

# Specific Uptake of Tumor Necrosis Factor- $\alpha$ Is Involved in Growth Control of *Trypanosoma brucei*

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**Abstract.** *Trypanosoma brucei* is lysed by tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) in a dose-dependent way, involving specific binding of the cytokine to a trypanosomal glycoprotein present in the flagellar pocket of the parasite. TNF- $\alpha$ -gold particles are endocytosed via coated pits and vesicles and are directed towards lysosome-like digestive organelles. The specific uptake of the cytokine by the parasite results in a developmentally regulated loss of osmoregulatory capacity. TNF- $\alpha$  specific lysis is prevented when lysis assays are performed at a temperature  $<26^{\circ}\text{C}$ , despite uptake of the cytokine. In-

hibition of lysis is also observed when a lysosomotropic agent is added during the first 2 h of incubation. Both monomorphic and pleomorphic trypanosomes are lysed but only when isolated during the peak of parasitaemia. Lysis is not observed with early infection stage parasites or procyclic (insect-specific) forms. Anti-TNF- $\alpha$  treatment of *T. brucei*-infected mice reveals a dramatic increase in parasitaemia in the blood circulation, the spleen, the lymph nodes, and the peritoneal cavity. These data suggest that in the mammalian host, TNF- $\alpha$  is involved in the growth control of *T. brucei*.

**A**FRICAN trypanosomes are extracellular parasitic protozoa, transmitted by the bite of the tsetse fly (44). *Trypanosoma brucei brucei*, *Trypanosoma vivax* and *Trypanosoma congolense* are the causative agents of Nagana, a cattle disease similar to sleeping sickness, caused in humans by *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. All these parasites need to survive a long time exposure to the immune system of their mammalian host, as they multiply predominantly in the bloodstream. Hence, well equilibrated growth regulation systems must exist, allowing the parasite to survive sufficiently long without killing its mammalian host, to ensure an effective transmission of the species. Such a system involves the variant-specific surface glycoprotein (VSG),<sup>1</sup> which is the major surface antigen and acts as a protective coat for the parasite (8, 28). During the ascending parasitaemia, the majority of the dividing parasites (e.g., long slender forms) belong to the same antigenic type, called the homotype. A peak of parasitaemia is reached when

long slenders differentiate into nondividing, short stumpy forms, which have a relatively short in vivo half life of 24–36 h and release VSGs in the circulation upon degeneration (5). These degenerating parasites allow the host to develop an antibody response to the homotype (34). Subsequently, the parasitaemia enters a descending phase as trypanosomes of the major variable antigen type (VAT) are eliminated. In contrast to the homotype VAT, spontaneous arising minor VATs or heterotypes continue to multiply during the descending phase of the parasitaemia. One of these VATs will overgrow the others and become the new homotype, giving rise to a new peak of parasitaemia (43). Although an effective anti-VSG response allows the host to regularly eliminate excessive numbers of parasites through phagocytosis of opsonized parasites (11), resistance and survival time of different mice strains cannot be directly correlated to the antibody response. Combined results from studies using different trypanosome strains in both resistant and susceptible mice, as well as their F1 descendants, showed that the ability to produce antibodies to the first variant antigen population is inherited as a dominant trait, while survival time during trypanosomiasis is inherited as a recessive trait (10, 33). Other in vivo studies have shown that by artificial control of the height of the parasitaemia level, susceptible mice become capable of clearing trypanosome infections (9), while irradiation of mice before infection does not influence the height of parasitaemia plateau (32). Collectively, these observations favor the hypothesis that factors other than the anti-VSG re-

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1. *Abbreviations used in this paper:* FSC, forward scatter; IFN- $\gamma$ , interferon- $\gamma$ ; TEM, transmission electron microscopy; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VAT, variable antigen type; VSG, variant-specific surface glycoprotein.

sponse may contribute to the control of the parasitaemia. Recently it has become clear that growth control of trypanosomes involves specific immunoregulatory molecules. Both EGF (15) and interferon gamma (IFN- $\gamma$ ; 27) were shown to enhance the growth of *Trypanosoma brucei*. As IFN- $\gamma$  synthesis was shown to be induced during trypanosomiasis, this growth regulation can be considered as active. Another cytokine shown to be induced during trypanosome infections is tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ; 16), a cytokine mainly produced by activated macrophages (42). Although the name of this cytokine is derived from its capacity to cause hemorrhagic necrosis of certain parenchymal organs and certain tumors, the molecule was initially isolated from the serum of *T. brucei*-infected rabbits as a factor called "cachectin," responsible for systemic suppression of lipoprotein lipase activity and trypanosome-induced cachectia (4, 29). In recent years TNF- $\alpha$  was found to be involved in the pathology of several parasitic diseases, including trypanosomiasis (24), Chagas' disease (39), leishmaniasis (40), schistosomiasis (1), and malaria (19), and in many of these cases this cytokine plays a bidirectional role (41). We have recently reported a direct interaction between TNF- $\alpha$  and *T. brucei*, mediated by the lectin-like domain of the cytokine (25). This domain was mapped at the upper side of the TNF- $\alpha$  molecule (determined the TIP domain) and found to be functionally and spatially distinct from the mammalian TNF- $\alpha$  receptor binding site. In this study we show that in vitro, TNF- $\alpha$  exerts a temperature- and pH-dependent lytic effect on certain developmental stages of *T. brucei*, involving the loss of osmoregulatory capacity. Furthermore the physiological relevance of these in vitro findings is confirmed by demonstrating a controlling role of TNF- $\alpha$  on parasite development in vivo.

