p15 Trypanosome Microtubule Associated Protein: Structure/Function Analysis and Vaccine Development for the Prevention of African Sleeping Sickness

By

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ABSTRACT

Trypanosomes are hemoflagellated protozoan parasites causing chagas disease in South America, Leishmaniasis throughout the world, and African sleeping sickness in humans and nagana in animals in Africa. About 55 million people and 25 million cattle have been estimated to be at risk of contracting African sleeping sickness or nagana respectively. Once injected into the blood stream via the bite of a tsetse fly, the parasite evades the host's immune response by repeatedly changing its surface antigens, thus making the development of a vaccine seem impossible. Furthermore, chemotherapy existing today can be toxic, suggesting that novel methods to prevent diseases caused by trypanosomes are essential.

All parasites of the Trypanosomatidae family contain unique microtubular structures called the subpellicular microtubules. Microtubules are made of tubulin and of microtubule associated proteins (MAPs). Unlike other microtubules, the subpellicular microtubules are crosslinked to one another and to the plasma membrane. The unique structure of the subpellicular microtubules has been attributed to unique trypanosome subpellicular MAPs which stabilize the microtubule polymers and crosslink them to one another.

Three unique types of subpellicular MAPs have been identified: MARP, which is a high molecular mass MAP that stabilizes microtubules, p52 that is a 52kDa MAP which crosslinks microtubules, and p15, which is a 15kDa protein which bundles microtubules. Because trypanosome MAPs have been shown to be unique to these parasites, these molecules could serve as useful target sites for therapy. In this study p15 was cloned and sequenced and shown to contain highly organized, nearly identical tandem repeats with a periodicity of 10 amino acids, rich in positively charged and in hydrophobic amino acids. It was shown that p15 can also bind phospholipids, suggesting that it may not only bundle the microtubule polymer through its positively charged amino acids but may also crosslink the microtubules to the plasma membrane through its hydrophobic regions, thus contributing to the stable structure of the subpellicular microtubules.

To test for the efficiency of p15 as a vaccine candidate, the recombinant p15 was cloned into an adenovirus, which was used as a vaccine delivery system for p15. Mice were vaccinated with the native purified p15, with the expressed recombinant p15 and with the adenovirus containing the recombinant p15 gene (Ad-p15). The results indicated that p15 protected 100% of the animals vaccinated with the recombinant molecule (8/8), and 87%

of the animals vaccinated with the native protein (7/8), while none of the control animals were protected. Animals that were vaccinated with the Ad-p15 were protected but so were the control animals vaccinated with an adenovirus containing the *lacZ* gene. We have shown that vaccination with the adenovirus is associated with an elevated $CD8^+$ T cell response which is known to be trypanostatic (86), suggesting that animals vaccinated with Ad-p15 may have been protected not only by the specific anti-p15 response but also by non specific immunity that was induced by the adenovirus itself.

The source of the native and recombinant p15 was from a different strain of *T. brucei* that was used for challenge. Since the subpellicular microtubules are common to all members of the Trypanosomatidae family, p15 may ultimately serve as a common target for therapy to all types of diseases caused by trypanosomes.

To my daughters Daphne and Sharon

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Abbreviations often used in the thesis

Ad-p15	adenovirus containing the recombinant p15 gene
Ad-lacZ	adenovirus containing the *lacZ* gene
β-gal	β galactosidase
BSA	bovine serum albumin
CFA	complete Freund's adjuvant
DMEM	Dulbeco modified Eagle's medium
EDTA	ethylene diamine tetra acetic acid
FBS	foetal bovine serum
GFP	Green Fluorescent Protein
GAPDH	glyceraldehyde 3 phosphate dehydrogenase
HEK	Human kidney cell line
IPTG	Isopropyl- β -D-Thiogalacto Pyranoside
ICFA	incomplete Freund's adjuvant
LB	Luria broth
MAP	Microtubule associated protein
MARP	high molecular weight subpellicular MAP
MEM	modified Eagle's medium
MOI	multiplicity of infection
PFU	plaque forming unit
PBS	phosphate buffer saline (pH 7.2)
PSG	phosphate saline glucose
PCR	polymerase chain reaction
PIPES	piperazine bis sulfonic acid
RACE	rapid amplification of cDNA end
SSC	Sodium citrate pH 7.0
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
T. brucei	*Trypanosoma brucei*
TB	terrific broth
TBS	Tris buffered saline
VSG	Variable surface glycoproteins
x gal	5-bromo-4-chloro-3-indolyl- β -D-galactosidase

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Chapter I. GENERAL INTRODUCTION

Members of the Trypanosomatidae family are hemoflagellated protozoan parasites, causing African sleeping sickness in Africa, Chagas disease in South America and Leishmaniasis throughout the world, affecting animals and man.

African trypanosomes are considered by the World Health Organization (WHO) to be one of the most important parasites affecting the people of Africa (1,2). The primary social and economic impact of the diseases caused by these parasites is its effect on livestock (3). Trypanosomiasis in livestock, Nagana (meaning poorly in Zulu), is closely related to the human disease. Animal trypanosomiasis is a constant economic problem in Africa (4) , rendering four million square miles of land in sub-Saharan Africa unfit for livestock. It is estimated that could this land be used, the cattle population in Africa could be more than doubled (5). The WHO estimated in 1995 that 55 million people in Africa, mostly from rural areas in 36 different countries, are at risk of contracting African sleeping sickness. Of only 3 million people that were under surveillance, 25,000 cases were diagnosed and treated annually, suggesting that over 300,000 people would have been found to be infected and if left untreated, would die.

Moreover, under certain conditions, there can be severe outbreaks of African sleeping sickness. It is estimated that the disease had reduced the population of Uganda from 6.5 million to 2.5 million in the first decade of the twentieth century (6). In some villages today the disease affects half of the population. Political, economic and social instabilities have disrupted medical services and caused population movements, fostering the outbreak of new epidemics in countries including the Democratic Republic of Congo (formerly Zaire) (7,8), Ethiopia (5), Uganda (9), Sudan (6), Angola (10), and Zimbabwe (11).

1.1. The discovery of the disease, the parasite and its vector, the tsetse fly

Sleeping sickness in humans was described as early as the 14th century by an Arab writer, al Qualquashaudi (cited in 12). The infectious parasitic agents causing disease in various animal species were isolated in the latter part of the 19th century. In 1880, Evans (cited in 13) found that *T. evansi* caused surra in camels and horses. In 1894, Bruce discovered while working in Zululand, *T. brucei* in the blood of cattle suffering from nagana. He then demonstrated its transmission by tsetse flies in 1897 (cited in 13). Five years later, Forde and Dutton reported the isolation of *T. gambiense* from the blood of a Gambian native (14, 15). In 1903, Castellani discovered *T. gambiense* also in the cerebrospinal fluid of patients

suffering from sleeping sickness in Uganda (17). Bruce and Nabarro (in 1903) (18), showed that *T. gambiense* could be transmitted to monkeys with a bite of a tsetse fly. Kleine (1909) found that the parasites underwent a cyclic development in the fly, making the fly infectious twenty days or more after ingesting an infected blood meal (19). Kinghon and York (cited in 15) demonstrated that trypanosomes are transmitted to man by the tsetse fly species *Glossina morsitans*, and that the parasite undergoes a cycle of development in the transmitting fly similar to that of *T. gambiense* in *G. palpalis* (15). Stephens and Fantham (1909) recovered trypanosomes from the blood of a patient in Rhodesia suffering from symptoms suggestive of sleeping sickness and named it *T. rhodesiense*, regarding it as a new species (16).

1.2. Systematics and classification of African Trypanosomes

The African trypanosome is a unicellular eukaryotic protozoan of the genus *Trypanosoma.* The genus *Trypanosoma* is split into two divisions (sterocoraria and salivaria) which differ primarily in the course of their development in their respective vectors (20). The sterocoraria division consists of species in which development is completed in the terminal gut and where transmission is by faeces from the vector. One well-known member of this division is *T. cruzi*, the South American trypanosome that causes Chagas disease. The second division, salivaria, is composed of species in which development is completed in the anterior part of the vector's digestive tract and where transmission is via the saliva of the fly. The African trypanosomes belong in this latter division. The pathogens of the salivaria division fall into three subgenera: Duttonella (type species *T. (D) vivax*), Nannomonas (type species *T. (N) congolense*) and Trypanozoon (type species *T. (T) brucei*).

1.3. Trypanosomiasis-transmission

In Africa, the tsetse fly is responsible for widespread distribution of the disease in humans and their domestic animals. The geographic distribution of African trypanosomiasis in humans and domestic animals is essentially similar to that of the tsetse fly zone (21), although the parasite can also be mechanically transmitted by other types of biting flies (22). Infected asymptomatic animals constitute a large reservoir of pathogenic parasites. Many African cattle-owning tribes like the Masai have been aware of African trypanosomiasis in livestock from very early times and therefore avoid the tsetse-infested areas.

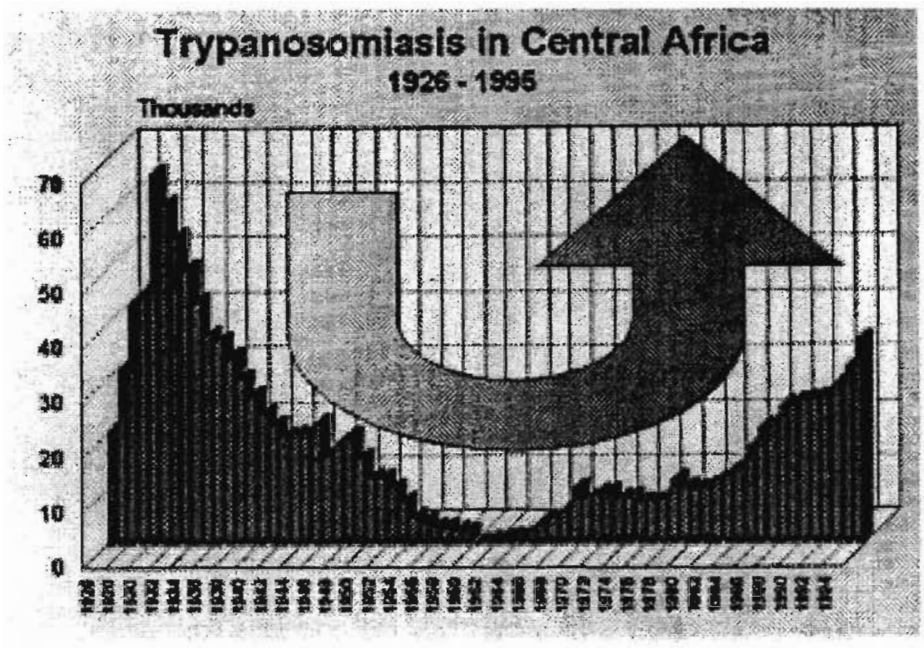

Diagram 1. Incidence of human trypanosomiasis in Central Africa from 1926-1995. The incidence of the disease was greatly reduced during the 1950s and 1960s', but began to increases from 1970 until today. The current increase of the disease is similar to that observed during the great epidemic of the thirties (adapted from 89).

During the 1950's and 60's the number of cases decreased as new tools for vector control emerged (Diagram 1). Insecticides, such as DDT and pyrethroid, had been developed and various forms of biological control had been used successfully (22). However, in the past 20 years the number of new cases has grown steadily (see diagram above).

1.4. Trypanosomiasis-the disease

African sleeping sickness progresses through three stages:

1. The initial stage - the parasite is localized at the site of the tsetse fly bite. There is a swelling at this site.

2. The systemic stage - the trypanosomes are distributed throughout the body by the bloodstream and lymphatic system. This stage is characterized by an intermittent fever and other nonspecific signs of infection including lymphadenopathy, joint pains and general malaise. Some of these symptoms could be associated with waves of parasitemia.

3. The neurological stage - the parasite invades the central nervous system (CNS). When the parasite invades the brain it causes a meningoencephalitis. This appears clinically as an irritable state at first and then the patient's level of consciousness progressively

deteriorates until the patient lapses into a stupor. In some cases the patient may die of starvation. As the disease progresses a state of immunodepression also develops. The lymphoid organs become depleted of lymphoid cells and a systemic neutropenia develops. The ability to mount specific antibody responses to new antigens decreases. Thus the cause of death is sometimes a secondary infection, such as pneumonia. Sleeping sickness patients infected with *T. brucei rhodesiense* may also die from heart failure. (3,23,24, 25).

1.5. Host-parasite relationships in Trypanosomiasis

The immune system plays an important role in both attempted control of the parasite and the pathogenesis of the disease. Upon infection, different populations of trypanosomes in the mammalian bloodstream possess different proteins on their surface called variable surface glycoproteins (VSG) (26). The immune response of the host appears to be largely directed against the VSG. Massive non-specific activation of polyclonal B cells results in a large production of IgM (27), resulting in antigen-antibody complexes that in turn cause hyperplasia, especially in the spleen and lymph nodes. Activation of $CD8^+$ T cells produces interferon-gamma, which then activates macrophages (28) but also promotes parasite growth (29). Activated macrophages release tumor necrosis factor and nitric oxide, which are trypanostatic (30). The parasites regularly change their VSG by trans-splicing the long precursor RNA of the VSG genes (31) more rapidly than new antibodies can be made, thus creating a situation that is similar to having infections by related but non-identical organisms. While most of the parasites are eliminated from the circulation by this antibody response, a small number of parasites in the population bear an antigenically different VSG and thus evade the host's immune response. These parasites proliferate and re-populate the host's blood stream. These, in turn, are eliminated by a new antibody response while new antigenic variants of the parasite arise to maintain the infection. This process may repeat itself for 100 or more sequential waves of parasitemia in the same infected host (27).

1.6. Current treatments

Available chemotherapy to African sleeping sickness has not advanced greatly in the last 50 years and is often highly toxic. For example, Pentamidine can be effective in some patients with early-stage nerve involvement. Melarsoprol is highly toxic and is fatal in about 5% of patients treated. New drugs (e.g. nitroimidazoles) may become available in the future, but development is slow (3).

It is estimated that over 300,000 new individuals are infected per year, suggesting an urgent need for a vaccine.

1.7. Vaccine development

Hope for a vaccine based on the variant surface glycoprotein coat was abandoned several years ago when the complexity of the parasite's antigenic repertoire was appreciated (see section 1.5). As a result, research is now focused on identifying invariant trypanosome components as potential targets for therapy (32,77). In the present study unique non-variable trypanosome microtubule-associated proteins (MAPs) were used as vaccine target sites. Although these proteins are intracellular, they have been shown to be protective against *T. brucei* infections (32).

1.8. Microtubules

Microtubules are cytoskeletal proteins, existing in all eukaryotic cells and are especially abundant in the Trypanosomatidae. Microtubules are composed of tubulin heterodimers and of microtubule-associated proteins known collectively as MAPs. Tubulin heterodimers polymerize to form microtubules by associating with MAPs (32). These molecules enhance tubulin polymerization into microtubule filaments and help stabilize the filaments (35) (see below).

1.9. Tubulin

Tubulin is composed of a heterodimer of two closely related 55kDa proteins called alpha and beta tubulin. These two proteins are encoded by separate genes, or small gene families, whose sequences are highly conserved throughout the eukaryotic kingdom Consequently, tubulin isolated from bovine brain is highly homologous to tubulin isolated from any other eukaryotic source. Because of this, tubulin cannot be used as a vaccine target site, but it can be used to assay MAPs originating from many diverse species, by testing the induction of polymerization of tubulin isolated from different sources (33,34).

1.10. Trypanosome microtubules

Trypanosomes contain 4 structurally distinct types of microtubules: mitotic, cytoplasmic, flagella, and subpellicular microtubules. The supramolecular arrangement of the subpellicular microtubules is unique to the trypanosome and consists of uniformly spaced crosslinked microtubules, lying in close apposition to the plasma membrane (35,36). The organization and stability of the subpellicular microtubules is due to the MAPs that crosslink tubulin polymers and link the microtubule network to the plasma membrane (35) (see below).

1.11. Trypanosome subpellicular MAPs

Three distinct classes of trypanosome subpellicular MAPs have so far been described. One class contains high molecular weight subpellicular MAPs called MARP (37) which contain tandem repeats of near-identical sequences which bind to microtubules via a tubulin domain other that the C-terminus used by mammalian MAPs. A second class of trypanosome MAPs includes p52 (38,39). This 52kDa protein is associated with a subpellicular fraction which is solubilized by high salt and induces crosslinking of microtubules when incubated in the presence of purified calf brain tubulin or microtubules (39). The third class includes p15 which is a 15kDa protein (39). p15 is also associated with salt–solubilized subpellicular microtubules, and induces bundling of microtubules when incubated in the presence of purified calf brain tubulin (40,41).

The aim of this study was to test if immunization with p15 can protect against a *T. brucei* infection. Chapter I describes the structure/function analysis of p15. Chapter II describes the use of a recombinant adenovirus as a vaccine delivery system. Chapter III describes the use of p15 (native, recombinant or transduced) as a vaccine for the prevention of infections caused by *T. brucei* .

II. MATERIALS AND METHODS

Some methods described here are in common use. Nevertheless, since several alternative protocols are often employed, the specific protocols used in the present study are described in detail. Basic procedures such as DNA digestion by restriction enzymes and ligation are not described and are carried out according to the enzyme manufacturer's instructions.

2.1. Materials

Primers, LipofectAMINE, DMEM, MEM, Optimem, FBS, Pen/Strep, Trypsin, Trizol, Super script II reverse transcriptase, Green Lantern vector, dNTPs and 1kb DNA ladder were obtained from Gibco-BRL-Life Technology. Grand Island NY.

E. coli DH10B and T4 ligase, were obtained from Gaithersburg MD.

Tubulin was obtained from Cytoskeleton, Inc. Denver, Colorado.

IPTG was obtained from BioNexus, Inc. San Leandro, CA.

cDNA *T. brucei* library was kindly provided by Dr. Pollakis, New York University, New York, NY.

Affi-gel 10, BioRad protein assay and Silver Stain Plus kit were obtained from BioRad, Hercules, C.A. α^{32}P dCTP, peroxidase conjugated antibodies, Rapid-hyb buffer, Readyprime kit, and ECL chemiluminescence detection kit were obtained from Amersham, Life Science Inc., Arlington Heights, IL.

Sea plaque agarose was obtained from FMC, Rockland, ME.

Zwittergent 3-14 detergent was obtained from Calbiochem-Behring, Corp. La Jolla, CA.

T. b. brucei SB 1 was kindly provided by Dr. Sam Black, Paige Laboratory, Amhurst, MA.

T. b. brucei subgroup formerly EATRO 1443, now KETRI 2693 was obtained from Kenya Trypanosomiasis Research Institute, Muguga, Kenya.

DEAE cellulose (DE-52) was obtained from Whatman Int. Ltd. Maidstone, UK.

PCR II plasmid vector was obtained from Invitrogen, Carlsbad, CA.

pFLAG-CMV-1 expression vector and anti-FLAG monoclonal antibodies M2 were obtained from Kodak. Rochester, NY.

DAKO labeled streptavidin-biotin reagents, DAB substrate kit, anti-CD4 and anti-CD8 antibodies were obtained from Novocastra Laboratories Ltd. Carinteria, CA.

HEK293 cells and Cos7 cells were obtained from ATCC, Rockville, MD.

pVS-galactosidase plasmid vector , Taq DNA polymerase, and Restriction enzymes were obtained from Promega, Madison ,WI.

picoBLUE Immunodetection kit was obtained from Stratagene, Lajolla, CA.

All other materials were obtained from Sigma, St. Louis, Missouri.

2.2. Buffers and media

Terrific broth (TB) media, Luria broth (LB), LB-agar plate, SSC and PBS were prepared according to Sambrook et al. (42).

2.3. Isolation of trypanosomes

For parasite propagation, 10^8 parasites were inoculated intraperitoneally into male Swiss mice, and blood was collected from anesthetized animals by cardiac puncture. Trypanosomes were separated from the blood by passage through a positively charged DEAE cellulose column pre-equilibrated with PSG. The blood cells that are negatively charged adhere to the column, while the trypanosomes, which have a thick positively charged glycoprotein surface coat, pass through the column and are collected (93).

2.4. Isolation and purification of subpellicular microtubules and MAP p15

Parasite pellets (*T. b. brucei* KETRI 2693) were resuspended in buffer A which consisted of 100mM 1,4-piperazinediethanesulfonic acid (PIPES) pH 6.9, 1mM EGTA, 0.1mM EDTA, 0.5mM $MgCl_2$, and 1mM dithiothreitol, supplemented with the protease inhibitors 1mM phenylmethylsulphonyl fluoride, 10mM Benzamidine, 10mM Aprotinin, 10ng ml^{-1} Leupeptin, 100ng ml^{-1} DNase and 100ng ml^{-1} RNase. Ten percent (wt/vol) Zwitterion detergent in PBS (pH 7.2) was added to the suspension to a final concentration of 0.25%. After a 30min incubation at 4°C, the suspension was centrifuged at 10,000 x g for 20 min at 4°C. The pellet, which contained axonemal and subpellicular microtubules, was washed once with buffer A. The pellet was resuspended in buffer A together with 0.75M NaCl to dissolve the subpellicular microtubules, leaving the flagellar microtubules intact. Flagellar microtubules were removed by centrifugation at 10,000 x g and the salt-solubilized subpellicular microtubules were retained in the supernatant and collected. Salt-solubilized subpellicular microtubules were run through 30kDa cutoff membranes (Amicon). The material which ran through the membrane and was smaller than 30kDa was concentrated on a cutoff membrane of 10kDa (Amicon). The fraction greater than 10kDa was washed in buffer A to remove the salt. The 15kDa MAP, p15, was purified from the 10-30kDa protein fraction by the use of a tubulin affinity column as described by Balaban et al., (39). Briefly, tubulin which was isolated from bovine brain (T238, Cytoskeleton, Inc.) was coupled to an affinity resin Affi-gel 10 (BioRad) (300µl of Affi-gel 10, 500µg tubulin). The 10-30kDa fraction was incubated with the resin for 1 h at 37°C in the presence of 1mM GTP (conditions known to be favorable for microtubule polymerization). The material which did

not bind to the column was washed away, and a 15kDa protein (p15) was eluted from the column by the addition of 0.75M NaCl at 4°C.

