokines decreased in $CD4^+$ T-cell-depleted mice. This suggested that the $CD4^+$ T-cell directly or indirectly involves IFN-γ and IL-4 production in the IL3253 infected mouse. IL-4, which is mainly produced by Th2 cells, plays an important role in B cell activation and differentiation, leading to production of IgG1 and IgE (23, 53). Although T-cell responses and cytokine profiles in mice infected with *T. brucei* are biased to Th1 type responses, the importance of Th2 type cytokines, especially IL-4, was recently reported (4, 126). Therefore, administration of anti-IL-4 mAb into

IL3253 infected mice was carried out. The anti-IL-4 mAb treated mice frequently relapsed in comparison with the control mice. Schopf et al. (1998) (126) reported that cryptic IL-4 production, which is not derived from antigen-specific Th2 cells, but from antigen-nonspecific cells, is important in VSG-specific immunoglobulin G1 isotype switch response. Moreover, mouse strain-dependent ability to produce IL-4 against *T. brucei* infection determines the susceptibility to the infection and is crucial to control of parasitemia and survival (4). The result (Figs. 21 and 22) are consistent with those of previous studies in terms of the importance of the IL-4 response in infected mice. Interestingly, IL-4 production in infected mice was not cryptic, but was present at levels detectable in the serum.

In conclusion, although the IL-4 producing cell was not identified, the $CD4^+$ T-cell and IL-4, regulated by the $CD4^+$ T-cell, have crucial role(s) in controlling *T. b. gambiense* IL3253 infection in mice.

3-5: Summary

The role of IL-4 was studied in relation to host defense during *T. b. gambiense* IL3253 infection in mice. BALB/c, nude and SCID mice were infected i.p.with 5×10^3 BSFs of the trypanosome. The BALB/c mice showed high resistance to IL3253 infection with sporadic parasitemia. The nude mice were also able to control IL3253 infection and experienced low, but persistent parasitemia. However, the SCID mice, which have no functional T- and B-cells, showed high susceptibility to IL3253 infection with more than 1 $\times10^8$ BSFs/ml. Serum IL-4 levels in the infected BALB/c mice were increased on days 12-18 PI. In BALB/c mice depleted of $CD4^+$ T-cells by mAb treatment, parasitemia was persistent, ranging from 1×10^4 to 1×10^6 BSFs/ml and was significantly higher than that of the other groups. IL-4 was not detected in the serum of $CD4^+$ T-cells-depleted mice. On the other hand, anti-IL-4-treated IL3253-infected BALB/c mice relapsed significantly longer than the control mice ($P<0.01$). These findings suggest that the $CD4^+$ T-cells may control the levels of parasitemia in IL3253 infection through the IL-4 pathway.

GENERAL DISCUSSION AND CONCLUSIONS

The studies reported in this thesis have established that *in vivo* and *in vitro* systems that support the growth of low virulent strains of trypanosomes (Chapter 1). Kinetoplast DNA (kDNA) of various trypanosome strains including low virulent strains of *T. b. gambiense* were then examined in order to evaluate the "*T. evansi* minicircle DNA specific polymerase chain reaction (PCR)", and the author found that *T. b. gambiense* Welcome strain and *T. b. rhodesiense* IL1501 possessed *T. evansi* type minicircle DNA. Moreover, it was demonstrated that the procyclic acidic repetitive protein (PARP) gene was highly conserved among various strains of *T. evansi* and *T. brucei*, and the potential use of the PARP primers as a diagnostic tools for *T. evansi* and *T. brucei* was demonstrated (Chapter 2). The *in vivo* propagation system established in Chapter 1 was applied to immunological studies on low virulent *T. b. gambiense* infection in mice, and it was demonstrated that $CD4^+$ T-cells and interleukin 4 (IL-4) play important role(s) in controlling the infection (Chapter 3).