2.5. Antibody preparation

Polyclonal antibodies directed against p15 were raised in guinea pigs which were injected three times subcutaneously (on days 0, 7, 21) with 5μg of p15. The first injection contained complete Freund's adjuvant (CFA) while the second and third injections contained incomplete Freund's adjuvant (ICFA).

2.6. Western blotting

Protein samples (1-5 μg pure proteins or 10-50μg of non-purified proteins) mixed with sample buffer (0.5 Tris pH6.8, 15% SDS, 5nM DTT, 50% glycerol, 0.01% bromophenol blue) were boiled for two min and separated by SDS-polyacryamide gel electrophoresis (SDS-PAGE). Proteins were western blotted onto nitrocellulose membranes at 100V for one hour in transfer buffer (25mM Tris base, 192mM glycine, 20% methanol). Subsequent steps were conducted at room temperature: after the transfer the membranes were blocked with 3% bovine serum albumin (BSA) in PBS with 0.05% Tween 20 for one hour and incubated in the presence of guinea pig anti-p15 polyclonal or anti-Flag monoclonal (Kodak) primary antibodies for two hours. The membranes were washed in PBS containing 0.05% Tween 20 (3 times 10 min each), and incubated for one hour with peroxidase conjugated protein A or anti-mouse secondary antibodies (Amersham). The membranes were washed as above, and bound antibody was detected using ECL chemiluminescence detection kit (Amersham).

2.7. Protein concentration

Protein concentration was determined using the Bio-Rad protein assay (BioRad) with bovine serum albumin as a standard according to the manufacturer's instructions.

2.8. Silver staining

Silver staining of the SDS-PAGE gel was carried out using Silver Stain Plus kit (BioRad) according to the manufacturer's instructions.

2.9. Extraction of DNA and RNA from *T. brucei*

RNA isolation

RNA and DNA were isolated using Trizol reagent according to the manufacturer's instructions (Gibco-BRL). Triazol reagent is a mono-phasic solution of phenol and gunidine

isothiocyanate that allows the purification of RNA and DNA in a single step. Briefly, Triazol reagent was added to trypanosomes that were separated from blood by passage through a DEAE cellulose column. Chloroform was then added in a ratio of 1:0.2 (v/v), and the suspension was vortexed and then centrifuged at 12,000 x g for 15 min. The lower phase containing DNA and protein was used for DNA purification (see below). The supernatant containing RNA was collected carefully into a second polystyrene tube. Isopropanol was added, vortexed briefly, and centrifuged as above for 15 min. The isopropanol was carefully decanted and the pellet was washed once with 0.5 ml of cold 75% ethanol, and air dried. The pellet was resuspended in DEPC-water. RNA concentration was determined by spectrophotometry at 260nm.

DNA isolation

DNA in the interphase and phenol phase (see above) was isolated by precipitation with 0.3ml of ethanol for each 1 ml of Trizol reagent that was initially used (see above). After gentle mixing, the samples were incubated at room temperature for three min and centrifuged at 2,000 x g for five min at 4°C. The aqueous phase was removed and the DNA pellet was washed twice in a solution containing 0.1 M sodium citrate in 10% ethanol. Following these two washes, the DNA pellet was washed in 75% ethanol, air dried and resuspended in 8mM NaOH.

2.10. Cloning p15

The polyclonal antibodies directed against p15 were used to screen a cDNA phage (l-ZAP) expressing library of *T. brucei* strain lab110 EATRO which was kindly provided by Dr. Pollakis (New York University). The screening was carried out as described in the picoBLUE Immunodetection kit manual (Stratagene).

2.11. Liposome binding assay

Unilamellar liposomes were prepared by solubilizing soybean asolectin in methanol:acetone {1:1 (v/v)}, evaporating the mixture and resuspending the lipids as a 20mg/ml suspension in 10mM Hepes and 100mM sucrose, pH 7.2. The suspension was then sonicated in a bath sonicator for 30min and the liposomes purified by gel filtration through Sephadex G-15. Microtubules (polymerized in the presence of mammalian MAPs or trypanosome MAPs) were mixed 1:1 (v/v) with asolectin liposomes. A drop of the mixture was applied to a formvar coated copper grid for one min and ten min after mixing and the grids negatively stained for ten sec with 1% uranyl acetate (in water). Grids were examined at 80kV in a Phillips EM 410 microscope.

2.12. PCR amplification.

PCR amplification of recombinant p15 was performed in 50 µl reaction mixtures containing 22mM Tris-HCl (pH 8.4), 55mM KCl, 1.65mM $MgCl_2$, 220µM dGTP, 220µM dATP, 220µM dTTP, 220µM dCTP, and 200nM primers. Taq DNA polymerase (2.5 units) and 0.5µg of template DNA were added. Reaction volumes were adjusted to 50µl with H_2O. Reactions were performed in a programmable thermal cycler (94°C for four min, followed by 29 cycles of 94°C for 30 sec, 51°C for 30 sec, 72°C for 60 sec, and a final elongation step at 72°C for ten min).

2.13 RT PCR

RNA (one µg) which was isolated from *T. b. brucei* using Trizol reagent (BRL) (see above) was denatured by incubation at 60°C for ten min, then immediately chilled on ice. cDNA was synthesized by incubating the denatured RNA, a primer corresponding to the antisense-specific sequence of the 3' end of the p15 recombinant gene (5'-CTGAAACGTGTGACAGCTCTC-3'), which corresponds to nucleotides 615-595 of the cDNA), together with super script II reverse transcriptase (Gibco-BRL), according to the manufacturer's instructions. The RNA template was removed by treatment with RNases H and T1 for 30 min. Unincorporated gene-specific primer and proteins were separated from the cDNA with a spin cartridge (Gibco BRL). PCR amplification was performed using primers corresponding to the 5' sense (5'- AGCAACTGCTGTCCCCAA-3' which correspond to nucleotides 116 through 134 of the cDNA) and the nested antisense 5'-CTGAAACGTGTGACAGCTCTC-3' (which correspond to nucleotides 447-426 of the cDNA). The PCR reaction was performed in a programmable thermal cycler (94°C for four min followed by 29 cycles of 94°C for 30 sec, 51°C for 30 sec, 72°C for 60 sec, and a final elongation step at 72°C for ten min). The PCR products were analyzed by electrophoresis in 1% agarose gels staining with ethidium bromide.

2.14. Cloning of PCR products

The PCR products, produced with a single deoxyadenosine (A) overhang in the 3' ends of PCR products (a feature resulting from the use of Taq polymerase, were ligated to PCR II plasmid vector (Invitrogen) that has single ovehanging 3' deoxythmidine (T) residues. This allows PCR inserts to ligate efficiently with the vector. Resulting plasmids were used to transform *E.coli* DH10B competent cells (BRL) by electroporation. White colonies were picked, grown, and plasmid DNA from these transformed colonies was isolated and analyzed for the presence of the p15 insert by digestion with EcoRI restriction enzyme. The

digested DNA was separated by electrophoresis on a 1% agarose gel. Plasmid DNA of positive clones was sequenced using T3 and T7 primers.

2.15. Southern blotting

DNA was separated by electrophoresis on 1% agarose gels and Southern blotted to a nylon membrane. The 528 bp of the p15 gene was radiolabeled by random priming using $\alpha^{32}P$ dCTP and the Readyprime kit (Amersham). Hybridization was carried out in Rapid-hyb buffer (Amersham) at 65°C for 2 h. Filters were washed twice in high stringency washes containing 2X SSC, 0.1% SDS at 65°C for 10min. Detection of the radiolabeled DNA fragment was performed by exposing the blot to autoradiography films with an intensifying screen at -70°C.

2.16. Growth of bacterial cells

The bacteria were streaked on LB-agar plates containing 80μg ml^{-1} ampicillin, and grown overnight at 37°C. Colonies were picked from the plate, resuspended in 3 ml TB plus ampicillin, and grown at 37°C for 6 hours. The culture was then added to 250ml TB medium containing antibiotics and incubated overnight.

2.17. Preparation of plasmid DNA by alkaline lysis

Bacterial cells were grown overnight at 37 °C in TB medium containing 80 μg/ml ampicillin, to increase the yield of plasmid DNA. The bacteria were harvested by centrifugation and resuspended in Lysis buffer (50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA). Freshly prepared alkaline solution was added to a final concentration of 0.2M NaOH and 1% SDS. After 5min incubation on ice, ammonium acetate solution (pH 7.8) was added to a final concentration of 3.7 M. The tube was maintained on ice for 10min to allow most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. After centrifugation, the clear supernatant was collected. Isopropanol (0.6 volumes) were added and incubated at room temperature for 10min. After centrifugation, the supernatant was removed by aspiration and the pellet was washed with 70% ethanol. The tubes were inverted on tissue paper for 15 min at room temperature to dry. The pellets were resuspended in 10mM Tris-HCl (pH 8.0).

2.18. Purification of plasmid DNA by equilibrium centrifugation in CsCl-ethidium bromide gradients

The plasmid DNA prepared by alkaline lysis was put in a clear Quick-Seal tube and for every milliliter of the DNA solution 1g of solid CsCl and 0.06 ml of ethidium bromide solution (5 mg/ml) was added, to make a final density of 1.55 g/ ml of the CsCl solution. Density gradients were centrifuged at 55,000 rpm for 12 hours in a Beckman vertical Ti65 rotor. After centrifugation, two bands of DNA were visible. The upper band consisted of linear and nicked circular plasmid DNA, and the lower band comprised of closed circular plasmid DNA. The lower band was collected using an 18 gauge needle. Ethidium bromide was removed by extraction with NaCl-saturated isopropanol. Cesium chloride was removed by dialysis against 10mM Tris-HCl, pH 8.0. DNA concentration was determined by spectrophotometry at 260nm.

2.19. Transfection of HEK293 cells for the rescue of recombinant adenovirus vectors

Human Kidney Cells, HEK293 (10^5 low passage cells) were seeded in two six-well 35mm tissue culture plates. Three ml of medium containing DMEM with 10% FBS and 100 units/ml of both penicillin and streptomycin were added. The cells were incubated at 37°C in 5% CO_2. After 24h the 70% confluent cells were washed with serum-free DMEM and co-transfected with Lipofectamine liposomes (BRL).

The following solutions were prepared in two sterile tubes.

Tube A : 1.1ml Optimen culture medium (BRL) and 120ml of Lipofectamine.

Tube B : 1.2ml Optimen and 36 ng of the pJM17 plasmid and 24 ng of the shuttle plasmid.

The two tubes were combined, mixed gently, and incubated at room temperature for 30 min to form lipid-DNA complexes. An additional 9.5ml Optimen was then added.

HEK293 cells, grown in 35mm tissue culture plates, were washed in DMEM (to remove the serum which could bind lipofectamine). The DMEM was removed, and 1 ml of the diluted lipid-DNA complexes was added to the cells. The Lipid-DNA complexes were replaced after 18 h with DMEM containing 10% FBS and 100 units/ml of both penicillin and streptomycin. After 7-14 days the cytopathic effect appeared, where transfected cells became round and detached from the plate. The culture medium, containing both cells and adenovirus, was collected. This medium was used to infect new HEK293 cells.

2.20. Extraction of adenovirus DNA

The adenovirus infected HEK293 cells were harvested. The cells were lysed with 10mM Tris HCl, pH 7.4, 1% SDS and 50µg/ml Proteinase K. After incubation at 56°C for 1h,

the viscous lysate was transferred to a microfuge tube and the DNA extracted with phenol chloroform:isoamyl alcohol (24:1) followed by chloroform:isoamyl alcohol (24:1). The DNA was precipitated with 0.25M NaAcetate pH 5.2 and 66% ethanol. The extracted DNA was washed with 70% ethanol and air dried. The DNA pellet was resuspended in 10mM Tris, pH 8.0, and digested with EcoRI and KpnI restriction enzymes and run on 1% agarose gels. Viral DNA bands were visible as sharper bands along with a background smear of cellular DNA.

2.21. Preparation of high-titre viral stocks

Since most of the virus remained associated with the infected cells until very late in the infection process, high-titre viral stocks were prepared by concentrating the infected cells. Tissue culture flasks (150mm) were seeded with HEK293 cells in DMEM containing 10% FCS and 100units/ml penicillin/streptomycin. When cells reached 90% confluency, they were infected with the virus at a Multiplicity of Infection (MOI) of 10. When the cytopathic effect was nearly completed (after 48-72 h) and most of the cells were rounded but not yet detached, the cells were harvested (they were easily dislodged by tapping). Cells were pelleted by centrifugation at 800 x g for 5 min at 4°C. Both cell pellet and supernatant were collected. Since most progeny viruses remain cell-associated, infected cells were disrupted by freeze-thaw cycles followed by lysis as described below. To every cell pellet collected from 18 flasks (175mm), 18ml of PBS/1mM $MgCl_2$/0.1% NP40/1mM $CaCl_2$, were added for hypotonic lysis followed by three rounds of freeze thawing. Crude lysates were then pelleted to remove cellular debris by centrifugation at 9,500 x g for 20 min at 4°C. The supernatants that contained the crude viral lysates were carefully removed. The viral supernatants were loaded onto CsCl step gradients made by layering three densities of CsCl (1.25, 1.33, and 1.45 g/ml) and centrifuged at 50,000 x g for 2h in a Beckman SW41 rotor at 14°C. The lower band containing the intact packaged virus was removed, and a second step density gradient of 1.33g/ml CsCl was centrifuged for 16 h at 48,000 x g at 14°C. The lower band containing the packaged virus was collected.

2.22. Plaque assays for purification and titration of the adenovirus

Plaque assays depend on the ability of the adenovirus to propagate in HEK293 cells.
Six 35mm tissue culture plates were seeded with HEK293 cells. The cells were incubated at 37°C in a CO_2 incubator until the cells were 90% confluent. Serial dilutions (10^{-8}-10^{-13} of the adenovirus stock) were made in DMEM supplemented with 2% FBS. The diluted virus was added to the cells. After 2h, the medium was removed and replaced with 1x Modified Eagle Medium (BRL) and 1% sea-plaque agarose (FMC). The agar overlay was added to

keep the virus localized after the cells had lysed. After 5 days plaques were visible and counted for titre determination after 7 days.

2.23. Detection of β-galactosidase

Detection of β–galactosidase within transduced cells in culture and in organs was performed using histochemical staining. The cells (grown on a coverslip) or frozen sections of tissue were fixed at 22°C with 0.05% glutaraldehyde for 5 min. The fixative solution was rinsed with PBS at 22°C. A solution of 15mM $K_3Fe(CN)_6$, 15mM $K_4Fe(CN)_6$, 1mM $MgCl_2$, and 1mg/ml of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (x-gal), was added and incubated from 1h to overnight at 37°C. Cells or tissue were observed using a light microscope. An intense blue halogenated indoxyl derivative indicates the presence of β–galactosidase.

2.24. Vaccination experiments

Swiss male mice were injected subcutaneously with the antigen (10μg pure p15; 10μg recombinant p15; 30μg partially purified subpellicular microtubules; or with buffer only as a negative control) 3 times on days 1, 7, and 21. The subcutaneous injections included complete Freund's adjuvant (CFA) on first inoculation and incomplete Freund's adjuvant on second and third inoculation. Both vaccinated and control animals were challenged intraperitoneally on day 31 with 500 parasites (which were previously isolated as described above), Parasitemia was followed by taking a drop of blood from the tip of the tail, applying it to a glass slide and observing it under a light microscope at 400 X magnification.

For vaccination with the adenovirus, Swiss male mice were injected subcutaneously and intramuscularly with $2x10^9$ recombinant adenovirus 3 times on days 1, 7, and 21. Animals were challenged on day 31 with 500 parasites (which were previously isolated as described above). Parasitemia was followed as described above.

2.25. Sera collection

Blood (100 μl) was collected from the tip of the tail into a heparinized micro-haematocrit capillary tube before vaccination and 7 days after the final vaccination. Sera were collected.by centrifugation.

2.26. *In vivo* transduction with adenovirus

Female Fischer 344 rats (Charles River Laboratories, Wilmington, MA; weight 150 to 175 gm) were anesthetized by inhalation of methoxyflurane. After a lower midline incision was made, dissection was carried down to the aorta. A 1.5-cm section of the aorta was clamped with an Acland clamp approximator (Microsurgery Instruments, Inc Bellaire, TX). A sample of adenovirus was injected into the aorta section between the clamps. After the removal of the Acland clamp, the abdomen was irrigated and closed with an absorbable suture. Sterile saline solution (10ml) was injected intraperitoneally to circumvent volume depletion. The skin was closed, and the animal was allowed to recover from the anesthesia. Food and water were made available immediately after the surgery. At 24h and 7 days after transduction, rats again underwent anesthesia. The abdomen was opened and the aorta was removed and flushed with a saline solution. The aorta was fixed with 0.5% glutaraldehyde and washed with a saline solution. Staining was performed with X-gal for 18h at 37°C. The aorta was then embedded in paraffin and serially sectioned.

2.27 Immunohistochemistry

Aortal blood vessels isolated from control and transduced rats were fixed at 22°C with 0.05% glutaraldehyde for 5 min and embedded in paraffin. Serial sections of the blood vessel were mounted onto glass slides. The slides were deparaffinized with xylene 3 times (5 min each), followed by rehydration in a descending series of ethanol concentrations (twice in 100% ethanol, 5 min each time, followed by 5 min treatments in 95% ethanol, 75% ethanol and 50% ethanol, followed by Tris buffered saline (TBS)). The sections were treated with 1mM EDTA, pH 10, and then microwave treated (twice for 2 min), agitated for 2 min in between, followed by microwaving for an additional 2 min. The sections were put in 1mM EDTA, pH 10, for 2min and agitated again. The sections were then rinsed twice in a wash solution (TBS, pH 7.6, containing 0.01% Triton X-100) for 5 min each time. The sections were treated with Methanol/H_2O_2 (60ml/1ml) for 30 min at room temperature and then washed again twice with the wash solution. To inhibit nonspecific immunostaining, sections were blocked in DAKO LSAB (0.25% casein in PBS, and carrier protein (Novocastra Laboratories Ltd.)) for 30 minutes, and incubated overnight in a humidified chamber at 4°C with the respective primary antibodies (anti-CD4, anti-CD8 (obtained from Novocastra Laboratories Ltd.) that were diluted 1:40 with TBS, pH 7.6, with 0.01% Triton X-100. The sections were then rinsed 3 times with TBS, pH 7.6, containing 0.01% Triton X-100 for 5 min each time. Sections were then incubated with biotinylated secondary anti-mouse antibodies for 10 min at room temperature. The sections were rinsed

twice with TBS, pH 7.6, containing 0.01% Triton X-100 and then incubated with LSAB Streptavidin (streptavidin conjugated to horse radish peroxidase, Novocastra Laboratories Ltd.) for 20 min at room temperature. The sections were rinsed twice with TBS, pH 7.6, plus 0.01% Triton X-100 and bound antibodies were detected colourimetrically, using DAB as a substrate according to the manufacturer's instructions (Novocastra Laboratories Ltd). After colour developed, the sections were washed for 5 min in Hematoxylin counter stain for 30 sec, in running water for 5 min, mounted with a coverslip using Permount (Fisher Scientific) and observed under light microscopy.

Chapter I

III STRUCTURE AND FUNCTION ANALYSIS OF p15

3.1. Introduction

Microtubules are cytoskeletal proteins, existing in all eukaryotic cells and are especially abundant in the Trypanosomatidae. Microtubules consist of tubulin and of microtubule-associated proteins (MAPs). Trypanosomes contain structurally distinct types of microtubules: flagellar, mitotic, cytoplasmic and subpellicular microtubules (36,43).

Trypanosome subpellicular microtubules are unique because, unlike other microtubules, they are arranged as uniformly spaced crosslinked microtubules, lying in close apposition to the plasma membrane (44). This organization has been attributed to the unique trypanosomal subpellicular MAPs that crosslink tubulin polymers and probably link the microtubule network to the plasma membrane (39,41). p15 is one of three classes of trypanosome subpellicular MAPs so far described (41). p15 has been shown to be specific to the subpellicular microtubules and to induce microtubule polymerization and bundling (41). Because p15 is unique to the trypanosome subpellicular microtubules and the subpellicular microtubules have been shown to be essential to the survival of the parasite (32), it is hypothesized that p15 may serve as a useful target for vaccine and therapy against trypanosomal infections.

Results

3.2. Affinity purification of p15

Commercially available tubulin, isolated from bovine brain, was coupled to an affinity resin. Solubilized subpellicular microtubules that were purified from *T. brucei* were applied to a tubulin affinity column and a protein of about 15kDa bound to the column and was eluted by salt. This protein was termed p15 (Figure 1, Lane 1). Polyclonal antibodies directed against p15 were raised in guinea pigs which were injected 3 times subcutaneously with 5μg of purified p15 as an antigen (Figure 1, Lane 2).

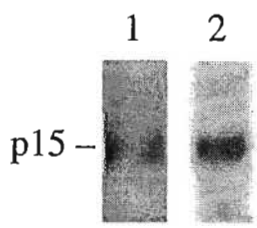

Figure 1. Affinity purification of p15. Proteins eluted from the tubulin affinity column were separated on SDS PAGE 15%, Silver stained (Lane 1) or western blotted and incubated with anti-p15 antibodies (Lane 2).