Sub-inoculation of infected blood and celebrospinal fluid into experimental rodents is a commonly used technique to detect and isolate salivarian trypanosomes. However, a virulence of trypanosome to the experimental rodents is widely diverse among isolates. Low virulent strains of trypanosomes develop chronic infection with low parasitemia in mice. Therefore, such low virulent trypanosomes are often scientifically neglected because of the difficulty in preparing sufficient number of trypanosomes for a study. Sub-lethally irradiated mice and immunosuppressed mice have been used for propagation of such low virulent trypanosomes. However, a special facility is needed to irradiate animals and it was reported that certain variable antigen types of trypanosomes could not grow well in irradiated mice (26). Moreover, it is not clear whether immunosuppressants

have some effects on trypanosomes or not. Therefore, a method which is surely support the growth of low virulent trypanosomes without any problems is needed. The studies reported in this thesis have demonstrated that a severe combined immunodeficient (SCID) mouse is a highly susceptible host of low virulent strains of *T. b. gambiense* and *T. evansi* (Chapter 1). Bosma et al. (1983) (17) firstly reported the SCID mice, which lacks functional T- and B-cells. Therefore, the SCID mouse is highly susceptible to various parasitic protozoa (135). All trypanosome strains tested were infectious to SCID mice with high parasitemia and there were no substantial differences in the highest parasitemia between high and low virulent trypanosomes.

An alternative way of the isolation of trypanosomes from infected animals is an *in vitro* cultivation of the parasites. Procyclic forms of trypanosomes are the only developmental stage of the parasites to be able to grow *in vitro* until establishment of the method supporting the growth of bloodstream forms (BSFs) by Hirumi et al. (1977) (46). At present, all the developmental stages of salivarian trypanosomes are able to grow *in vitro*. Nevertheless, in most cases, it is necessary to prepare a large number of BSFs in order to initiate the *in vitro* cultivation of BSFs and procyclic forms of trypanosomes. Therefore, the authors used BSFs of a low virulent strain of *T. b. gambiense*, which were isolated from SCID mice, for a starting material of *in vitro* cultivation (Chapter 1). Except for the difference in serum requirement, RPMI-1640 and IMDM well supported the growth of both high and low virulent strains of *T. brucei in vitro*. Although, both high virulent *T. b. rhodesiense* IL2343 and low virulent *T. b. gambiense* IL3253 were isolated from human cases of sleeping sickness, only the IL3253 grew in the medium supplemented with human serum (HuS) or plasma. In consideration of an unstable feature of HuS resistance in trypanosomes, the IL3253 might retain its original features, such as infectivity

to human (72, 117). The shortest population doubling time of the IL3253 (8.7 h) was 1.9 h longer than that of the IL2343 (6.8 h). Thus an *in vitro* growth speed of BSFs may have relation to a virulence of a parasite to its natural host.

Further application of the SCID mouse system on a molecular biological study of low virulent trypanosomes was made in Chapter 1 and Chapter 2. A pulsed-field gel electrophoresis (PFGE) is one of the useful molecular epidemiological tools for salivarian trypanosomes, although a large number of BSFs of the parasite are needed (65). Thus, there are no report of the PFGE analysis of low virulent trypanosomes, except for the parasites grow well *in vitro* as procyclic forms. According to the result, there were no specific PFGE pattern of low virulent strains but 4 strains of *T. b. gambiense* could be distinguished by their less staining intensity of mini-chromosomes. This may suggest that the number of the variable surface glycoprotein genes, most of which reside at mini-chromosomes, are fewer than those of the other species of trypanosomes (28).

PCR technique was first reported by Mullis et al. (1986) (85). Since then, the PCR technique has been contributed to advances in molecular biology and the development of PCR-based diagnosis. Although further improvements are required, the PCR-based diagnosis is theoretically the most sensitive and specific diagnostic method. The PCR-based diagnosis of African trypanosomoses was firstly reported by Moser et al. (1989) (80). They demonstrated that *T. congolense* and *T. brucei* spp. were specifically detected by the specific PCR amplification of 369 bp nuclear DNA repeats and 177 bp nuclear DNA repeats, respectively. Furthermore, trypanosomal DNA could be amplified from blood of infected animals, even though parasitemia levels were below detectable level under a microscopical examination. In case of *T. evansi*, a kinetoplast DNA minicircle specific primer pair, which specifically amplified *T. evansi* minicircle DNA, was reported by