3.3. Cloning and sequencing of p15

Antibodies directed against p15 were used to screen a cDNA phage (l-ZAP) library of *T. brucei* strain lab 110 EATRO (kindly provided by Dr. Georgio Pollakis). Immunopositive phage contained a p15 gene of trypanosomal DNA, which was subcloned into the Bluescript vector (Stratagene). The subclone was sequenced using T3/T7 specific pBluescript primers. The nucleic acid sequence is shown below (Figure 2). The start of recombinant p15 and the stop codon are indicated in bold.

```
                                    ATG ACC ATG ATT ACG CCA AGC TCG      24

AAA TTA ACC CTC ACT AAA GGG AAC AAA AGC TGG AGC TCC ACC GCG      69

GTG GCG GCC GCT CTA GAA CTA GTG GAT CCC CCG GGC TGC AGG AAT      114

TCG GCA CGA GCA ACT GCT GTC CCC AAG AAA GCC GTG GCC AAA AAG      159

GCC GCT CCA AAG AAA ACT GTG GCC AAA AAG GCT GCT CCA AAG AAG      204

GCT GTC GCT AAG AAG GTT GCT CCC AAA AAG GCT GTC GCC AAG AAG      249

GTT GTC GCC AAA AAG GCT GTC GCC AAG AAG GTT GTC GCC AAA AAG      294

GTT GCC CCC AAG AAG GTT GTC GCC AAA AAG GTT GCC CCC AAG AAG      339

GTT GCC GGC AAG AAG GCC GCC GCT AAG AAG GCG TGA GCG CAT CCG      384

CTG CTG CCC GCT ATT AGA CAC GCT ATG AGG TTT ACC TGA GTG TGG      429

GAG AGA GCT GTC ACA CGT TTC AGG ACG TCC TCG TGC GTC CCT CCA      474

GGA CGG AGT TAG AAT TTT CTT ATC TTA CTT GGT TAG TTC CCT TCT      519

ACC GNT TGT AAT GGA AAT GTT TCG TTT GTG GGN TGC GTC TTA TGT      564

ACC CCC ANN CGG TGT TGG TTT CGT ANC GTT ACC AAG ANC AAA TCA      609

TCC TGA TGT GTG GCT AAT TTA ACT TCC TGT GTA TGG TTG TGG TCC      654

CAA AAA AAA AAA AAA AAA AAC TCG AGG GGG GGC                      687
```

Figure 2. cDNA sequence of recombinant p15. The ligation site (end of pBluescript vector) and stop codon of recombinant p15 gene are indicated in bold.

The p15 gene was then amplified by PCR using primers 5' AGCAACTGCTGTCCCCAA 3' which correspond to nucleotides 123 through 140 (of sequence in Fig. 2), and 5' CTGAAACGTGTGTGACAGCTCTC 3' which correspond to nucleotides 431 through 452 (of sequence in Fig. 2). The DNA used as a template was extracted from *T.b.brucei* (which was kindly provided by Dr. Sam Black, Paige Laboratory, Amhurst, MA). The PCR products (Fig. 3A lane 2) were then cloned into PCR II plasmid vector (Invitrogen) prior to transforming the plasmid into *E.coli* DH10B competent cells (BRL). White colonies were picked, grown, and plasmid DNA from these transformed colonies was isolated and analyzed for the presence of the p15 gene insert by digestion with EcoRI restriction enzyme. The digested DNA was separated by electrophoresis on 1% agarose gel (Fig. 3B lane 2), and plasmid DNA of positive clone was sequenced using M13 forward and reverse primers. The sequence of the recombinant PCR product is shown below (Fig. 4).

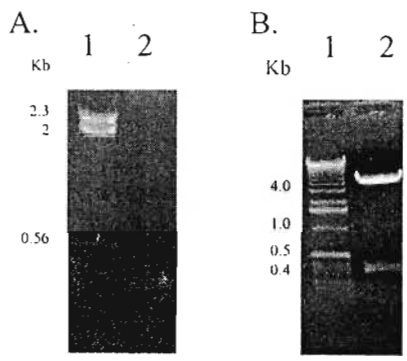

Figure 3A. PCR DNA amplification of p15 gene, using p15-specific primers.
Lane 1. λ HindIII DNA size marker.
Lane 2. p15 gene was amplified by PCR, using p15 specific primers and *T. brucei* DNA as a template. Amplified product was separated by 1% agarose gel and gel stained by ethidium bromide

Figure 3B. The digested PCR II plasmid that contained the p15 gene was separated by 1% agarose gel and stained by ethidium bromide.
Lane 1. 1KB DNA ladder as size marker.
Lane 2. PCR II plasmid that contained the p15 gene and digested with EcoRI.

```
      A GCA ACT GCT GTC CCC AAG AAA GCC GTG GCC AAA AAG GCC GCT   166
         A   T   A   V   P   K   K   A   V   A   K   K   A   A

CCA AAG AAA ACT GTG GCC AAA AAG GCT GCT CCA AAG AAG GCT GTC GCT   213
P   K   K   T   V   A   K   K     A   A   P   K   K   A   V   A

AAG AAG GTT GCT CCC AAA AAG GCT GTC GCC AAG AAG GTT GTC GCC AAA   261
K   K   V   A   P   K     K   A   V   A   K   K   V   V   A   K

 AAG GCT GTC GCC AAG AAG GTT GTC GCC AAA AAG GTT GCC CCC AAG AAG   309
 K   A   V   A   K   K   V   V   A   K   K   V   A   P   K   K

GTT GTC GCC AAA AAG GTT GCC CCC AAG AAG GTT GCC GGC AAG AAG GCC   357
V   V   A   K   K   V   A   P   K   K   V   A   G   K   K   A

GCC GCT AAG AAG GCG TGA                                         375
A   A   K   K   A   *
```

Figure 4. Nucleotide and deduced amino acid sequence of p15 gene from *T.b.brucei* amplified by PCR.

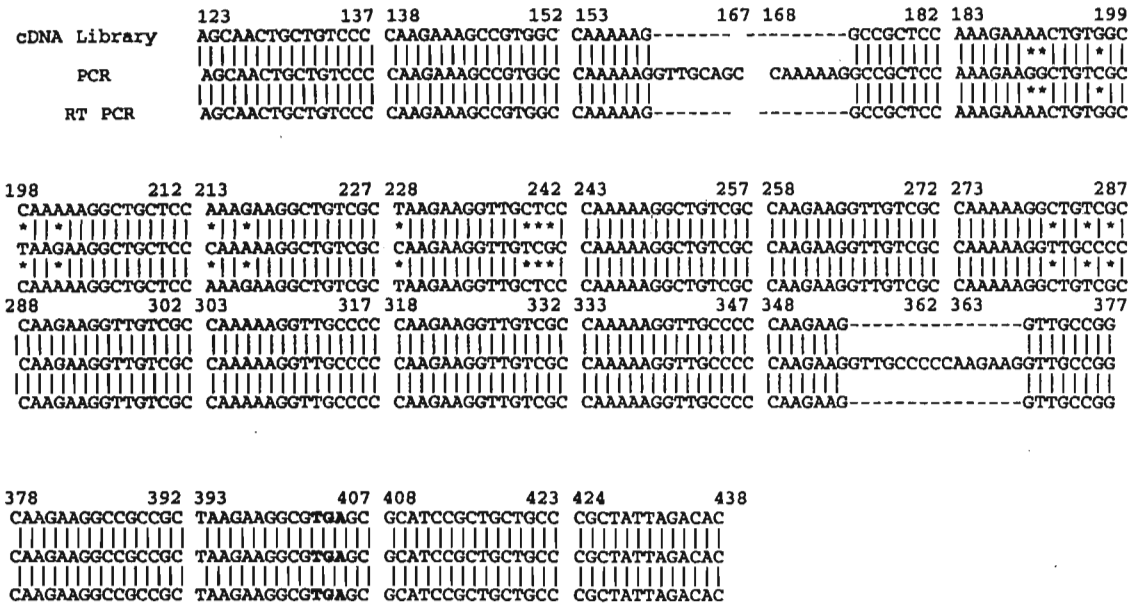

Figure 5. Alignment of DNA sequences of p15. Stop codon of p15 gene is indicated in bold. Non identical sequences are indicated by *.

3.4. Specific detection of trypanosomes by PCR using a set of primers targeted to a p15-specific DNA sequence

PCR is a valuable technique due to its potential to detect infectious agents with high specificity and sensitivity (99, 100). PCR could also be used to detect the presence of parasites in the vector (102), thus obtaining better epidemiological data in endemic areas (98).

The choice of appropriate primers in the PCR technique is crucial for obtaining more specific and more sensitive data. Because p15 is unique to trypanosome (no similar sequences from other species were found in the National Center for Biotechnology information database) and the subpellicular microtubules exist in all parasites of the Trypanosomatidae family, it is hypothesized that primers specific to p15 may be useful for the PCR detection of not only *T. brucei* but also of other trypanosome species.

To test this hypothesis, cell pellets of *T. cruzi* and *T. rangeli* epimastigotes that were grown in Noguchi-Weyon medium (kindly provided by Dr. Theis, UC Davis and contains 0.85% NaCl, 2% nutrient agar, 10% sheep blood, and brain heart infusion medium) were resuspended in water, boiled for 5min, and used as templates for p15 DNA amplification by PCR, using p15-specific primers. As a positive control, *T. brucei* genomic DNA was used as a template, and as a negative control DNA extracted from the human cell line HEK293 was used as a template.

As shown in Figure 6, DNA was amplified in all trypanosome species tested. The DNA that was amplified from *T. brucei* and *T. cruzi* was 329 bp long, and the DNA that was amplified from *T. rangeli* was approximately 1kb long. p15 did not amplify DNA from HEK239 cells). The results suggest that in fact primers specific to p15 DNA could be used to detect trypanosomes.

The different sizes of DNA which were amplified from *T. rangeli* and *T. cruzi* or from *T. brucei* may allow specific diagnosis in endemic areas. In the case of *T. rangeli and T. cruzi* the different sizes of amplified DNA may be of special importance regarding diagnosis, because these parasites are present in highly overlapping geographic areas. In Panama, *T. rangeli* is found six times more frequently than *T. cruzi*, in a population in which the average combined infection rate is 3.4% (23).

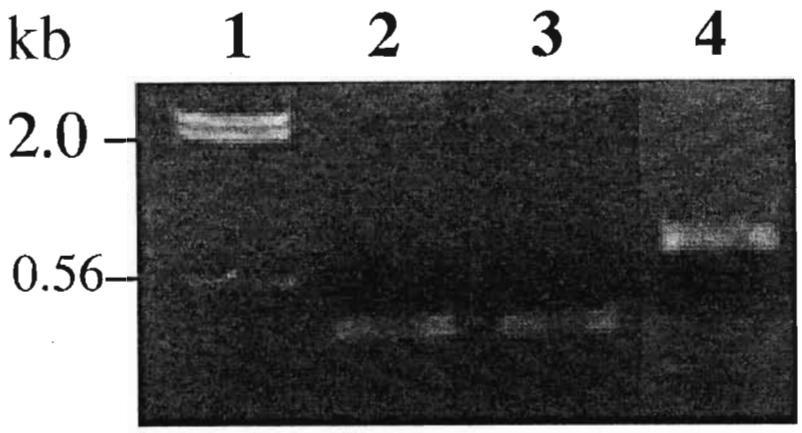

Figure 6. PCR DNA amplification, using p15-specific primers. Amplified DNA was separated by electrophoresis on 1% agarose gel and stained with ethidium bromide.
Lane 1. λ HindIII DNA size marker.
Lane 2. *T. brucei* DNA amplified by PCR.
Lane 3. *T. cruzi* DNA amplified by PCR.
Lane 4. *T. rangeli* DNA amplified by PCR.

3.5. Amino acid sequence analysis of p15

The deduced amino acid sequence shows that p15 contains highly organized, nearly identical tandem repeats with a periodicity of 10 amino acids, rich in positively charged and in nonpolar (hydrophobic) amino acids. For clarity, the sequence of the recombinant p15 is shown again below (Figure 7).

A				B
	GCACGAGC	AACTGCTGTC	118	AR ATAV
CCCAAGAAAG	CCGTGGCCAA	AAAGGCCGCT	148	PKK AVA KKAA
CCAAAGAAAA	CTGTGGCCAA	AAAGGCTGCT	178	PKK TVA KKAA
CCAAAGAAGG	CTGTCGCTAA	GAAGGTTGCT	208	PKK AVA KKVA
CCCAAAAAGG	CTGTCGCCAA	GAAGGTTGTC	238	PKK AVA KKVV
GCCAAAAAGG	CTGTCGCCAA	GAAGGTTGTC	268	AKK AVA KKVV
GCCAAAAAGG	TTGCCCCCAA	GAAGGTTGTC	298	AKK VAP KKVV
GCCAAAAAGG	TTGCCCCCAA	GAAGGTTGCC	328	AKK VAP KKVA
GGCAAGAAGG	CCGCCGCTAA	GAAGGCGTGA	358	GKK AAA KKA

Figure 7. Nucleotide (A) and corresponding amino acid (B) sequence of recombinant p15.

Analysis of p15 sequence data with the programmes of Garnier et al (40) and Chou and Fasman (45) suggests that the secondary structure of p15 may consist largely of α-helices, interrupted by the presence of prolines. Construction of a helical wheel diagram (46) predicts that unlike in mammalian MAPs where charged amino acids are predicted to cluster on one side of the helix (47), charged amino acids of p15 are distributed more uniformly around the helix (Figure 8).

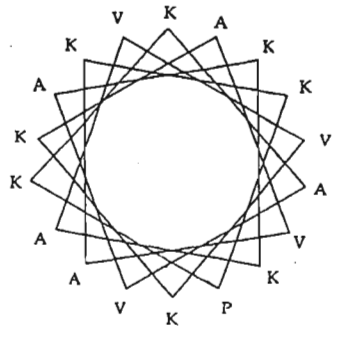

Figure 8. Helical 'wheel diagram' of the α-helix for p15 amino acid residues 37-55, assuming 3.6 amino acids per turn (47).

3.6. Function analysis of p15

The high content of hydrophobic amino acids suggests the possibility that p15 contains hydrophobic domains available for interaction with phospholipids and thereby crosslinking of the subpellicular microtubules to the plasma membrane. To test for this hypothesis, microtubules were polymerized in the presence of p15 and with soybean asolectin liposomes which were used as a model system for the plasma membrane. A sample of the mixture was observed under the electron microscope one and 10 min after mixing. The results shown in Figure 9 indicate that while calf brain microtubules (containing both tubulin and endogenous MAPs) only had minor associations with the liposomes (Figure 9A,B), microtubule polymers made of calf brain tubulin and p15 interacted strongly with liposomes, crosslinking them and creating tangles between liposomes and microtubule polymers (Figure 9C after one min and Figure 9D after 10 min). These results suggest that p15 may indeed crosslink microtubules to the plasma membrane. Inset in Figure 9C shows the bundling of microtubules in the presence of p15.

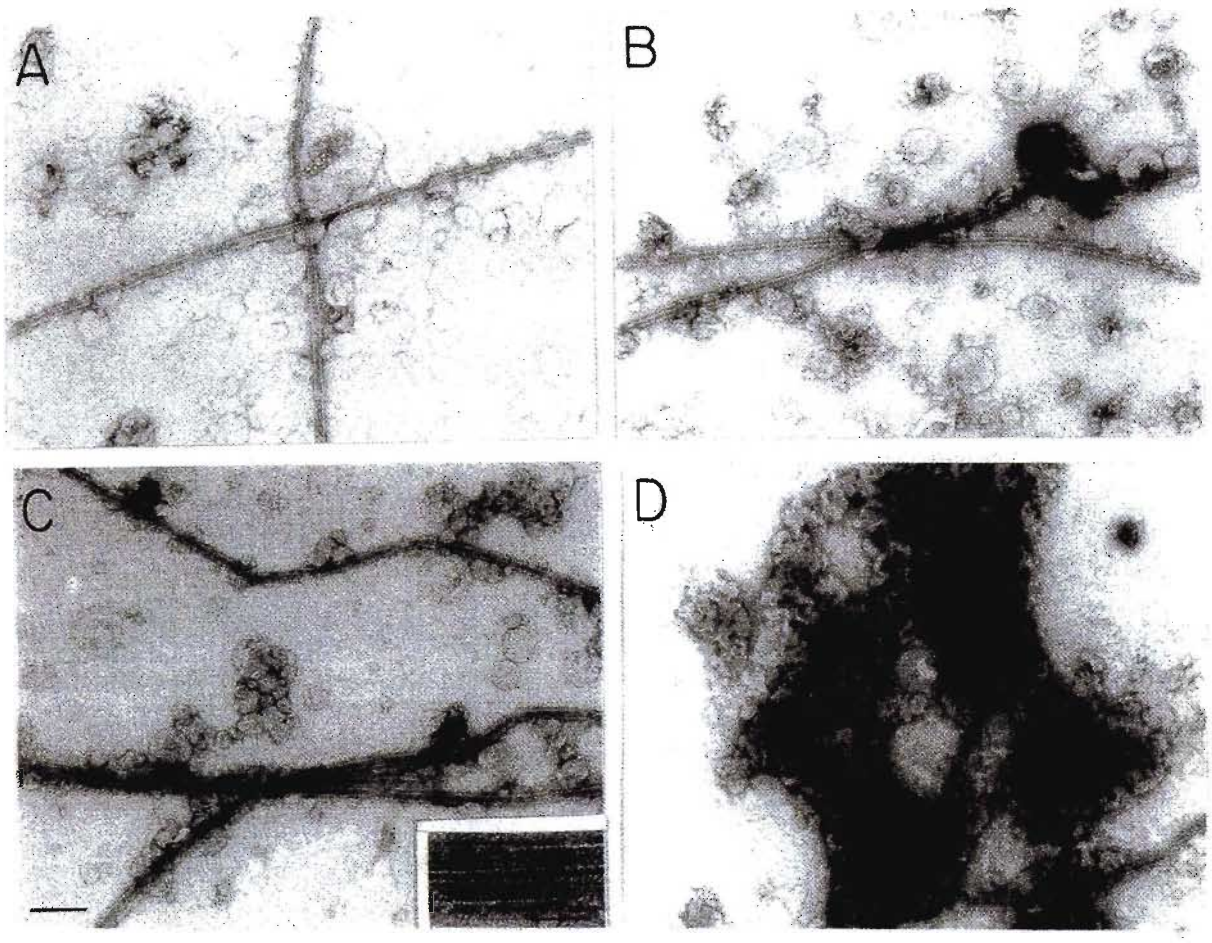

Figure 9. Crosslinking of liposomes to microtubule polymers – negative staining. Calf brain tubulin was incubated with calf brain MAPs and with liposomes for one min (A) and 10 min (B). Purified calf brain tubulin was incubated in the presence of p15 and liposomes for one min (C) and 10 min (D). At the indicated times a drop of liposomes was applied to a formvar coated grid, negatively stained for 10 sec with 1% uranyl acetate, and grids were examined at 80kV in a Phillips EM 410 microscope (bar, 168nm. Inset bar, 59nm).

3.7. Discussion

The aim of the experiments described in this chapter was to study the structure and function of p15. p15 is a unique 15kDa MAP which was purified from the subpellicular microtubules of *T. brucei.* p15 binds tubulin and has been shown to bundle the microtubule polymers (41).

p15 was cloned by screening a cDNA library of *T. brucei* using anti-p15 antibodies. The sequence data of this clone was used to amplify the p15 gene by PCR, using *T. brucei* genomic DNA as a template. Comparison of the two sequences reveals that the two are essentially identical. However, the p15 genomic DNA sequence is 30bp longer than the cDNA sequence, where two stretches of 15bps each are present in the genomic DNA but are absent in the cDNA, suggesting that the mRNA has been spliced. Alternative splicing is a common feature not only in trypanosomes (47, 48) but also in MAPs, where for example developmentally regulated alternative splicing of mRNAs encoding the mammalian MAP tau has been demonstrated (49). Interestingly, each p15 spliced region encodes five residues that, if translated, would have contributed to an additional repeat unit and to an additional helix turn. It is tempting to speculate that different lengths of p15 are expressed at different stages of the parasite's life cycle and that the length of the p15 helix can contribute to the level of its interaction with tubulin and as a consequence to the stability of the subpellicular microtubules.

Search of the NCBI data base show that p15 is unique to trypanosomes. The deduced amino acid sequence of p15 shows that it contains 8 highly organized, nearly identical tandem repeats with a periodicity of 10 amino acids. A repetitive motif has been shown to be a common feature in MAP architecture (50, 51, 53, 54, 55, 56, 57, 58), thus strengthening the hypothesis that p15 is indeed a MAP.

Analysis of p15 sequence data with the Programs of Garnier et al. (40) and Chou and Fasman,(45) suggests that the secondary structure of p15 consists largely of α-helices, interrupted by the presence of prolines. Construction of a helical wheel diagram (40) predicts that unlike in mammalian MAPs where charged amino acids are predicted to cluster on one side of the helix (45), charged amino acids of p15 are distributed more uniformly around the helix. The binding of MAPs to tubulin occurs via positively charged MAPs that bind to the negatively charged C terminus of tubulin. If the charged amino acids are clustered on one side of the helix, only that side of the molecule can interact with the

negatively charged tubulin. Because in p15 the charged amino acids are distributed on either sides of the helix, it is possible that this charge distribution enables the molecule to bind tubulin on either side of the molecule, thus bundling the microtubule polymers to one another.

Unlike other microtubules, the subpellicular microtubules are unique to the trypanosomes both by the fact that the microtubules are crosslinked to one another and by their unique localization, lying right beneath the plasma membrane. It had been suggested that unique trypanosomal MAPs may not only crosslink the microtubule polymers to one another but may also crosslink the polymers to the plasma membrane, thus creating a highly organized stable cytoskeletal structure.

Unlike other MAPs (including MARP), the sequence of p15 suggests that it is of an amphipathic nature, containing repetitive sequences of both charged and hydrophobic amino acids. It is therefore hypothesized that not only may p15 be able to crosslink microtubule to one another via the charged amino acids, but may also crosslink the polymers to the plasma membrane via its hydrophobic domains, which would be available for interaction with phospholipids (40). To test for this hypothesis, microtubules were polymerized in the presence of p15 and mixed with soybean asolectin liposomes which were used as a model system for the plasma membrane (38). The results indicate that while calf brain microtubules (containing both tubulin and endogenous MAPs) did not associate with the liposomes, microtubule polymers made of calf brain tubulin and p15 interacted strongly with liposomes, crosslinking them and creating tangles between liposomes and microtubule polymers. These results suggest that p15 may indeed crosslink microtubules to the plasma membrane.

The p15 genomic DNA sequence is 30bp longer than the cDNA sequence, where two stretches of 15bps each are present in the genomic DNA but are absent in the cDNA, suggesting that the mRNA has been spliced. Alternative splicing is a common feature not only in trypanosomes but also in MAPs, where for example developmentally regulated alternative splicing of mRNAs encoding the mammalian MAP tau has been demonstrated Interestingly, each p15 spliced region (at positions nt 160 and 355) encodes five residues that if translated would have contributed to an additional repeat unit and to an additional helix turn. It is tempting to speculate that different lengths of p15 are expressed at different stages of the parasites life cycle and that the length of the p15 helix can contribute to the

level of its interaction with tubulin, and as a consequence, to the differential stability of the subpellicular microtubules.

p15 DNA sequences may serve as a useful tool for future diagnosis of various trypanosome species by PCR, because repetitive target sequences have been shown to be very suitable for sensitive and specific diagnosis of infective agents (97). Diagnostic methods of trypanosomiasis by direct parasitological techniques like haematocrit centrifugation, mini-anion-exchange centrifugation and microscopic examination (101, 103), have shown high sensitivity in the acute phase of the infection, when large numbers of bloodstream parasites are present. But in the chronic phase, when few parasites are present, these techniques are not sensitive enough. A more sensitive and potentially specific technique would be the use of PCR (98,99,100,103).

PCR is a valuable technique due to its potential to detect infectious agents with high specificity and sensitivity. PCR could also be used to detect the presence of parasites in the vector, thus obtaining better epidemiological data in endemic areas (98).

The detection rate of PCR has been shown to be about two times higher than that of the direct parasitological techniques, suggesting a higher sensitivity (103). These studies used trypanosome species-specific oligonucleotide primers (59). Primers used for the detection of *T. brucei* ssp. incldude primers specific to the varient surface glycoprotein gene (90), primers specific to the kinetoplast minicircle DNA (91), and primers specific to the mini-exon repeat DNA (92). Other studies used primers specific to the kinetoplast DNA for the detection of *T. cruzi* (60).

The choice of appropriate primers in the PCR technique is crucial for obtaining more specific and more sensitive data. Because p15 is unique to trypanosome subpellicular microtubules and the subpellicular microtubules exist in all parasites of the Trypanosomatidae family, it is hypothesized that primers specific to p15 may be useful for the PCR detection not only of *T. brucei* but of other trypanosome species as well.

When DNA preparations from cell pellets of *T. cruzi* and *T. rangeli* epimastigotes were used as templates for DNA amplification by PCR using *T. brucei* p15-specific primers, DNA was specifically amplified in all trypanosome species tested. The DNA that was amplified from *T. cruzi* was 310 bp long (like the one amplified from *T. brucei*), while DNA that was amplified from *T. rangeli* was approximately 1kb long. These results

suggest that in fact primers specific to p15 DNA could be used to detect a range of trypanosomes. In addition, the different sizes of DNA, which were amplified from *T. rangeli* and *T. cruzi* or from *T. brucei* may allow specific diagnosis of specific trypanosome species in endemic areas. In the case of *T. rangeli and T. cruzi*, the different sizes of amplified DNA may be of special importance regarding diagnosis, because these parasites are present in highly overlapping geographic areas. In Panama, for example, *T. rangeli* is found six times more frequently than *T. cruzi*, in a population in which the average combined infection rate is 3.4% (23).

To summarize, structure/function analysis of p15 indicates that it is a unique trypanosome MAP which can bundle microtubule polymers to one another and also to the plasma membrane, thus contributing to the stable and unique structure of the subpellicular microtubules. p15 has also been shown here to be of potential use for the detection, and perhaps diagnosis, of various trypanosome species in infected individuals.

Chapter IV

RECOMBINANT p15 ADENOVIRUS AS AN EXPRESSION SYSTEM IN MAMMALIAN CELLS

4.1. Introduction

The aim of the experiments described in this chapter was to develop a delivery system of p15 into mammalian cells

Adenoviruses are non-enveloped viruses with their genomes packaged in an icosahedral protein capsid. The wild type viruses can cause a mild upper respiratory disease in humans. Adenoviruses were first cultured in 1953 during attempts to establish tissue culture lines from adenoidal tissue surgically removed from children. Since then, 42 distinct adenovirus serotypes have been identified in humans (62).

It was observed that adenoviruses administered orally to humans resulted in an asymptomatic intestinal infection and in immunity against adenoviral respiratory diseases (61,62). Millions of US military recruits were successfully immunized with the adenovirus and were shown to be protected from upper respiratory infections (61,62). This suggested that the adenovirus is safe for use and is effective in inducing an immune response. The success of these experiments also made the adenovirus a popular recombinant transfer vector for the expression of foreign genes, to elicit a specific host's immune response (63).

A number of studies in animal models have shown that recombinant adenovirus vectors are efficient in inducing protective immunity against various pathogens. For example, when recombinant adenoviruses encoding the hepatitis-B surface antigen (HBsAg) were used as vaccine delivery systems, a high antibody response to HBsAg was induced (63). When recombinant adenoviruses expressing the HIV Env and Gag proteins were used as vaccine delivery systems, high-titred IgG responses to Env and Gag proteins were induced (64,65). When recombinant adenoviruses expressing vesicular stomatitis virus (VSV) were used as a vaccine delivery system, the animals were protected against a subsequent intravenous challenge of lethal doses of VSV (66). When recombinant adenoviruses expressing the herpes simplex virus (HSV) glycoprotein B (gB) were injected into mice, the mice were protected from a lethal challenge of HSV (67,68,69,70).

The recombinant adenovirus was selected as a delivery system to express p15 in mammalian cells because this system has emerged as a highly promising method for vaccine development. The major advantages are its ability to induce the host's immune response, its wide host range and its high transduction efficiencies.

The use of the recombinant adenovirus as an expression system in mammalian cells

To test the efficiency of the adenovirus as an expression system in mammalian cells, an adenovirus with reporter genes was constructed. The Green Fluorescent Protein (GFP) and the enzyme β-galactosidase were used as markers. Using such markers enabled the efficiency of the delivery of the gene and its expression to be demonstrated, and to determine in which cells the recombinant protein was expressed.

The adenovirus

The adenovirus contains a linear double stranded DNA of about 36 kb packaged in an icosahedral protein capsid. The adenovirus genome contains early (E1-E4) and late (L1-L5) genes which are expressed before and after replication of the viral chromosome, respectively. Replication-defective viruses which cannot replicate independently can be created by deleting the E1 region of the virus. This defective virus is very safe because it cannot replicate unless E1 is provided in trans. It can, however, replicate *in vitro* in human embryonic kidney cell lines (e.g. HEK293) that have been transformed by E1 sequences (66).

The adenovirus enters the cell via receptor mediated endocytosis into the lysosomal compartment from which the viral genome escapes. The adenovirus genome is released into the cytoplasmic compartment and is efficiently translocated to the cell nucleus. The DNA that remains episomally is transcribed and translated, causing the expression of the virus encoded proteins, including the foreign protein which is under the control of a suitable promoter (71).

4.2. Results

The adenovirus cloning system is a binary system, made of two plasmids, pJM17 (71) and a shuttle vector (72), containing the gene of interest. The plasmid pJM17 contains the full length of the adenovirus genome, including a 4.4 kb sequence of antibiotic resistance genes. Even though this plasmid contains the whole adenovirus genome, it cannot be packaged and become an infective virus because of its large size (resulting from the 4.4kb

insert). The plasmid is not stable and it often undergoes rearrangements during propagation in the host bacterium (Figure 10,11).

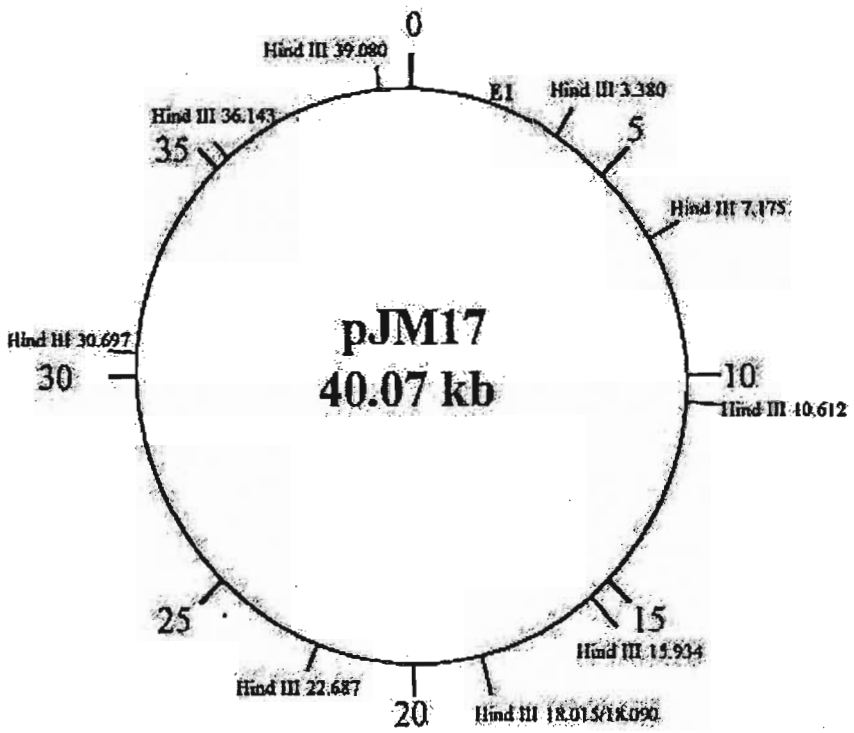

Figure 10. Map of pJM17 plasmid contains the full length of the adenovirus genome. Sites of HindIII cleavage are indicated (72).

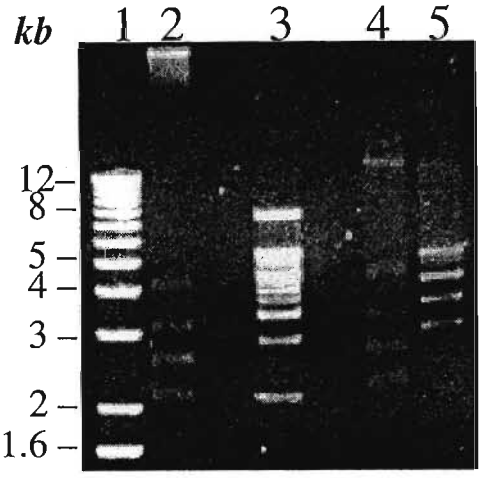

Figure 11. Restriction enzyme analysis of the 40.07kb DNA plasmid pJM17 that encodes the full-length of the adenovirus type 5 genome. The plasmid DNA was separated by electrophoresis on a 1% agarose gel.

Lane 1. 1kb DNA ladder (BRL) as a marker.

Lane 2. Undigested pJM17 that did not undergo any detectable rearrangements.

Lane 3. pJM17 that was digested with HindIII restriction enzyme, that did not undergo any detectable rearrangement.

Lane 4. Undigested pJM17 that rearranged during propagation in the host bacteria.

Lane 5. pJM17 that rearranged during propagation in the host bacteria digested with HindIII restriction enzyme.

4.3. Homologous recombination between the shuttle plasmid and pJM17

To generate recombinant adenovirus of a packageable size, pJM17 and a shuttle plasmid was co-transfected in HEK293 cells (Figure12A). The target gene that was cloned into the shuttle vector integrated into pJM17 by deleting the E1 region, including the 4.4 kb insert. The recombination events occur in two steps. The first step is a single cross-over event (Figure 12B), resulting in integration of the circular plasmid and the creation of tandem duplication (Figure 12C). The second step is a recombination event that occured between the tandem repeats, resolving the structure into a small circular DNA molecule and a recombinant viral genome. The recombinant viral genome resulted in an infectious recombinant adenovirus with the gene insert replacing the native E1 region and the 4.4 kb insert. This recombinant genome was packaged (Figure 12D), and replicated in HEK293 cells (Figure 12E) as indicated by plaque formation within 7-14 days.

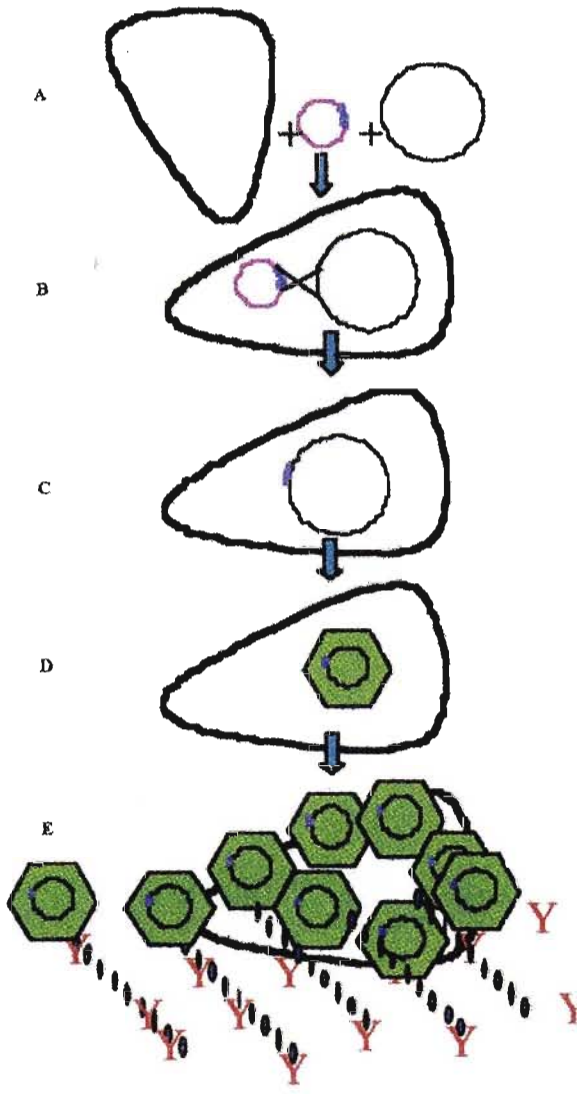

Figure 12. A diagram illustrating the adenovirus binary cloning system. Homologous recombination between the two plasmids in HEK293 cells (A,B), resulted in the reconstruction of a full-length recombinant adenovirus genome of packageable size in which the foreign gene is integrated into pJM17 and replaces the native E1 region (C,D,E). (Y) antibody directed against p15.

4.4. Construction of the adenovirus shuttle vector plasmid pRR1

pRR1 (Figure 13) was constructed by cloning the cytomegalovirus (CMV) promoter for constitutive expression, the polylinker cloning site for easy cloning of target genes and the polyadenylation signal (from bovine growth hormone), for efficient RNA processing. This expression cassette is flanked by the adenovirus E1 sequences that determine the site of homologous recombination in HEK293 cells.

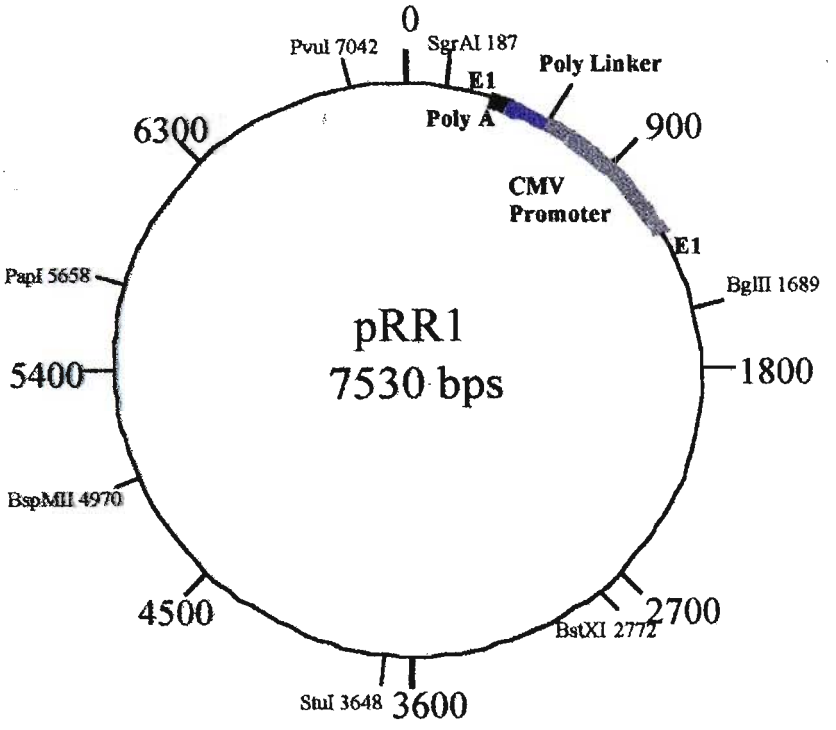

Figure 13. A map of the adenovirus shuttle vector pRR1.

pRR1 is an adenovirus shuttle vector for expression of recombinant genes in the recombinant adenovirus binary cloning system. pRR1 contains an expression cassette with a CMV constitutive promoter, polylinker cloning site for easy cloning of target genes, and bovine growth hormone polyadenylation signal for efficient RNA processing. The expression cassette is flanked by adenovirus E1 sequences that determine the site of homologous recombination.

Fluoresent Protein (GFP)

To construct a recombinant adenovirus containing the reporter gene expressing GFP, the GFP gene was cloned into pRR1 shuttle vector (pRR2, Figure 14). The GFP gene was recently cloned from the jelly fish *Aequorea victoria* and has added an additional tool for visualizing gene expression in living eukaryotic cells. This naturally fluorescent protein requires no added substrates. GFP absorbs UV light and emits green light at 509nm similar to fluorescein and is therefore used as a reporter gene (73).

In this study the GFP gene was isolated from the Green Lantern vector (BRL) by digestion with the NotI restriction enzyme. The 750bp fragment was purified from the gel using a Qiagen kit and was subcloned into pRR1 shuttle plasmid into the NotI site between the Cytomegalovirus immediate-early promoter (CMV) and the polyadenylation signal from bovine growth hormone.

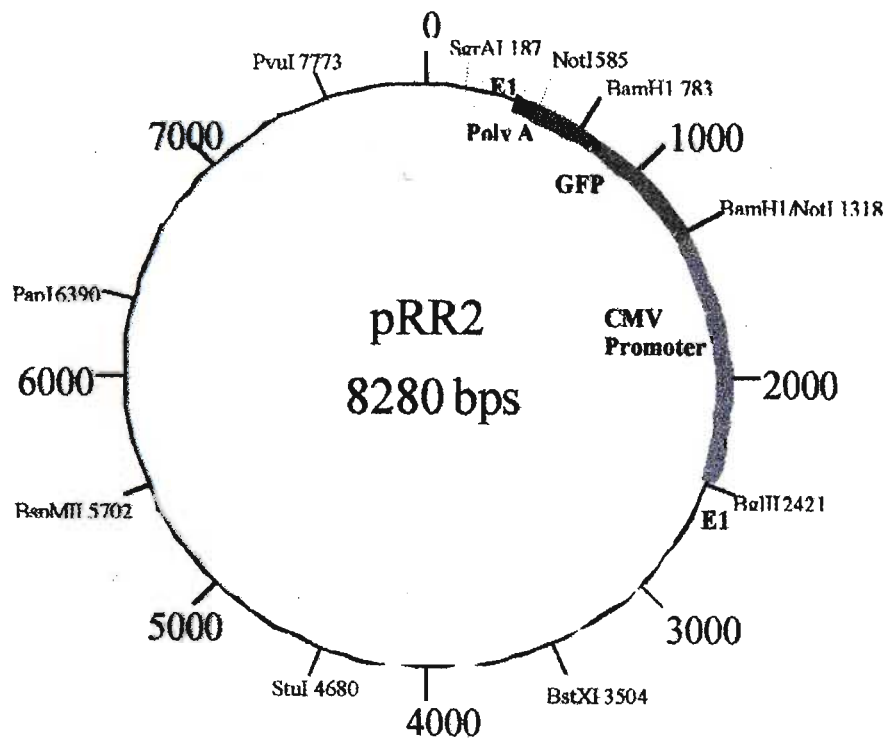

Figure 14. A map of pRR2, the adenovirus shuttle vector that contains the green fluorescent protein (GFP) gene. pRR2 is a pRR1 derivative vector, which contains the GFP gene under the control of a CMV constitutive promoter, thus enabling visualization of gene expression in living mammalian cells.