Artama et al. (1992) (2). Since akinetoplastic *T. evansi* was isolated from mule and buffalo in China, and capybara in Brazil (65, 71, 104), the *T. evansi* specific primer pair may fail to detect such parasites from infected animals. Moreover, the author demonstrated that *T. b. gambiense* Welcome and *T. b. rhodesiense* IL1501 possess the same or nearly the same minicircle DNA as that of *T. evansi* by using the *T. evansi* specific PCR (Chapter 2). This may suggest that the *T. evansi* specific PCR not only overlook but also misdiagnose *T. evansi* infection both tsetse-infested and tsetse-free areas. On the other hand, PCR using the PARP A-α primer pair showed the identical amplification pattern from *T. evansi* and *T. brucei* genomic DNAs (2). The authors newly determined nucleic acid sequences of the PARP A-α gene from 5 strains of *T. evansi* (4 kinetoplastic and an akinetoplastic strains) and 3 strains of *T. brucei*. Predictive amino acid sequences of the PARP A-α of these trypanosomes were almost identical, although the number of pentapeptide repeats (GPEET) varied from 4 to 6 among them (Chapter 2). Thus the PARP A-α gene was highly conserved among *T. evansi* and *T. brucei*.

The PARPs are the major surface protein of *T. brucei* procyclic and epimastigote forms. There were the 4 PARP expression sites in *T. brucei*, namely *Pro A*, *Pro B*, and 2 copies of *Pro C* (123). Each expression site begins with a pair of PARP genes called α and β. The PARP β gene encodes protein with internal dipeptide (EP) repeats in all the four expression sites, while the PARP α gene encodes protein with the EP repeats or pentapeptide (GPEET) repeats (120, 123). The function of the PARP was thought to protect the parasite from lytic components in the midgut of tsetse fly (121). Therefore, the PARP is not necessary in *T. evansi*, which does not have any developmental stages in its insect vector. Further analysis of the PARP A-α gene of *T. evansi* revealed that the gene was transcribed in both *T. evansi* and *T. brucei* BSFs. This result strongly suggests

close evolutional relationship between *T. evansi* and *T. brucei*. Moreover, a specificity of the PARP A-α gene primer to *T. evansi* and *T. brucei* was clearly demonstrated in Chapter 2. Since two-thirds of *T. evansi* endemic area is free of *T. brucei*, the PARP A-α primer may be useful tool for diagnosis of Surra and reduce possibility of overlooking akinetoplastic *T. evansi* infection.

T. b. gambiense causes chronic African sleeping sickness in man and patients die unless they are treated. In general, *T. b. gambiense* show extremely low virulence in experimental rodents such as mouse and rat (27). Mice infected with such parasite shows sporadic parasitemia lasting more than 60 days but appears to be health. Except for long lasting infection, clinical signs of *T. b. gambiense* infection in man and mice were quite different each other. Nevertheless, elucidation of protective immune mechanisms in infected mice may give useful information in controlling African trypanosomoses in man. In Chapter 3, the author demonstrated that the SCID mouse system, established in Chapter 1, was useful for not only propagation of low virulent trypanosomes but also analysis of host-parasite interactions. High susceptibility of $CD4^+$ T-cell subset-depleted mice against *T. b. gambiense* IL3253 infection clearly indicated that the T-cell subset plays important role(s) in controlling the infection. $CD4^+$ T-cell subset stimulates B-cell proliferation and differentiation to antibody producing cell via cytokine pathway. Recently, two kinds of $CD4^+$ T-cell subsets, namely T helper 1-type (Th1) cell and T helper 2-type (Th2) cell, were identified (81). According to the results, IL-4 appeared to be contribute to control of parasitemia. Since IL-4 is mainly produced by Th2 cell (81, 82), it is possible that $CD4^+$ T-cell produces IL-4 responding to *T. b. gambiense* infection. Identification of IL-4 producing cell may help to understand protective immune responses against *T. b. gambiense*.