Escherichia coli DH10B competent cells (Life Technology Inc.) were transformed with the ligated DNA by electroporation. Colonies were picked, grown, and plasmid DNA from these transformed colonies was isolated and analyzed for the presence of the GFP insert and its orientation by digestion with BamHI restriction enzyme that cut once in the insert and once in the vector. The insert may exist in one of two different orientations. 535bp fragment demonstrated the presence of the GFP gene in the correct orientation. The wrong orientation would have resulted in a 215bp fragent.

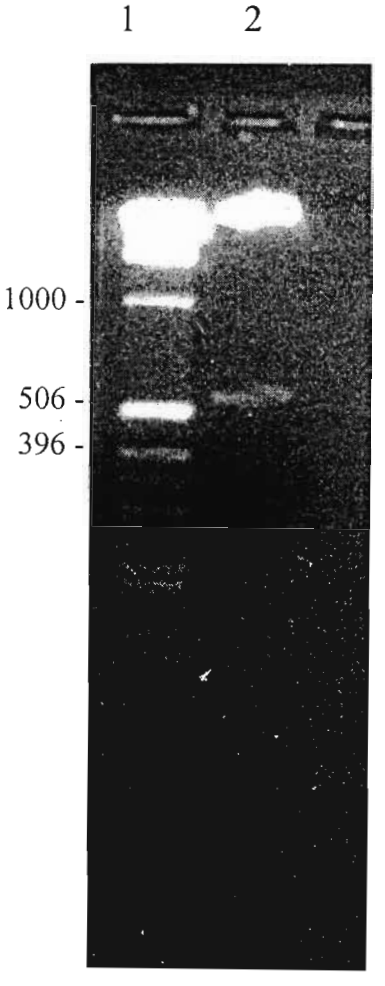

Figure 15. Restriction enzyme analysis of the shuttle plasmid pRR2 that was digested with BamHI and separated by electrophoresis on a 3% agarose gel and stained by ethidium bromide.
Lane 1. 1kb DNA ladder (BRL) as a size marker.
Lane 2. BamHI digested pRR2 plasmid that generated two fragments: 7748 and 535bp fragments that demonstrated the presence of the GFP gene in the correct orientation.

4.6. Detection of recombinant GFP gene in mammalian cells

To demonstrate that pRR2 is capable of expressing the GFP gene in mammalian cells, HEK293 cells were transfected by Lipofectamin liposome with pRR2A and pRR2C. The transfected cells were analyzed by fluorescence microscopy. Only the shorter insert in pRR2C expressed the GFP and, therefore, the other construct, pRR2A, was not characterized further.

4.7. Construction of an adenovirus expressing GFP

The plasmid pJM17 and the shuttle plasmid pRR2B were co-transfected into HEK293 cells by Lipofectamin liposome. After 10 days a recombinant adenovirus containing the GFP gene, which replaced the native E1 region and the 4.4kb additional insert, was isolated. This recombinant adenovirus became apparent as a plaque on HEK293 cells within 10 days. To test the host range and the transduction efficiencies in mammalian cells, five different types of mammalian cells were transduced with Ad-GFP. After 12 h cells were analyzed for the expression of the GFP gene by fluorescence microscopy (Figure 16). In Figure 16 A-E, cells were transduced with a multiplicity of infection (MOI) of 100 of the recombinant adenovirus GFP. In Figure 16 F, G, cells were partially transduced with MOI of 1 of the recombinant adenovirus GFP. As shown in Figure 16, all types of cells were successfully transduced with Ad-GFP (as detected by fluorescence). As shown in Figure 16F, only partial transduction took place. HEK293 cells were analyzed by phase microscopy to observe all cells. The cells were then analyzed by fluoresence microscopy to detect GFP-expressing cells. The two pictures were merged (Figure 16F) and show that not all the cells were transduced. In all cell lines tested, non transduced cells showed no autofluoresence at all, and the microscope field was completely dark (not shown).

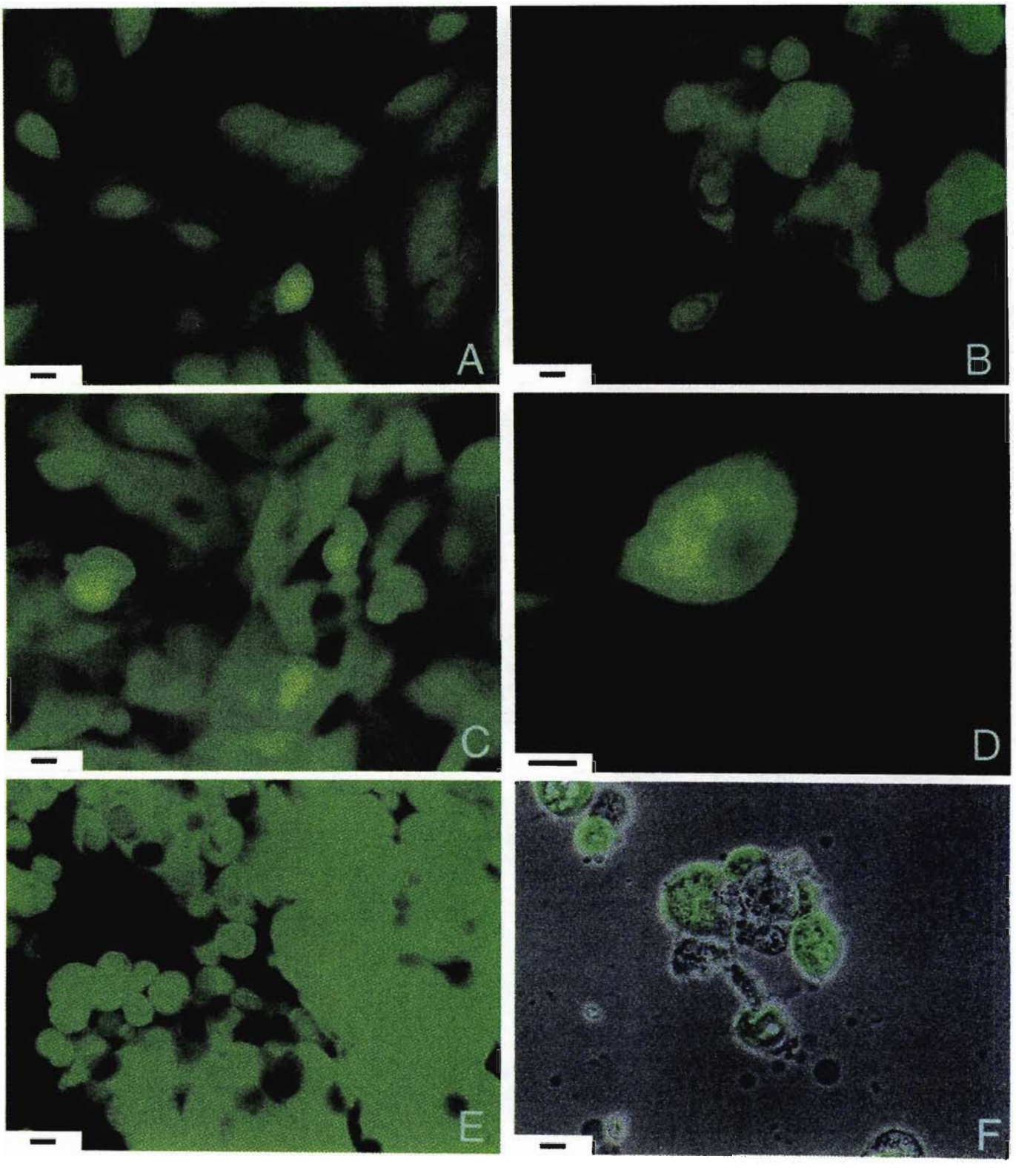
A
B
C
D
E
F

Figure 16. Expression of the GFP gene in mammalian cells analyzed by fluorescence microscopy (A-E, G) and by both fluorescence and phase contrast microscopy (F) (bar,10.μm.)

A. Human bronchus airway epithelial cells (HBE1).

B. Human unibillical vein endothelial cells (HUVEC).

C. Human epithelial retinal pigment cells line (ARPE-19).

D. Human prostate adenocarinoma cells (PC-3).

E. Human embryonic kidney cell line (HEK293).

F. HEK293 cells were partially transduced with recombinant adenovirus GFP (fluorescence and phase contrast microscopy).

G. HEK293 cells were partially transduced with recombinant adenovirus GFP. Same image as F (fluoresence microscopy).

4.8. Construction of a recombinant shuttle plasmid containing the bacterial enzyme *lacZ* gene

As shown in Figure 16A,B, the presence of a functional GFP protein was demonstrated in adenovirus-GFP transduced mammalian cells. However, in many cases it was difficult to distinguish between GFP fluorescence and endogenous tissue autofluorescence which limited the usefulness of this reporter gene. Such autofluorescence was not observed in the cell lines. To overcome this problem, an adenovirus expressing β galactosidase as a marker was used instead.

The bacterial *lacZ* DNA sequence was isolated from a pVS-β-galactosidase plasmid vector (Promega) by digestion with HindIII and BamHI. To construct the shuttle vector pRR3, the 3.7 kb fragment was purified from the gel using a Qiagen kit and was ligated to the pRR1 shuttle plasmid (which was digested with the same enzymes) between the CMV immediate-early promoter and the polyadenylation signal from the bovine growth hormone (Figure 17).

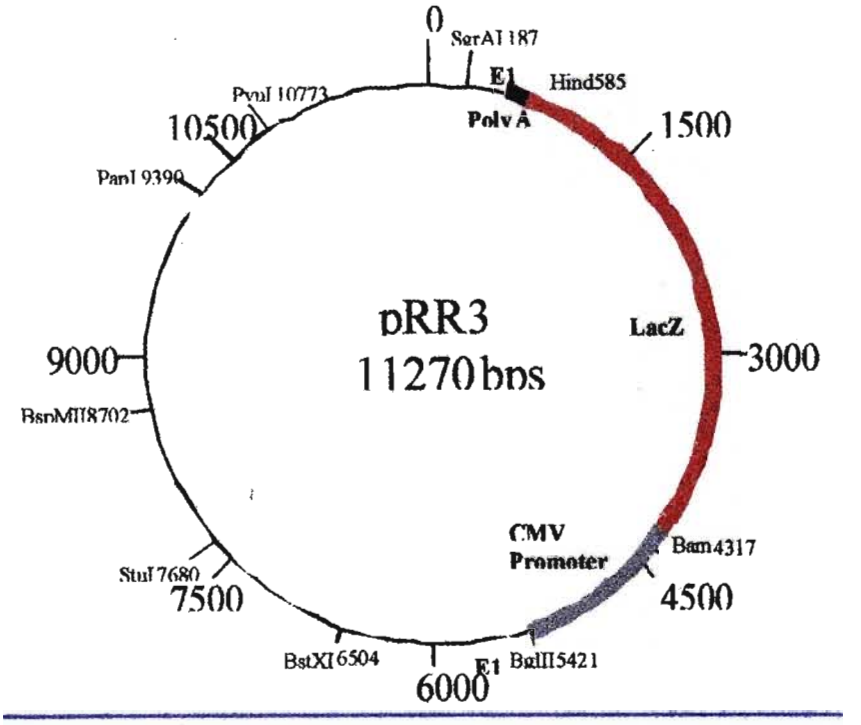

Figure 17. Map of the shuttle vector pRR3 carrying the *lacZ* DNA sequence flanked by the E1 region. pRR3 is a pRR1 derivative vector which contains the *lacZ* gene under the control of the CMV constitutive promoter

Escherichia coli DH10B competent cells (Life Technology Inc.) were transformed with the ligated DNA by electroporation. Plasmid DNA from these transformed colonies was isolated and analyzed for the presence of the *lac-Z* insert by digestion with HindIII and BamHI restriction enzymes. The digested DNA was separated by electrophoresis on agarose gels (Figure 18) and the clones containing a 3.7 kb fragment were collected. One of the clones was selected for further analysis. This construct contained the (CMV) promoter for constitutive expression of *lac-Z* and a polyadenylation signal (from bovine growth hormone) for efficient RNA processing, flanked by adenovirus E1 sequences that determine the site of homologous recombination to facilitate recombination into the adenovirus vector.

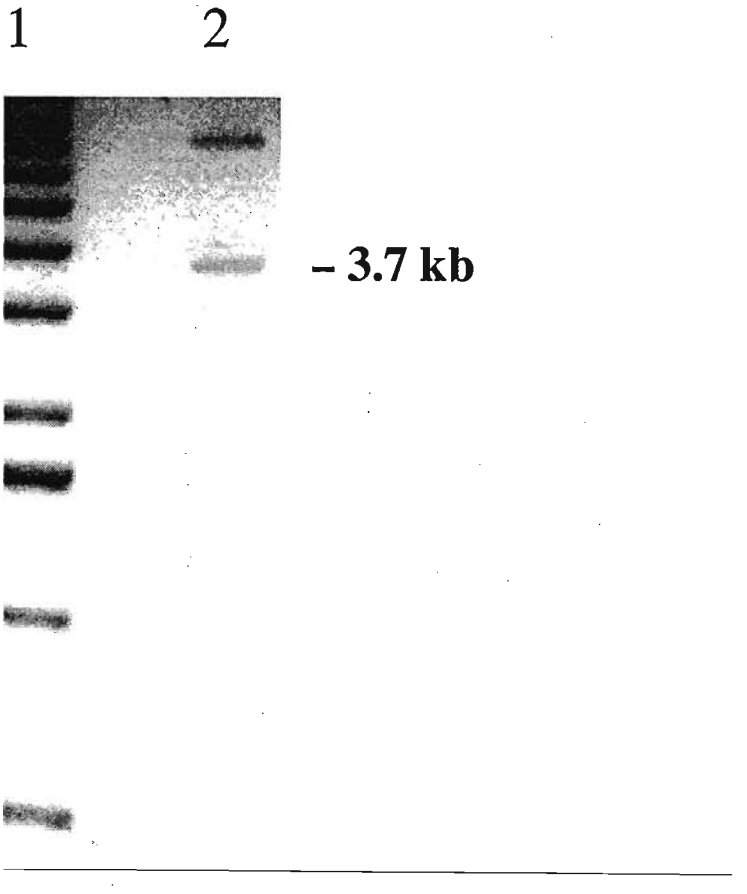

Figure 18. Restriction enzyme analysis of the shuttle plasmid pRR3 that was digested with BamHI and HindIII. The plasmid was separated by electrophoresis on a 1% agarose gel and stained by ethidium bromide.

Lane 1. 1 kb DNA ladder (BRL) as a size marker.

Lane 2. BamHI, HindIII digested pRR3 plasmid that generated two fragments: 7500bp (the shuttle plasmid) and 3737bp (the *lacZ* gene).

4.9. Analysis of the shuttle plasmid pRR3 containing the recombinant *lac-Z* gene in mammalian cells

To demonstrate that pRR3 is capable of expressing the *lac-Z* gene in mammalian cells, HEK293 cells were transfected by Lipofectamin liposome with pRR3. The cells were fixed by glutaraldehyde and stained with x-gal for the detection of β-gal . The results indicated that cells transfected with pRR3 can express β-gal (not shown).

4.10. Construction of recombinant adenovirus-*lacZ* virus

HEK293 cells were co-transfected with pJM17 and the shuttle plasmid pRR3 by Lipofectamin liposome. The cytopathic effect of the virus appeared after 14 days (the infected cells became more round and detached from the plate). The medium containing the virus at MOI of 10 was collected and used to infect HEK293 or Cos7 cells. The cells were fixed by glutaraldehyde and stained with x-gal for the detection of β-gal (Figure 19). Of note is the fact that cells that were not transduced did not express any detectable amounts of β-gal (not shown).

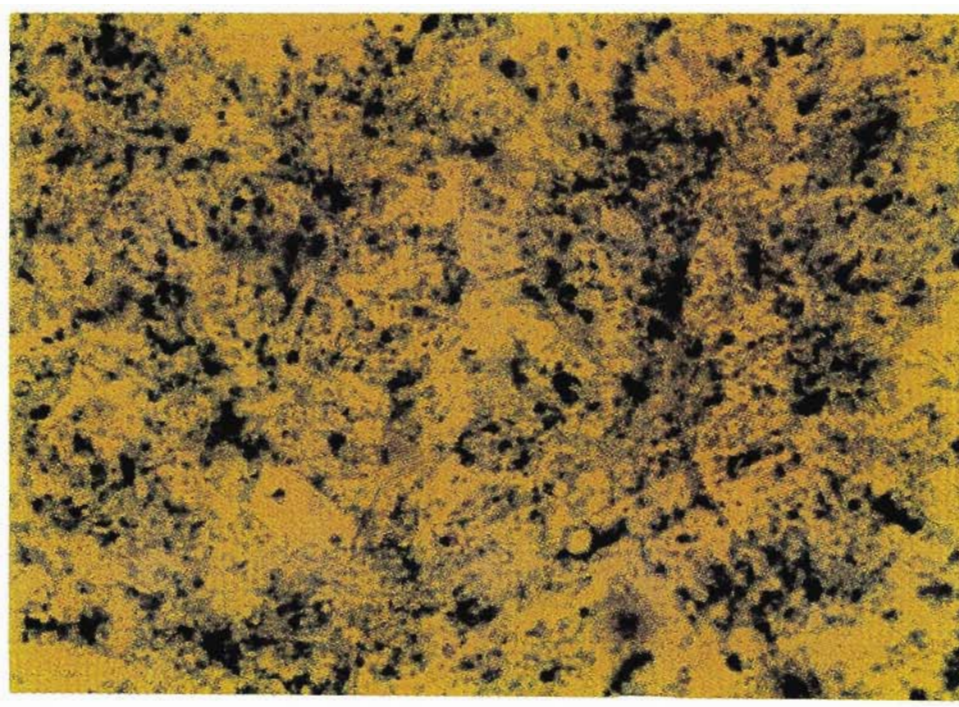

Figure 19. Expression of *lacZ* gene in Cos7 cells that were transduced with adenovirus-*lacZ*. The cells were examined by bright field light microscopy. Untransdused cells appear yellow; transduced cells appear blue.

4.11. Dose response of the adenovirus-*lacZ* in HEK293 cells

To test how many viruses are needed per cell for β-gal to be detected, cells were transduced with increasing amounts of virus.

HEK293 cells were transduced with MOI of 1, 10, 10^2, 10^3,10^4,10^5 viruses per cell. After 24h, the cells were fixed with glutaraldehyde and stained with x-gal. After 24h the cells were examined by light microscopy. As seen in Figure 20, the level of the cells infected is proportional to the MOI, where the higher the virus titre, the higher the expression of *lacZ*, reaching a plateau level at 10^3 (visualized as an increase in the intensity of the blue color produced). Of note is the fact that cells that were not transduced did not express any detectable amounts of β-gal (not shown).

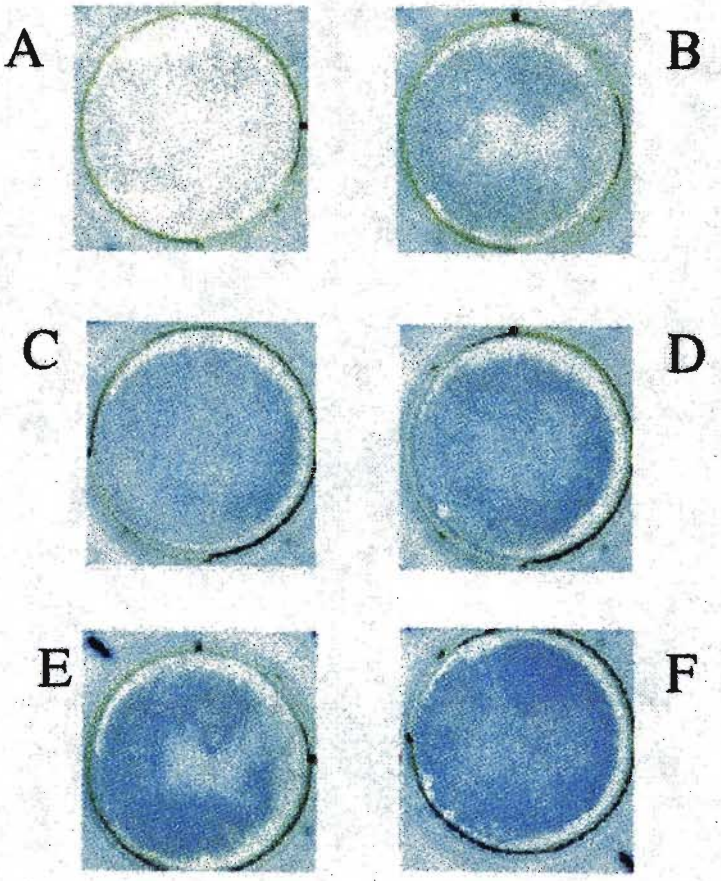

Figure 20. *In vitro* dose response of HEK293 cells transduced with MOI of 0, 1, 10, 10^2, 10^3,10^4,10^5 (A-F respectively) adenovirus-*lacZ* viruses per cell. The monolayers (in 35mm plates) were fixed in glutaraldehyde and stained with x-gal 24h after infection

4.12. *In vivo* dose response of the virus

To optimize the conditions for in vivo transduction, we injected increasing amounts of virus to blood vessels. We injected $5x10^7$,10^8,$5x10^8$,10^9 viruses to the blood vessels. The tissue was exposed to the virus (Fig. 21 B) or with PBS (Fig. 21 A) as a negative control for 20 min. After 24hrs, the tissue was fixed with glutaraldehyde, stained with x-gal and the activity of β-gal was examined after 24hrs. Our results indicate that the higher the titer the higher the expression of the *lacZ* gene. These results demonstrate the ability of the virus to express the inserted gene in vivo.

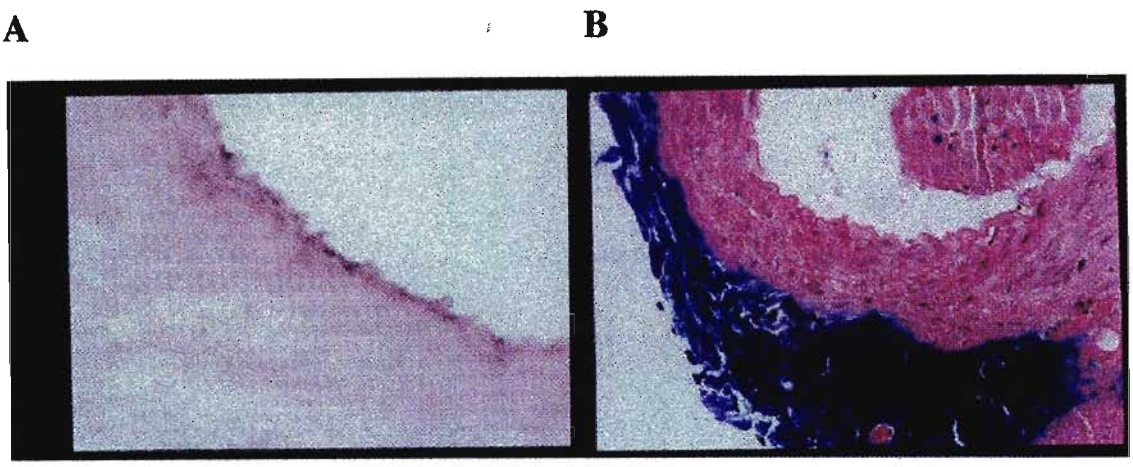

Figure 21. In vivo expression of *lacZ* gene in blood vessels that were transduced with PBS (A) as a negative control or with 10^9 adenovirus-*lacZ* (B). After 24 hrs, the tissue was fixed with glutaraldehyde, stained with x-gal, sectioned and stained with hematoxylin/Eosin (H&E) stain. Tissue sections were examined by light microscopy (bar, 4 μm.). The transduced adventitia appears blue while the non transduced media remained red.

4.13. The effect of exposure time on tissue infection

In this study, rat arteries exposed to 10^9 viruses for different lengths of time (2.5min, 5min, and 20min) were compared using PBS as a negative control. The *lacZ* gene expression was examined after 24h. No apparent difference between exposure of tissue to the virus for 2.5 min or longer was observed (not shown), suggesting that the infection of the tissue is very rapid and an exposure time of 2.5 min is sufficient.

4.14. Transgene expression time

It is essential to know for how long the recombinant gene is expressed in the tissue. For this purpose 10^9 viruses were injected into rat arteries. Tissue was exposed to the virus for 20 min and the *lacZ* gene expression was examined after 1 day, 7 days and 2 weeks. By the end of the first week, β-gal staining was undetectable, suggesting that the transduced cells had disappeared, probably by the induction of the host's immune response (see Chapter III).

4.15. Construction of pRR4, a shuttle plasmid containing a signal peptide preprotrypsin and the FLAG epitope

In preparation for the construction of a recombinant p15 adenovirus, the signal peptide preprotrypsin was cloned into the adenovirus shuttle plasmid pRR4 for the secretion of the recombinant protein. The FLAG octapeptide (DYKDDDDK) (74) was also cloned into pRR4. Using commercially available monoclonal anti-FLAG antibodies, FLAG can be used as a marker to trace the expressed recombinant protein.

pRR4 was constructed by cloning the expression cassette from the pFLAG-CMV-1 Expression vector (Kodak). The pRR4 plasmid contains the cytomegalovirus (CMV) promoter for constitutive expression, the signal peptide preprotrypsin for secretion of the recombinant protein and the polyadenylation signal at the C-terminus to allow efficient RNA processing (Figure 22).

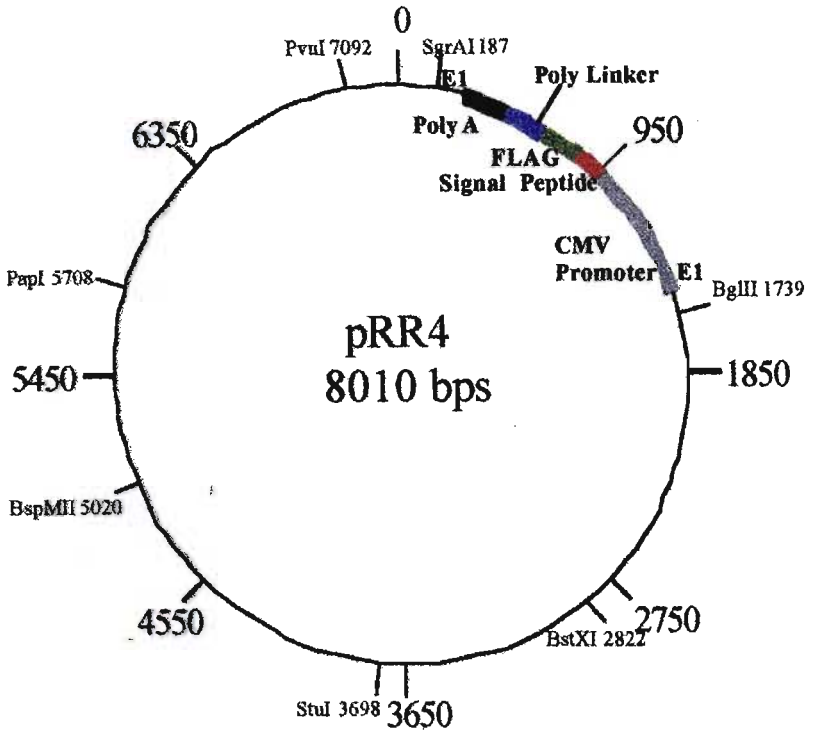

Figure 22. Map of the shuttle plasmid pRR4 . The shuttle vector pRR4 is under the control of the CMV promoter and contains a signal peptide for secretion of the recombinant protein and a FLAG epitope tag allowing the immunodetection of the recombinant protein

Construction of the shuttle plasmid containing the p15 gene (pRR5)

To study the use of the adenovirus as a delivery vehicle for vaccine development against *T. brucei*, the p15 gene was subcloned into the shuttle vector pRR4.

The 528 bp fragment containing the p15 DNA sequence which was subcloned from a *T. brucei* cDNA library (see results, chapter I) was subcloned into the shuttle vector pRR4 between EcoRI and KpnI, downstream to the CMV promoter and the signal peptide. The FLAG epitope tag was fused to the 5` end of p15 gene in frame, to allow immunodetection of p15 in cells and tissues (pRR5, Figure. 23).

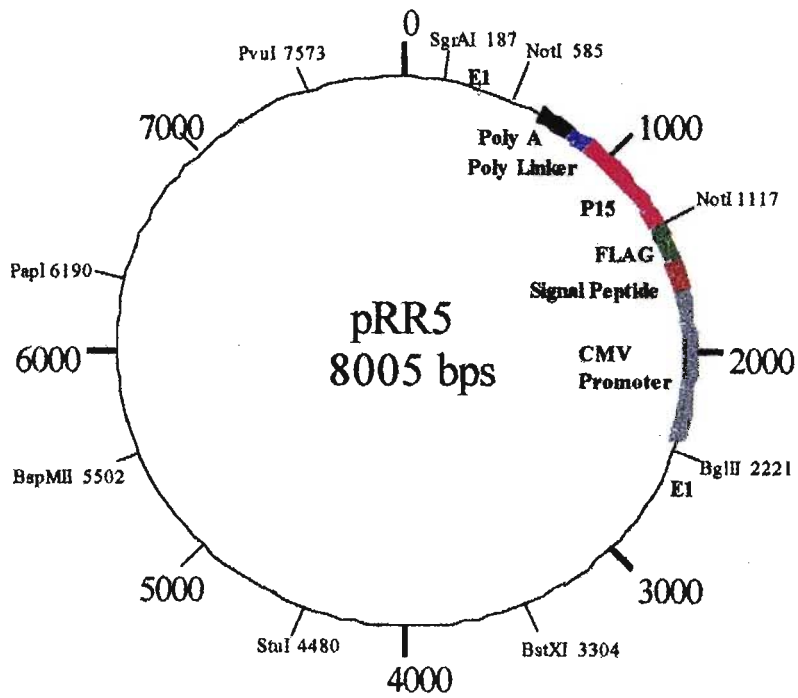

Figure 23. Map of the shuttle plasmid pRR5 containing the gene encoding for p15.
p15 DNA was subcloned into the shuttle vector pRR4 under the CMV promoter. The N-terminus of expressed recombinant p15 contains a signal peptide and FLAG epitope tag to allow for the secretion of p15 and for the immunodetection of p15.

E. coli DH10B competent cells were transformed with the ligated DNA by electroporation. Plasmid DNA from these transformed colonies was isolated and analyzed for the presence of the p15 gene insert by digestion with EcoRI and KpnI restriction enzymes. The digested DNA was separated by electrophoresis on agarose gels (Figure. 24). The shuttle plasmid containing the 528bp fragment was cotransfected with pJM17 in HEK293 cells to make infectious adenovirus expressing the recombinant p15 (Ad-p15).

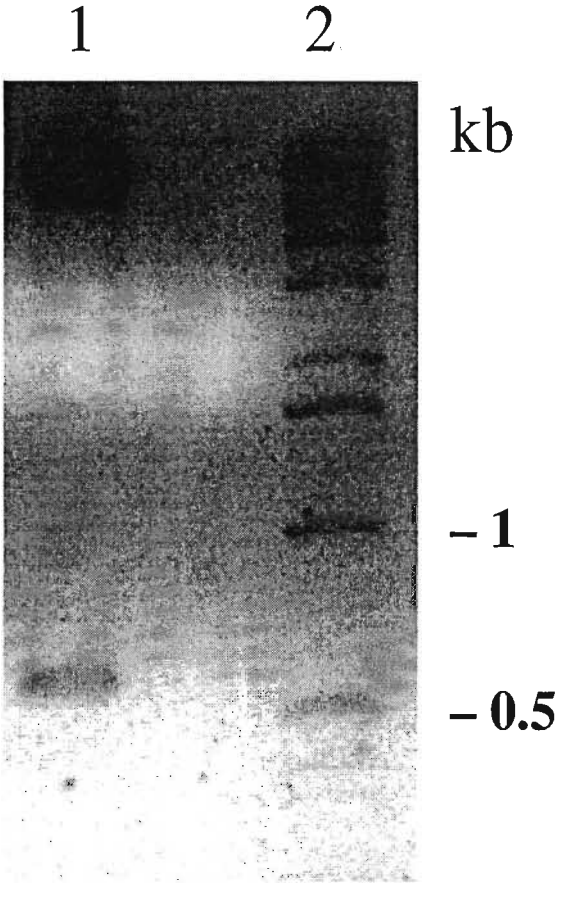

Figure 24. Restriction enzyme analysis of the shuttle plasmid pRR5 that was digested with KpnI and EcoRI restriction enzymes. The plasmid DNA was separated by electrophoresis on a 1% agarose gel and stained with ethidium bromide.
Lane 1. pRR5 digested with KpnI and EcoRI restriction enzymes generating two fragments: 7500bp of the shuttle plasmid and 528bp of the p15 gene insert.
Lane 2. 1kb ladder as a molecular weight marker.

4.16. Sequence analysis of pRR5

It is essential that p15 is in frame with the FLAG epitope for the proper expression of the recombinant p15 gene. Unlike the reporter genes cloned in the previous sections, in this case it was necessary to verify that p15 is cloned in frame with the FLAG epitope used as a reporter gene. DNA sequence analysis of pRR5 (Figure 25) demonstrates that p15 and FLAG are indeed in frame.

translation initiation || preprotrypsin
ACC ATG TCT GCA CTT CTG ATC CTA GCT CTT GTT GGA GCT GCA GTT/
T M S A L L I L A L V G A A V
Flag GCT GAC TAC AAA GAC GAT GAC GAC AAG/
A D Y K D D D D K
HindIII Not I EcoRI
CTT GCG GCC GCG AAT TC/ G
L A A A N S
P15 GCA CGA GCA ACT GCT GTC CCC AAG AAA GCC GTG GCC AAA AAG
A R A T A V P K K A V A K K
GCC GCT CCA AAG AAA ACT GTG GCC AAA AAG GCT GCT CCA AAG AAG
A A P K K T V A K K A A P K K
GCT GTC GCT AAG AAG GTT GCT CCC AAA AAG GCT GTC GCC AAG AAG
A V A K K V A P K K A V A K K
GTT GTC GCC AAA AAG GCT GTC GCC AAG AAG GTT GTC GCC AAA AAG
V V A K K A V A K K V V A K K
GTT GCC CCC AAG AAG GTT GTC GCC AAA AAG GTT GCC CCC AAG AAG
V A P K K V V A K K V A P K K
GTT GCC GGC AAG AAG GCC GCC GCT AAG AAG GCG TGA GCG CAT CCG
V A G K K A A A K K A
CTG CTG CCC GCT ATT AGA CAC GCT ATG AGG TTT ACC TGA GTG TGG

GAG AGA GCT GTC ACA CGT TTC AGG ACG TCC TCG TGC GTC CCT CCA

GGA CGG AGT TAG AAT TTT CTT ATC TTA CTT GGT TAG TTC CCT TCT

ACC GNT TGT AAT GGA AAT GTT TCG TTT GTG GGN TGC GTC TTA TGT

ACC CCC ANN CGG TGT TGG TTT CGT ANC GTT ACC AAG ANC AAA TCA

TCC TGA TGT GTG GCT AAT TTA ACT TCC TGT GTA TGG TTG TGG TCC

CAA AAA AAA AAA AAA AAA AAC TCG AGG GGG GGC

Figure 25. Nucleotide sequence analysis of pRR5. Sites of translation initiation site, signal peptide, FLAG, cloning sites of p15 and p15 are indicated.

4.17. Construction of recombinant Ad-p15 virus and detection of recombinant p15 gene in mammalian cells

HEK293 cells were co-transfected with pJM17 and the shuttle plasmid pRR5 by Lipofectamin liposome. The cytopathic effect of the virus appeared after 14 days. The medium from these cells was collected and used to infect HEK293 cells. After three consecutive plaque purifications, HEK293 cells were infected with the Ad-p15. The cells were incubated at $37^{o}C$ (in a CO_2 incubator) for 24h and both the cells and the medium were harvested. DNA from the cells was extracted, digested by EcoRI and KpnI, separated by electrophoresis on agarose gels, southern blotted and hybridized with radiolabeled p15-specific DNA. As shown in Figure 26, the presence of the p15 gene in the Ad-p15 is demonstrated.

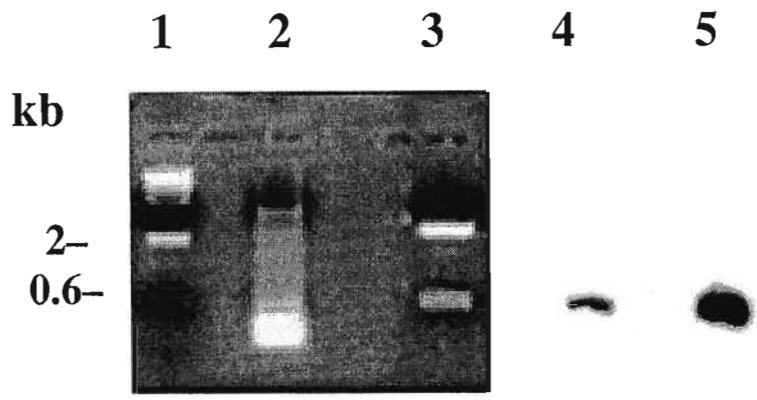

Figure 26. Southern blot analysis. DNA from the HEK293 infected cells was extracted, digested by EcoRI and KpnI, separated by electrophoresis on agarose gels, southern blotted and hybridized with radiolabeled p15-specific DNA.

Lane 1. λ HindIII DNA marker.

Lane 2. DNA extracted from HEK293 cells infected with the Ad-p15 and digested with EcoRI and KpnI (ethidium bromide staining).

Lane 3. DNA extracted from pBluescript plasmid that contains the p15 gene and digested with EcoRI and KpnI (as a positive control) (ethidium bromide staining).

Lane 4. Southern blotting of lane 2.

Lane 5. Southern blotting of lane 3.

4.18. p15 expression and secretion in transduced mammalian cells

The ability of Ad-p15 to express and secrete the recombinant p15 protein in mammalian cells was analyzed. HEK293 cells, which were infected with Ad-p15, were analyzed by western blotting for the expression and secretion of p15 using both anti-p15 and anti-Flag antibodies. Infected HEK293 cells were harvested, resuspended in sample buffer, boiled and separated by SDS 15% PAGE and western blotted. Following growth of infected cells, the medium was collected to test for the presence of secreted recombinant p15. Recombinant p15 was partially purified from the medium by running the medium through a 30kDa cut off membrane (Amicon) followed by concentration on a 10kDa cut off membrane (Amicon). The 10-30kDa fraction was suspended in sample buffer, boiled and separated on SDS 15% PAGE and western blotted. Nitrocellulose membranes were incubated with anti-p15 polyclonal or anti-flag monoclonal (anti-FLAG M2 IgG, from Kodak) primary antibodies. After washing, the membranes were incubated with peroxidase-conjugated protein A or anti-Mouse secondary antibodies (Amersham). The reaction was developed by a chemiluminescence detection Kit. As shown in Figure 27 and 28, the infected cells expressed and secreted the recombinant p15. These data suggest that Ad-p15 is functional and is able to express and secrete the recombinant p15 protein. As shown in Figure 28 lane 4, Cos7 cells transduced with Ad-p15 also expressed the recombinant p15, even though the virus cannot replicate in these cells. This suggests that even if the Ad-p15 will not replicate in the host, it will still be able to express detectable levels of recombinant p15. Of note is that some non specific binding of anti-p15 antibodies is observed in Fig. 28 where the antibody also binds to a 30kDa protein as well as to one of the molecular markers. This, however, does not detract from the clear observation that transduced cell lines over express p15.

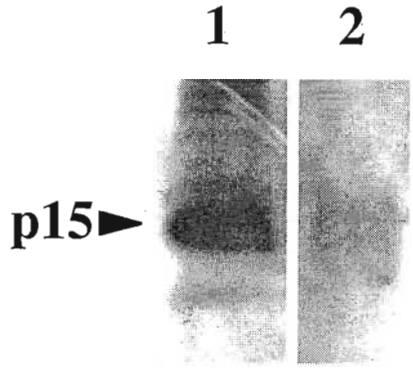

Figure 27. Western blot analysis (using anti-FLAG antibodies) of cells transduced with Ad-p15. Total cell homogenate (lane 1) and culture medium (lane 2) of Ad-p15 transduced HEK293 cells were separated on SDS 15% PAGE and western blotted.

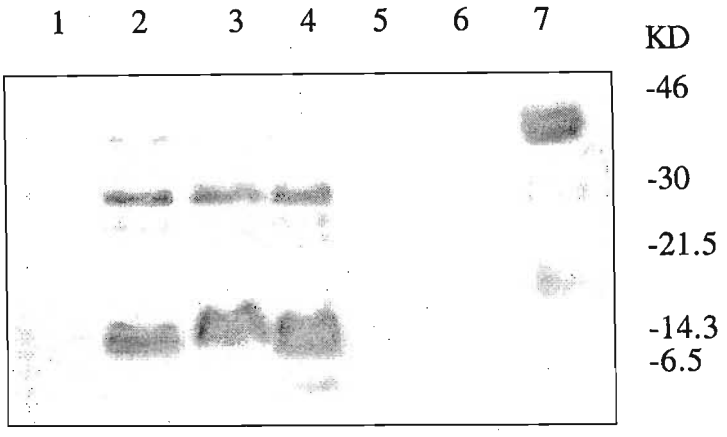

Figure 28. Western blot analysis of p15 using anti-p15 antibodies. Proteins were separated on SDS 15% PAGE and western blotted.

Lane 1 & 5. Non transduced HEK293 cells as a negative control.

Lane 2. Total cell homogenate of Ad-p15 transduced HEK293 cells (after 24hrs).

Lane 3. Total cell homogenate of Ad-p15 transduced HEK293 cells (after 12hrs).

Lane 4. Total cell homogenate of Ad-p15 transduced Cos7 cells.

Lane 6. Non transduced Cos7 cells as a negative control.

Lane 7. Rainbow protein molecular weight marker.

4.19. Discussion

The aim of the experiments described in this chapter was to develop an adenovirus delivery system of recombinant p15 into mammalian cells.

To test the efficiency of the adenovirus as an expression system in mammalian cells, an adenovirus with reporter genes was constructed. The Green Fluorescent Protein (GFP) and β-galactosidase were used as markers. Using such markers enabled the efficiency of the delivery of the gene and its expression to be demonstrated, and determination of the cells in which the recombinant protein is expressed.

The presence of a functional GFP protein was demonstrated in adenovirus-GFP transduced mammalian cells. The advantage of using this system is that GFP can be observed in real time, in live cells, with no addition of substrate. This method is very useful in testing GFP expression in cell lines. However, in some cases when expression of GFP in whole tissue was tested, it was difficult to distinguish between GFP fluorescence and endogenous tissue autofluorescence, which limited the usefulness of this reporter gene. To overcome this problem, an adenovirus expressing β galactosidase as a marker was used. This allowed the determination of the titre of the virus necessary for expression of the recombinant protein, the level of expression of the recombinant protein, and the duration of its expression.

The results indicate that the level of the cells infected by the virus is proportional to the MOI, where the higher the titre the higher the expression of β-galactosidase, reaching a plateau level at 10^3 viruses per cell.