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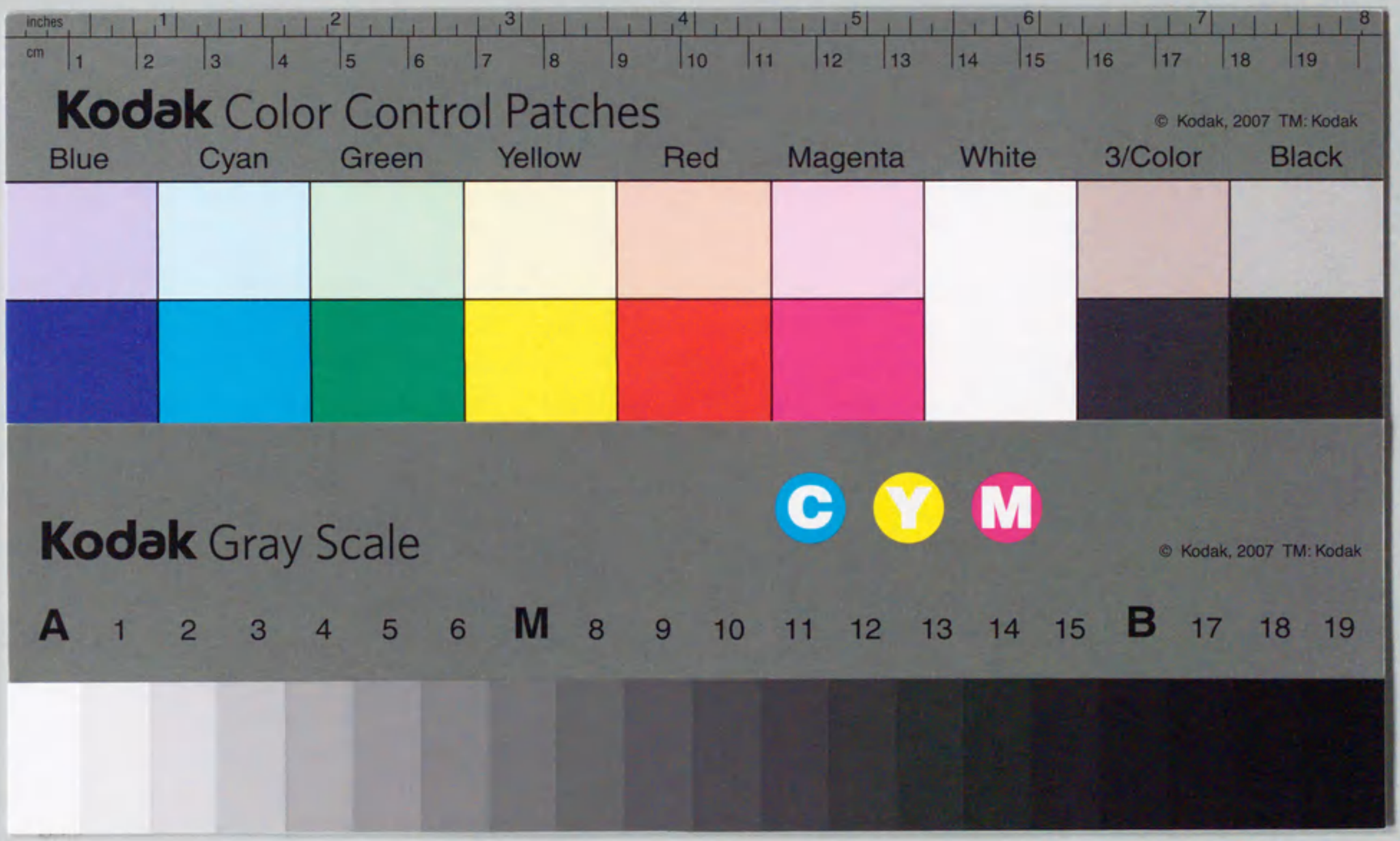
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