To optimize the conditions for *in vivo* transduction, increasing amounts of virus (up to 10^9) were injected into the tissue. The results indicate that the higher the titre the higher the expression of the *lacZ* gene.

To test the effect of exposure time *in vivo*, tissue was exposed for 2.5-20 min to the virus. There was no apparent difference between exposure of tissue to the virus for 2.5 min or longer, suggesting that infection of the tissue is very rapid.

It is essential to know for how long the recombinant gene is expressed in the tissue. To test for the transgene expression time, 10^9 viruses were injected into the rat arteries and the

lacZ gene expression was examined up to two weeks post exposure. The results indicate that by the end of the first week, β-gal staining was undetectable, suggesting that within a week, the transduced cells were eliminated. In fact, it has been recorded (75,76,77) that the adenovirus causes upregulation of the host's immune response which eliminates the infected transduced cells. Indeed in this study (see chapter 3) it was shown that the recombinant adenovirus induced primarily $CD8^+$ T cells, which appeared specifically at the site of infection.

In preparation for the construction of a recombinant p15 adenovirus (Ad-p15), the signal peptide preprotrypsin was cloned into the adenovirus shuttle plasmid for the secretion of the recombinant protein. The FLAG octapeptide (DYKDDDDK) was also cloned into the shuttle plasmid to be used as a marker to trace the expressed recombinant protein by the use of commercially available anti-FLAG antibodies. p15 was cloned into the shuttle vector and the adenovirus containing the recombinant p15 gene (Ad-p15) contained the cytomegalovirus (CMV) promoter for constitutive expression, the signal peptide preprotrypsin for secretion of the recombinant protein, FLAG to be used as a marker, recombinant p15 in frame with FLAG, and the polyadenylation signal at the C-terminus to allow efficient RNA processing.

The ability of Ad-p15 to express and secrete the recombinant p15 protein in mammalian cells was analyzed by western blotting using both anti-p15 and anti-Flag antibodies which indicated that in fact the infected cells expressed and secreted the recombinant p15. The data obtained suggest that Ad-p15 is functional and is able to express and secrete the recombinant p15 protein.

Cos7 cells were also transduced with Ad-p15. Cos7 cells can be transduced by the virus but the virus cannot replicate within them. Interestingly, Cos7 cells also expressed the recombinant p15, even though the virus cannot replicate in these cells. This suggests that even if the Ad-p15 will not replicate in the host, it will still be able to express detectable levels of recombinant p15, suggesting that Ad-p15 could serve as a useful delivery system of the cloned antigen.

Chapter V

p15-BASED VACCINE FOR *T. brucei*

5.1. Introduction

Trypanosomes enter the bloodstream where they exhibit a remarkable power of adaptation, allowing them to escape the host's immune response. The parasites have evolved a mechanism of antigenic variation, where they regularly change their surface antigens (variable surface glycoproteins, VSG) by trans-splicing the long precursor RNA of the VSG genes (31) more rapidly than new antibodies can be made against them. While most of the parasites are eliminated from the circulation by this antibody response, a small number of parasites in the population bear an antigenically different VSG and thus evade the host's immune response. The large repertoire of variable surface antigens which can be expressed by a single parasite made vaccination appear as a goal beyond reach. Some vaccination studies have been done using flagellar pocket nonvariant trypanosome proteins. However, only partial protection was achieved (77). Since the development of a vaccine appeared beyond reach, emphasis has been placed on the development of improved drug therapy directed against vital metabolic processes that are unique to the trypanosome. However treatment of African sleeping sickness has not advanced greatly in the last 50 years and is often highly toxic. Trypanosomiasis control programmes aimed at eradication of the most common vector, the tsetse fly, though adequate if well managed, have not been fully effective (78).

It is therefore essential to find a new specific target site for therapeutic attack.
All parasitic trypanosomes contain a prominent subsurface array of microtubules unique in localization and apparent stability. This suggests that the functional integrity of trypanosome microtubules may be important in the control and maintenance of membrane-located physiological processes that are essential for the survival of the parasite in the host (44). The unique trypanosomal MAPs could serve as therapeutic target sites, taking advantage of the importance of microtubule integrity to the parasite and the uniqueness of these MAPs to trypanosomes (32).

Unique trypanosomal MAPs could not only serve as chemotherapeutic target sites, but may also serve as unique antigens for immunological suppression of the disease. The use of intracellular antigens as vaccines has been tested successfully using unique subpellicular MAP p52 together with the glycosomal enzymes aldolase and GAPDH (32). Sera of protected animals caused the aggregation of trypanosomes *in vitro*, and immunoelectron microscopy of these aggregated trypanosomes revealed the presence of antibodies within the parasites (32).

The aim of this study was to test if immunization with p15 can protect against a *T. brucei* infection. In previous studies (32) it was shown that immunization with a trypanosomal MAP (P52) along with two glycosomal enzymes (aldolase and GAPDH) had a protective effect, but it was not clear whether protection was due to vaccination with the trypanosome MAP, to the glycosomal enzymes or to a combination of the two. In this chapter studies showing that p15 can protect mice in its native form, recombinant form or transduced form, from a *T. brucei* infection are described.

Results

5.2. Immunization with native or recombinant p15

Mice were chosen as model systems for protection experiments because of their sensitivity to a trypanosome infection. Mice were immunized three times subcutaneously with one of the following: 10μg native p15 that was isolated from *T. brucei* subpellicular microtubules on a tubulin affinity column. With 10μg recombinant p15 (rp15) that was secreted to the medium by Ad-p15 trancduced HEK293 cells. With a subpellicular fraction greater than 30kDa used as a positive control (+C, containing 30μg of equal concentrations of p52, aldolase and GAPDH, but not containing p15 (32)), or with buffer only as a negative control. To test the protective potential of the recombinant viral vaccine, mice were injected by intramuscular injection with the adenovirus containing the gene for p15 (Ad-p15) or with adenovirus containing *lacZ* (Ad-lacZ) as a control. Vaccinated and control animals were challenged with 500 live *T. b. brucei* SB 1 that had been freshly isolated from infected mice. Of note is the fact that the tsetse fly injects the host with only 50 parasites per bite. Parasitemia was followed for the next 60 days by removing a drop of blood from the tail and observing it by light microscopy.

As seen in Table 1, vaccination of mice with p15 (native or recombinant) effectively protected animals from a *T. brucei* infection. Of the group of animals that were vaccinated with the native p15, only one out of eight animals (Figure 29 E) was infected, while all control animals (Figure 29 A-D) injected with buffer only were infected (protection was followed for over three months). None of the animals vaccinated with recombinant p15 (rp15) or with the positive control, +C (p52 aldolase and GAPDH) were infected, suggesting 100% protection. All negative control animals vaccinated with buffer only were infected, suggesting 0% protection (0/4).

5.3. Table 1: Protection from *T.b.brucei* strain SB1 infection:

Mice were vaccinated with one of the following: p15 purified from *T. b. brucei* KETRI 2693 (Native p15). Recombinant p15 isolated from culture media of Ad-p15 trancduced HEK293 cells (rp15). Adenovirus containing the gene for p15 (Ad-p15). Adenovirus containing *lacZ* (Ad-lacZ). A *T. b. brucei* KETRI 2693 subpellicular fraction containing p52, aldolase and GAPDH as a positive control (+C). Pipes buffer, as a negative control. Mice were challenged with 500 *T.b.brucei* strain SB1 and % protection and parasitemia recorded.

Group	(n)	% protection (no. protect/total)		Day parasitemia detected (n)	Day of death (n)
Native p15	(8)	87.5%	(7/8)	7 (1)	57 (1)
Pipes buffer	(4)	0%	(0/4)	7 (4)	31 (1) 38 (2) 41 (1)
rp15	(7)	100%	(7/7)	–	–
Ad-p15	(8)	100%	(8/8)	–	–
Ad-lacZ	(4)	100%	(4/4)	–	–
+ C	(4)	100%	(4/4)	–	–

As shown in Figure 29, it seems that the pattern of infection in the one native p15 immunized mouse that contracted the disease was somewhat different from that observed in the non immunized mice. The parasite load was lower, and the animal lived longer.

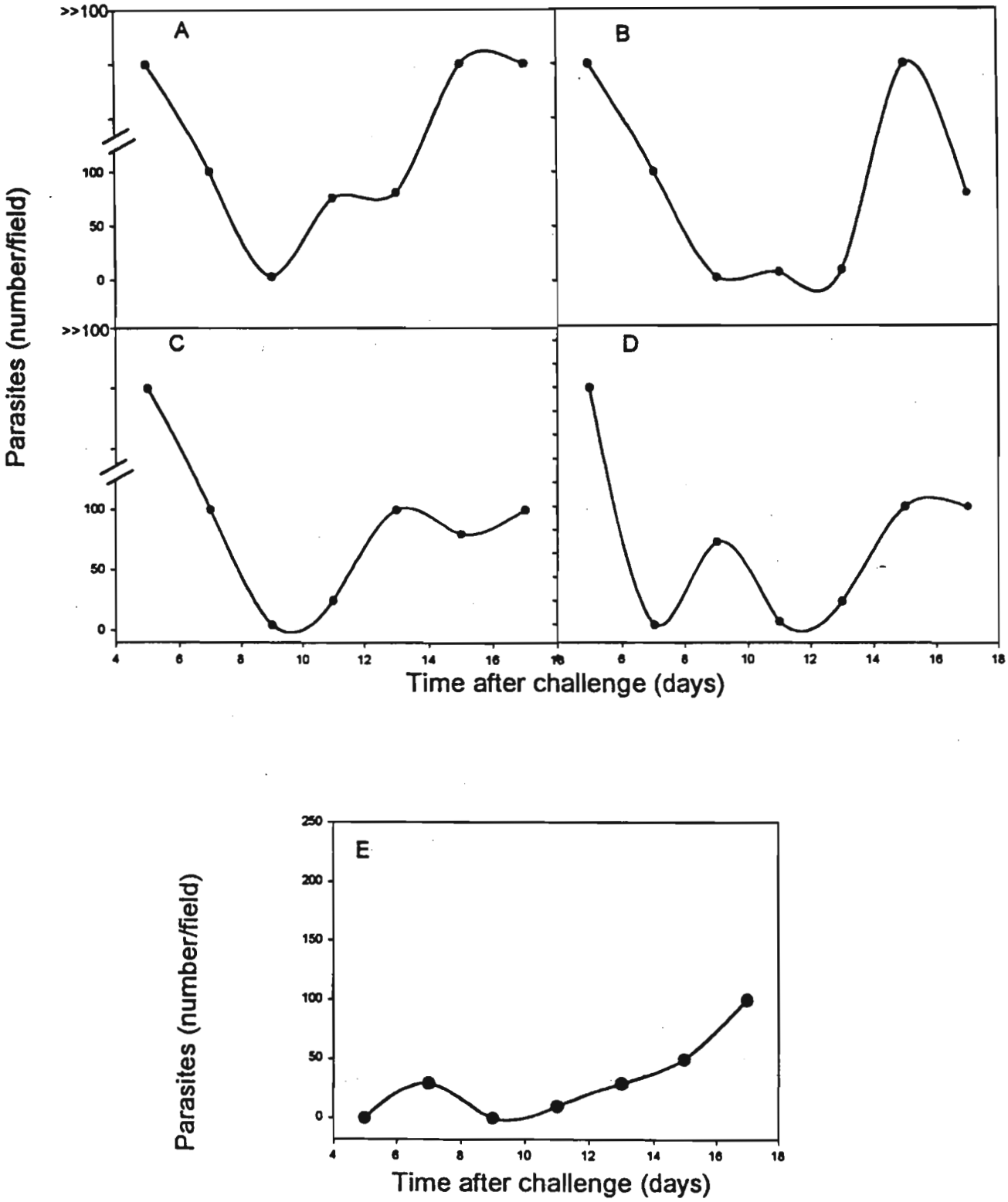

Figure. 29. Course of parasitemia in the infected mice. Each peak of parasitemia represents a new antigenic variant. Parasitemia in control mice vaccinated with buffer (A-D). Parasitemia of mouse vaccinated with native p15 (E).

5.4. Immunization with the recombinant viral vaccine (Ad-p15)

Eight animals were injected with $2x10^9$ Ad-P15 virus particles capable of expressing and secreting the recombinant p15 protein (with no adjuvant). None of the eight animals became infected even after eight weeks, suggesting 100% protection by the recombinant viral vaccine (Table 1).

As a control, four mice were immunized with $2x10^9$ Ad-*lacZ* (with no adjuvant) to test for the non-specific immune response induced by an adenovirus injection. The *lacZ* gene is helpful for tracing the presence of the virus and localizing it. None of these control animals became infected even after eight weeks (Table I). This result suggests that nonspecific activation of the immune system by the adenovirus effectively protected the animals from a *T. brucei* infection.

5.5. Cell-mediated immune response to the adenovirus

Protection against a *T. brucei* infection by the control adenovirus may have been caused by the non-specific activation of the $CD8^+$ T cells. To analyze the non-specific immune response to the adenovirus itself, the effect of the adenovirus on $CD4^+$ T cell proliferation and on $CD8^+$ T cell proliferation was tested. Both types of T cells have already been shown to play a role in the cell mediated response to the adenovirus (75, 76). $CD4^+$ T helper cells express on their surface both the $CD4^+$ molecule as well as the major histocompatibility complex (MHC) class II molecule. $CD8^+$ T cells express on their surface both the CD8 molecule as well as the MHC class I molecule and are cytotoxic. Rat arteries were chosen as a model system for testing cell-mediated immune response to the adenovirus because rat arteries are large enough to handle, and because rats survive such experiements better than mice and get infected less readily (Rasooly, unpublished). Adenovirus-infected tissues (rat arteries) were analyzed immunohistochemically using anti-CD4 and anti-CD8 antibodies. Bound antibodies were detected by biotinilated secondary antibodies, which allows the colourimetric identification (brown color) of the cells which express these antigens.

The results indicate that while the adenovirus only slightly induces $CD4^+$ T cell proliferation (Figure 30), it strongly induces $CD8^+$ T cell proliferation (Figure 31).

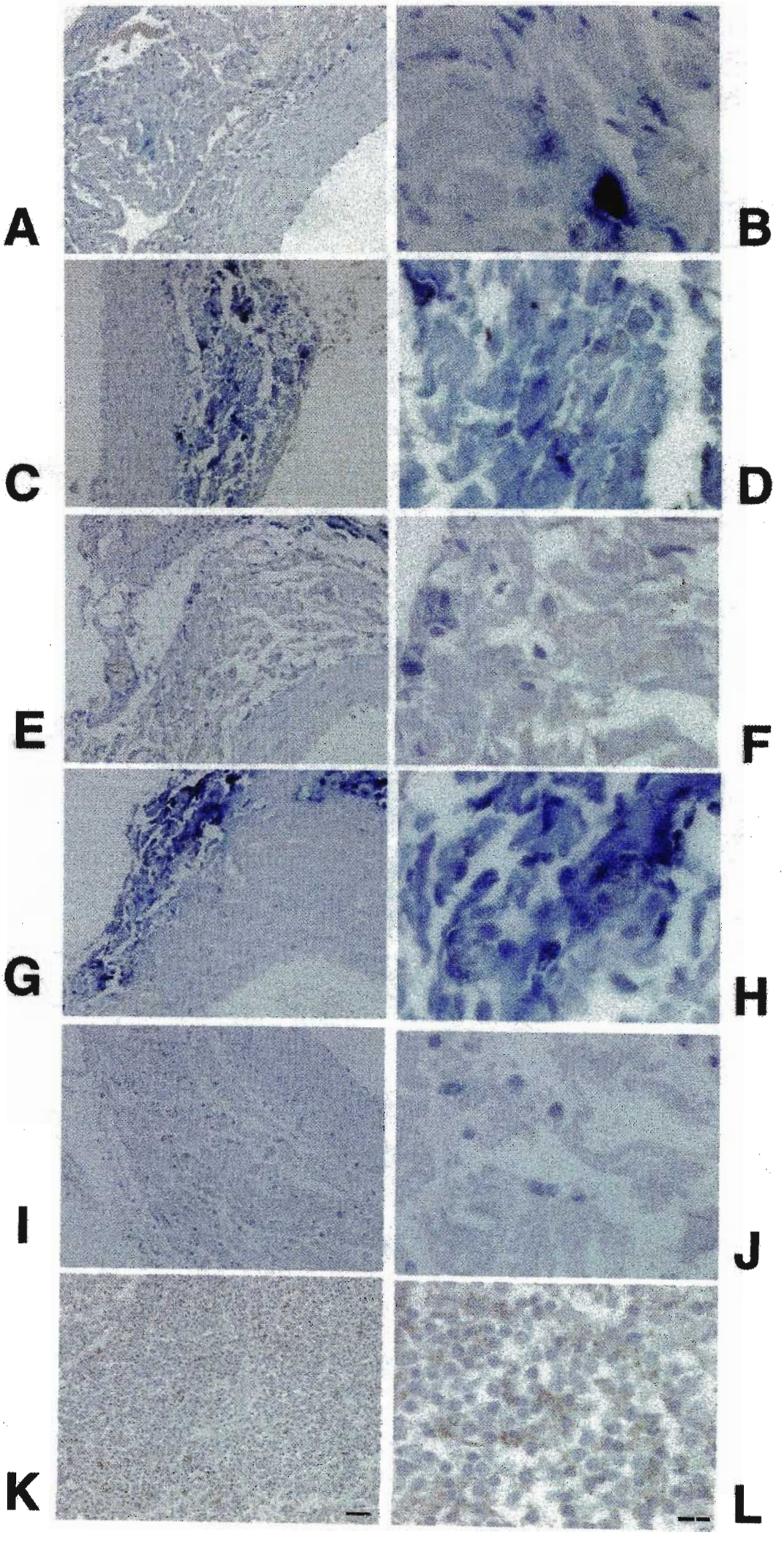
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Figure 30. $CD4^+$ T cell proliferation induced by increasing numbers of Ad-*lacz* virus particles. Rat arteries were injected with increasing amounts of Ad-*lacZ* virus. After 24h, the tissue was fixed with glutaraldehyde and serial sections were stained with x-gal to detect the expression of *lacZ* in infected cells and analyzed immunohistochemically, using anti CD4 antibodies, to detect $CD4^+$ T cell proliferation. Sections were observed by bright-field light microscopy. Brown staining indicate positions of the $CD4^+$ cells.

A and B: injection of $5x10^7$ PFU.
C and D: injection of 10^8 PFU.
E and F: injection of $5x10^8$ PFU.
G and H: injection of 10^9 PFU.
I and J: injection of PBS only as a negative control.
K and L: Tonsil tissue used as a positive control for anti CD4 antibodies.
A,C,E,G,I,K: bar, 30 μm.
B,D,F,H,J,L: bar, 10 μm.

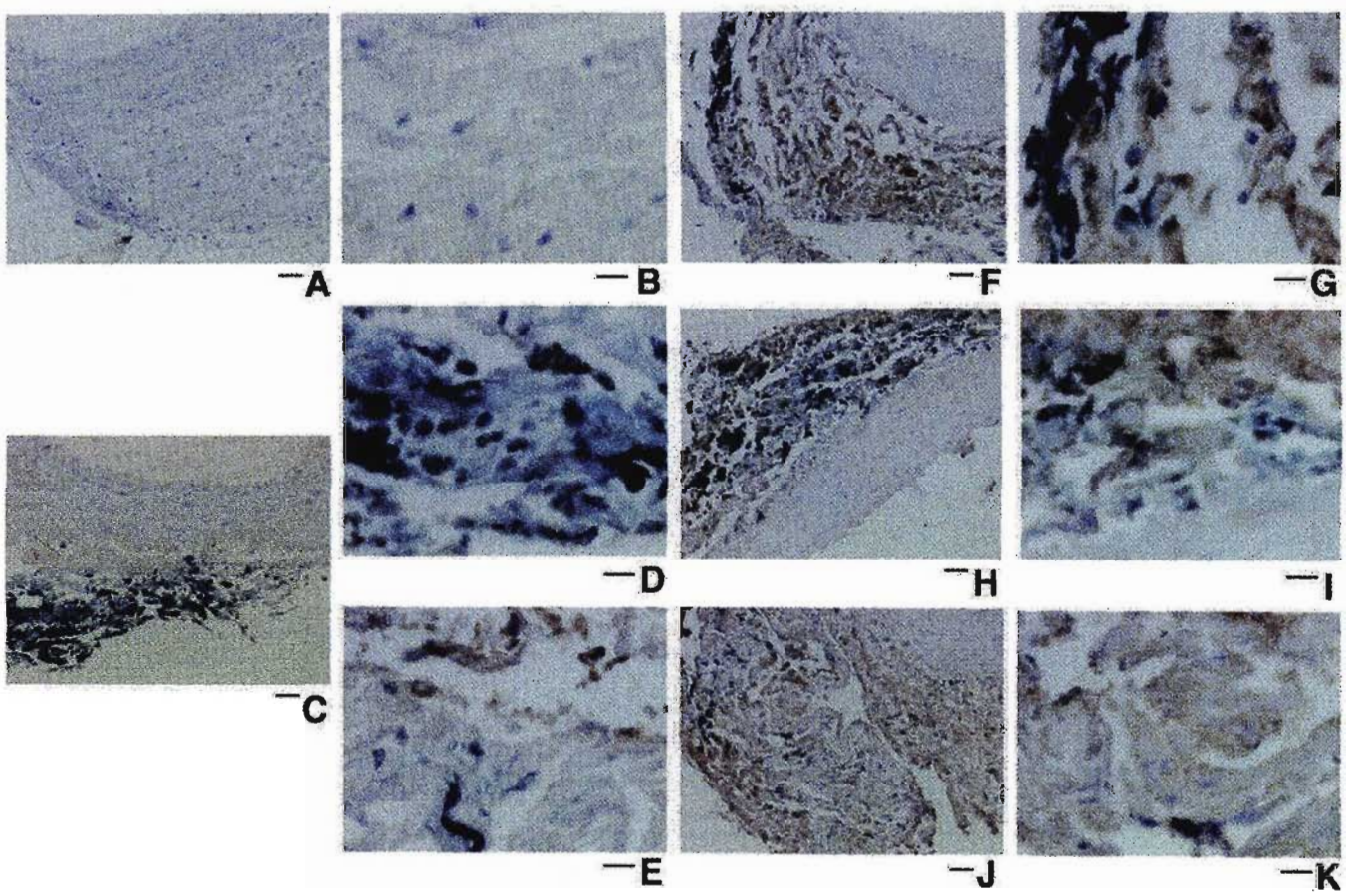
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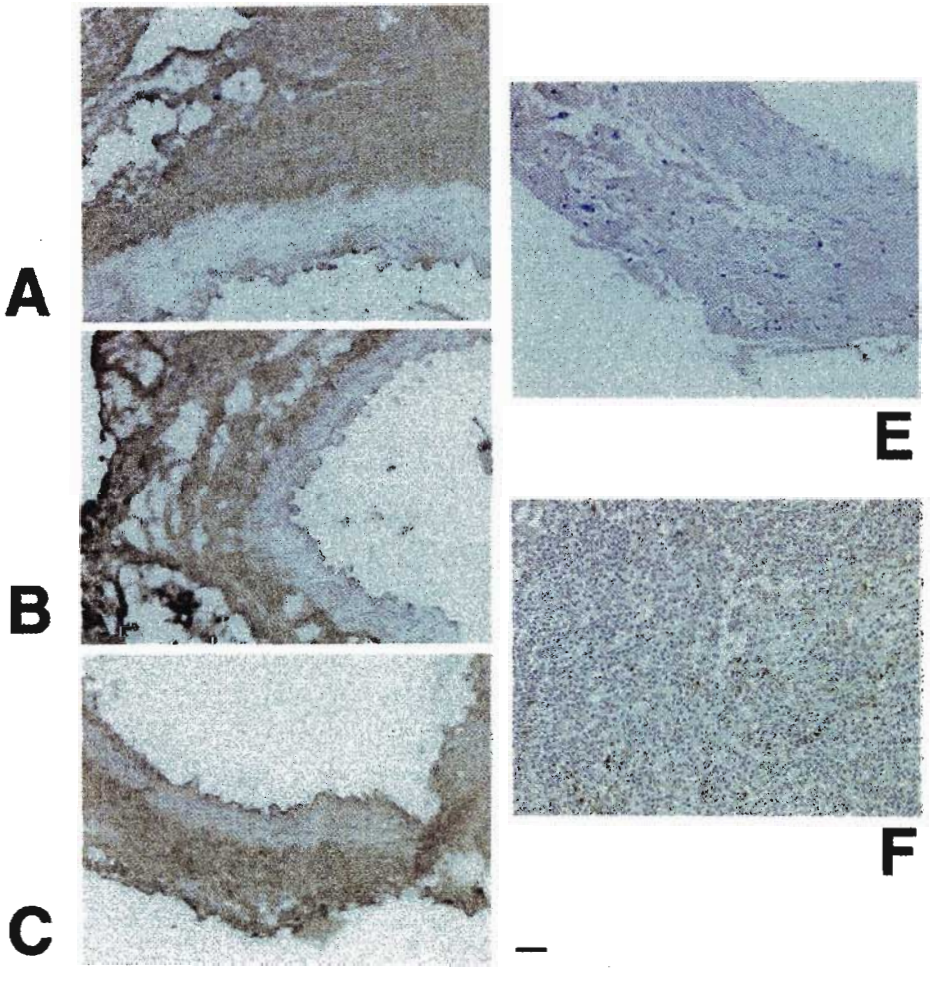
Figure 31. $CD8^+$ T cell proliferation induced by increasing numbers of Ad-*lacZ* virus particles.
Rat arteries were injected with increasing amounts of Ad-*lacZ* virus.
After 24h, the tissue was fixed with glutaraldehyde and serial sections were stained with x-gal to detect the expression of *lacZ* in infected cells and analyzed immunohistochemically, using anti CD8 antibodies, to detect $CD8^+$ T cell proliferation. Sections were observed by bright-field light microscopy. Brown staining indicate positions of the $CD8^+$ cells.

C, D, E: injection of $5x10^7$ PFU.
F, G: injection of 10^8 PFU.
H, I: injection of $5x10^8$ PFU.
J,K: injection of 10^9 PFU.
A,B: injection of PBS only as a negative control.
A,C, F, H, J: bar, 30 μm.
B,D,E, G,I,K: bar, 10 μm.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5.6. The effect of exposure time on tissue infection

To test $CD8^+$ T cell proliferation and compare it with *lacZ* gene expression after 24h, samples were exposed to 10^9 viruses for different lengths of time (2.5min, 5min, and 20min) using PBS as a negative control. No apparent difference between exposure of tissue to the virus for 2.5 min or longer was observed (Fig. 32). The results suggest that infection of the tissue is very rapid and an exposure time of 2.5 min is sufficient.

Figure 32. $CD8^+$ T cell proliferation as a result of increasing exposure time to Ad-*lacZ* virus particles. Rat arteries were injected with 10^9 PFU of Ad-lacZ for increasing lengths of time. After 24h, the tissue was fixed with glutaraldehyde and serial sections were analyzed immunohistochemically, using anti CD8 antibodies, to detect $CD8^+$ T cell proliferation. Sections were observed by bright-field light microscopy. Brown staining indicate positions of the $CD8^+$ cells.

A. 2.5min

B. 5min

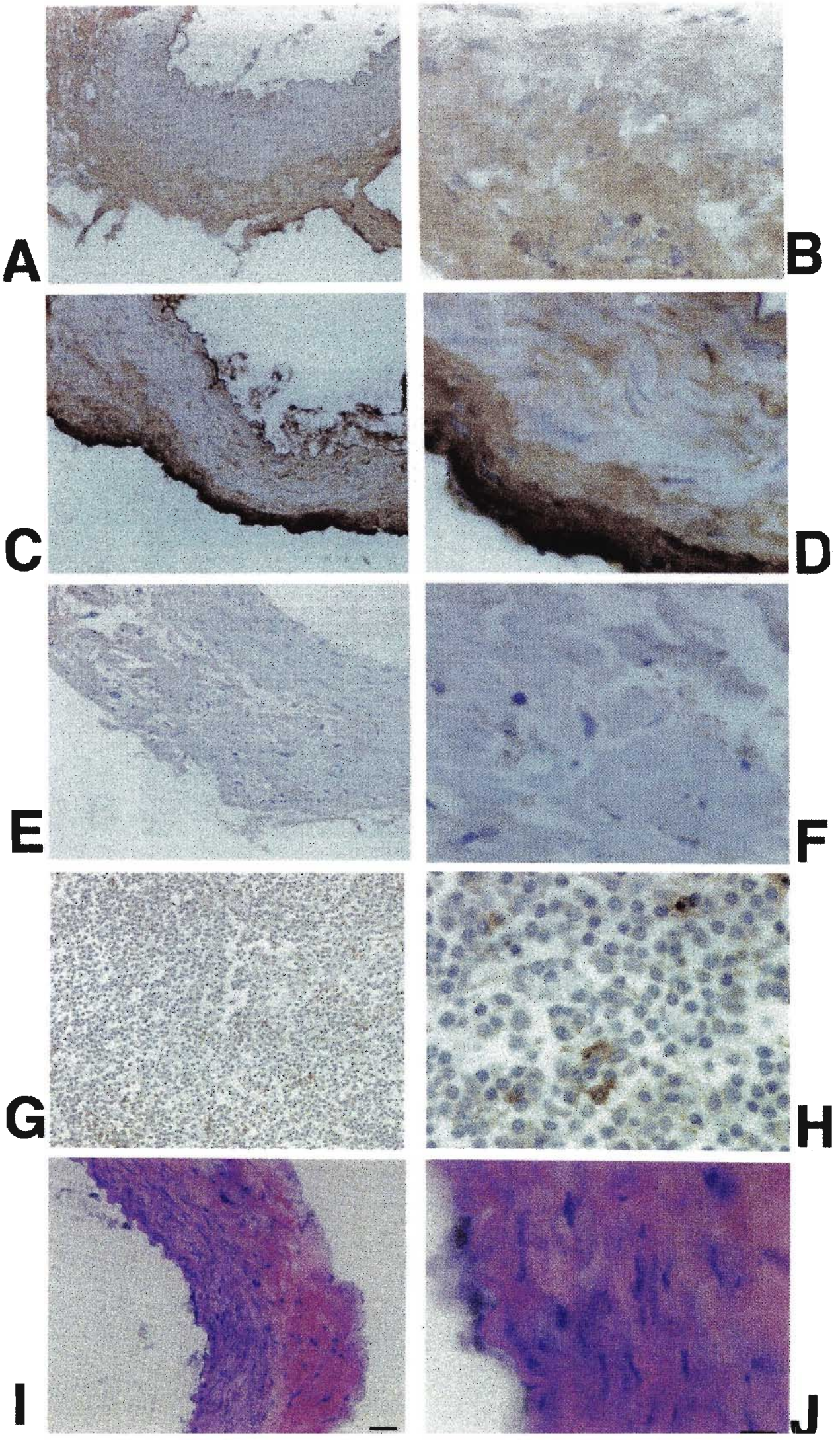
C. 20min

E. PBS as a negative control

F. Tonsil tissue used as a positive control for anti CD8 antibodies.

A-E: bar, 30 μm.

F: bar, 10 μm.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5.7. Transgene expression time

It is essential to know for how long the recombinant gene is expressed in the tissue. For this purpose 10^9 viruses were injected into the arteries of rats. Tissue was exposed to the virus for 20 min and the *lacZ* gene expression was examined after 1 day, 7 days and 2 weeks. As shown in Fig. 33, by the end of the first week, β-gal staining was undetectable.

Figure 33. Length of time $CD8^+$ T cell proliferation is observed as a result of exposure to Ad-*lacZ* virus particles. Rat arteries were injected with 10^9 PFU of Ad-lacZ virus for 20min. After 7-14 days the tissue was fixed, and serial sections were analyzed immunohistochemically using anti CD8 antibodies, to detect $CD8^+$ T cell proliferation. Sections were observed by bright-field light microscopy. Brown staining indicate positions of the $CD8^+$ cells.

A and B: 7 days.

C and D: 14 days.

E and F: PBS as a negative control.

G and H: Tonsil tissue used as a positive control for anti CD8 antibodies.

I and J: H and E staining.

A, C, E, G, I: bar, 30 μm.

B, D, F, H, J: bar, 10 μm.

5.8. Discussion

The aim of the studies described in this chapter was to test whether p15 can protect against a *T. brucei* infection. Because p15 is unique to trypanosomes, it can serve as a target site of therapy. The possibility of using p15 as a unique target site for vaccine development, although it is an intracellular antigen, was also investigated.

Previous studies have shown that immunization of mice with paraflagellar rod proteins present in the flagellum of *T.cruzi* induces an immune response that results in reduction in the level of circulating parasites and 100% survival against an otherwise lethal inoculum of *T.cruzi* trypomastigotes (95). intracellular antigens can serve as vaccine target sites against trypanosomal infections. For example, animals vaccinated with a *T. brucei* p52 MAP preparation containing the glycosomal enzymes aldolase and GAPDH were protected against a *T. brucei* infection (32). Vaccination of animals with a flagellar pocket fraction of *T. brucei* caused a partial protection against natural trypanosomiasis (77). The reason internal antigens may be protective has been attributed to the observations that there is uptake of macromolecules by the trypanosomes (through the flagellar pocket) (82,83). Internalization of antibodies was described in live *Crithidia fasciulata* that were incubated in the presence of antibodies directed against intracellular antigens (84), and immunization with p41 (aldolase) of *Plasmodium falciparum* induces immunity to malaria (85). Sera of animals vaccinated (and subsequently protected) with p52 MAP, together with aldolase and GAPDH, caused the aggregation of trypanosomes *in vitro*, where antibodies were detected inside the cell (32).

The present studies show that mice vaccinated either with the native purified p15 or with the expressed recombinant p15 were protected from a challenge of *T. brucei*. all the animals (8/8) vaccinated with the recombinant protein were protected and 87.5% of the animals (7/8) were protected when vaccinated with the native protein. The one animal that was not protected had decreased parasitemia and increased survival time. None of the control animals which were injected with buffer only were protected.

Also tested was whether the adenovirus could be used as a vaccine delivery system for p15. The recombinant adenovirus was chosen as a delivery system to express p15 in mammalian cells because this system has been emerging as a highly promising method for vaccine development. It's advantages are its ability to induce the host's immune response, its wide host range and its high transduction efficiency.

The results indicate that 100% of the animals (8/8) injected with the adenovirus containing the recombinant p15 gene were protected from a challenge of *T. brucei*. As a control, animals were injected with an adenovirus containing the *lacZ* gene. Interestingly, these animals were also 100% protected (4/4). It is hypothesized that this could be due to nonspecific activation of the host's immune response. Previous studies have shown that when control mice immunized with adenovirus containing the *Lac-Z* geneafter chalenge with a lethal inoculum of the highly virulent strain of *T. cruzi* 2/4 of the mice were protected from *T. cruzi* infection. All 6 mice that vaccinated with Freund's adjvvant were infected suggesting 50% protection (95).

These studies indicate that while the adenovirus only slightly induces $CD4^+$ T cell proliferation, it highly induces $CD8^+$ T cell proliferation. It has been demonstrated that $CD8^+$ T cells have a major role in the natural immune response against *T. brucei* (86). Activated $CD8^+$ T cells produce INFγ which activates macrophages. Activated macrophages then release tumor necrosis factor and nitric oxide (NO). Nitric oxide is one of the reactive nitrogen intermediates generated during the conversion of arginine to citrulline by arginase; it is strongly cytotoxic to a variety of parasites, and has been shown to be trypanostatic (87). Arginase can also cause damage by leading to deprivation of arginine, which is an essential amino acid for the parasites (88). It has been demonstrated that $CD8^+$ T cells have a major role in the natural immune response against *T. brucei* (79,80,81). The results suggest that protection against the p15 recombinant adenovirus may not have only been due to the expression of the recombinant p15 but also due to an induction of a non-specific cellular immune response to the adenovirus itself.

The source of the native and recombinant p15 was from a different strain of *T. brucei* that was used for challenge, and the subpellicular microtubules are common to all members of the Trypanosomatidae family. This suggests that p15 may ultimately serve as a common target of therapy to all types of diseases caused by trypanosomes.

5.9. General discussion

The trypanosome subpellicular microtubules are unique both by the fact that unlike other microtubules, they are crosslinked to one another and also by their unique localization, lying right underneath the plasma membrane. It had been suggested that unique trypanosomal MAPs crosslink the microtubule polymers to one another and also crosslink the polymers to the plasma membrane, thus creating a highly organized stable cytoskeletal structure (39). The deduced amino acid sequence of p15 shows that it contains 16 highly organized, nearly identical tandem repeats with a periodicity of 5 amino acids. A repetitive motive has been shown to be a common feature in MAP architecture (50), including other trypanosome MAPs like MARP (51), making p15 a legitimate member of the MAP family. As compared to other MAPs, however, the structure of p15 is unique in its organization. Not only does p15 contains repetitive sequences, but also the repeat domains themselves are highly organized and contain a pair of positively charged and non polar (hydrophobic) amino acids, thus enabling the molecule to bind not only tubulin but also phospholipids. The binding of MAPs to tubulin occurs via positively charged MAPs, which bind to the negatively charged C terminus of tubulin (50). If the charged amino acids are clustered on one side of the helix as is the case in mammalian MAPs (47), only that part of the molecule can interact with the negatively charged tubulin. Because in p15 the charged amino acids are distributed on both sides of the helix, it may be possible that this charge distribution enables the molecule to bind tubulin on both sides of the molecule, thus bundling the microtubule polymers to one another. Furthermore, being amphipathic, p15 may serve as a duel function, both bundling the microtubule polymers and also enabling the complex to bind to the cell membrane.

The p15 genomic DNA sequence is 30bp longer than the cDNA sequence, where two stretches of 15bps each are present in the genomic DNA but are absent in the cDNA, suggesting that the mRNA has been spliced. Alternative splicing is a common feature not only in trypanosomes but also in MAPs, where for example developmentally regulated alternative splicing of mRNAs encoding the mammalian MAP tau has been demonstrated (49). Interestingly, each p15 spliced region encodes five residues that if translated would have contributed to an additional repeat unit and to an additional helix turn. It is tempting to speculate that different lengths of p15 are expressed at different stages of the parasites life cycle and that the length of the p15 helix can contribute to the

p15 helix can contribute to the level of its interaction with tubulin, and as a consequence, to the differential stability of the subpellicular microtubules.

Repetitive sequences have been shown to be suitable for sensitive and specific detection of infective agents (97). Because p15 is a repetitive target sequence, is unique to trypanosome subpellicular microtubules and the subpellicular microtubules exist in all parasites of the Trypanosomatidae family, we hypothesized that primers specific to p15 may be useful for the PCR detection not only of *T. brucei* but also of other trypanosome species. PCR detection can provide a highly sensitive tool for the detection of trypanosomes, especially in early or chronic infections when the level of parasitemia is low or when trypanosomes are sequestered (60). The early diagnosis of trypanosomiasis is vital for successful treatment. When DNA of *T. cruzi* and *T. rangeli* epimastigotes were used as templates for DNA amplification by PCR using *T. brucei* p15-specific primers, DNA was specifically amplified in all trypanosome species tested. The DNA that was amplified from *T. cruzi* was as large as the one amplified from *T. brucei* (329bp) while DNA that was amplified from *T. rangeli* was larger (approximately 1kb). These results suggest that in fact primers specific to p15 DNA could be used to detect various trypanosome species. In addition, the different sizes of DNA, which was amplified from *T. rangeli* and from *T. cruzi* or from *T. brucei,* may allow specific diagnosis of specific trypanosome species in endemic areas. In the case of *T. rangeli and T. cruzi,* the different sizes of amplified DNA may be of special importance regarding diagnosis, because these parasites are present in highly overlapping geographic areas (23).

p15 could serve as therapeutic target sites, taking advantage of the importance of the microtubule integrity to the parasites and the uniqueness of MAPs to trypanosomes. p15 may also serve as unique antigen for immunological suppression of the disease. The use of intracellular antigens as vaccines has already been tested successfully using unique subpellicular MAP p52 together with the glycosomal enzymes aldolase and GAPDH (39). Sera of protected animals caused the aggregation of trypanosomes in vitro, and immunoelectron microscopy of these aggregated trypanosomes revealed the presence of antibodies within the parasites (32). Furthermore, host antibodies were shown to exhibit specific cross-reactivity to the trypanosome protein I/6, an internally repetitive cytoskeletal protein (58). Our studies show that mice vaccinated either with the native purified p15 or with the expressed recombinant p15 were protected from a challenge of *T. brucei.* The source of the native and recombinant p15 was from a different strain of *T. brucei* that was used for challenge, and the subpellicular microtubules are common to all members of the Trypanosomatidae family. This suggests that p15 may ultimately serve as a common target of therapy to all types of diseases caused by trypanosomes. Our studies show that mice vaccinated

either with the native purified p15 or with the expressed recombinant p15 were protected from a challenge of *T. brucei*. All the animals (8/8) vaccinated with the recombinant protein were protected and 87.5% of the animals (7/8) were protected when vaccinated with the native protein. The one animal that was not protected had decreased parasitemia and increased survival time. None of the control animals, which were injected with buffer only, were protected and all died.

Also tested was whether the adenovirus could be used as a vaccine delivery system for p15. The recombinant adenovirus was chosen as a delivery system to express p15 in mammalian cells because this system has been emerging as a highly promising method for vaccine development. It's advantages are its ability to induce the host's immune response, its wide host range and its high transduction efficiency. The results indicate that 100% of the animals (8/8) injected with the adenovirus containing the recombinant p15 gene were protected from a challenge of *T. brucei*. As a control, animals were injected with an adenovirus containing the *lacZ* gene. Interestingly, these animals were also 100% protected (4/4). It is hypothesized that this could be due to nonspecific activation of the host's immune response.

To test whether the adenovirus induces a cellular response, transduced tissue was assayed by immunohistochemistry. These studies indicate that while the adenovirus only slightly induces $CD4^+$ T cell proliferation, it highly induces $CD8^+$ T cell proliferation. It has been demonstrated that $CD8^+$ T cells have a major role in the natural immune response to *T. brucei* (86). Activated $CD8^+$ T cells produce INFγ that activates macrophages. Activated macrophages then release tumor necrosis factor and nitric oxide. Nitric oxide is one of the reactive nitrogen intermediates generated during the conversion of arginine to citrulline by arginase; it is strongly cytotoxic to a variety of parasites, and has been shown to be trypanostatic (87). Arginase can also cause damage by leading to deprivation of arginine, which is an essential amino acid for the parasites (88). It has been demonstrated that $CD8^+$ T cells have a major role in the natural immune response against *T. brucei* (79,80,81). Put together, these results suggest that protection against the p15 recombinant adenovirus may not have only been due to the expression of the recombinant p15 but also due to an induction of a non-specific cellular immune response to the adenovirus itself.

To summarize, p15 was cloned sequence and characterized and shown that it can protect animals from *T. brucei* infections. We hope that due to the similar structure of microtubules in all trypanosome species, p15 may serve as a useful vaccine target site in other trypanosome species as well.

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