## Materials and Methods

### Parasites

Two clonal lines of *T. brucei AnTat 1.1* were used in all experiments. The first *AnTat 1.1* line produces a pleomorphic infection in laboratory rodents and was kindly provided to us by Dr. N. Van Meirvenne (Institute of Tropical Medicine, Antwerp, Belgium). The second *AnTat 1.1* line used produces a monomorphic infection and was obtained by syringe passing the parasite. For analysis of the in vivo parasitaemia of *T. brucei*, F1 mice (C57bl6 X BALB/e) were infected with  $5 \times 10^3$  parasites. The number of trypanosomes present in the blood was counted every 2 d using a light microscope.

For in vitro TNF- $\alpha$ -mediated lysis experiments and TNF- $\alpha$ -gold binding and uptake experiments, trypanosomes were grown in F1 mice to  $10^7$  or  $2 \times 10^8$  cells/ml of blood for the pleomorphic line and  $10^7$  or  $10^9$  cells/ml blood for the monomorphic line. Whole blood was collected and diluted in an equal volume of PBS (pH 8.0) supplemented with 1% glucose (PSG) and 200 U heparin (Sigma Chemical Co., St. Louis, MO). Trypanosomes were separated from blood by DE52 chromatography (22), using PSG, pH 8.0, for equilibration and elution. Further in vitro incubation was performed in the equilibration buffer. Procyclic EATRO 1125 *AnTat 1.1* trypanosomes were cultured in vitro and incubated in the PSG equilibration buffer during all in vitro experiments.

### Preparation of Trypanosome Lysate

Washed DE52 purified trypanosomes were resuspended in PSG, pH 8.0, to a final cell density of  $2 \times 10^8$ /ml. Total cell lysate was obtained by three freezing/thawing cycles in the presence of 1 mM Pefablock<sup>®</sup> protease inhibitor (Boehringer Mannheim Corp., Mannheim, Germany). The total protein concentration of the lysate was determined by the BioRad detergent compatible protein assay kit (BioRad, Hercules, CA).

Endoglycosidase digestion of total lysate was performed with recombinant *Flavobacterium meningosepticum* N-glycosidase F (Boehringer Mannheim Corp.) using 5 U/ml in PBS, pH 8.0. The digestion was performed during 24 h at 37°C and followed by dialysis against PSG.

### Recombinant Murine TNF- $\alpha$

Commercially available recombinant murine TNF- $\alpha$  was supplied in 50 mM Tris/acetate, pH 7.0, 350 mM NaCl, sterile filtered (0.22  $\mu$ m) without addition of preservatives, and was kindly provided by Innogenetics NV (Gent, Belgium). The protein concentration was 1,200  $\mu$ g/ml as determined by means of UV protein measurement using 1.62 as extinction coefficient for a 1 mg/ml solution. The cytokine purity was >98%, as determined by SDS-PAGE analysis under nonreducing conditions, using both silver and Coomassie brilliant blue staining. The biological activity, determined with the mouse Lewis lung carcinoma cell line L929, was  $2.3 \times 10^8$  U/mg. Endotoxin levels were <0.25 EU/ $\mu$ g as determined by kinetic quantitative chromogenic LAL assay using an *Escherichia coli* standard, standardized against the EC-5 reference standard.

### Trypanolysis Assays

All lysis assays analyzed by light microscopy were performed with DE52-purified trypanosomes, washed, and resuspended in PSG (pH 8.0) to a final cell density of  $2 \times 10^9$ /ml. 100  $\mu$ l of the suspension was mixed with 50  $\mu$ l of different TNF- $\alpha$ /PSG dilutions and 50  $\mu$ l PSG in a 96-well flat bottom culture plate. The percentage of lysis was calculated using light microscopy counts of remaining parasites after TNF- $\alpha$  incubations, compared to the counts of remaining parasites incubated for the same period of time in the absence of TNF- $\alpha$ . Background lysis, i.e., lysis of trypanosomes within 5 h of incubation at 30°C in the absence of TNF- $\alpha$ , never exceeded 5%. Light microscopy images from a BX50 microscope (Olympus Corp., Tokyo, Japan) were photographed on Agfapan 100 ASA film (Agfa-Gevaert AG, Leverkusen, Germany).

To analyze dose- and stage-dependent, TNF- $\alpha$ -mediated lysis, trypanosomes were incubated at 30°C for up to 8 h in the presence of different final concentrations of TNF- $\alpha$ , ranging from 1 to  $10^6$  U/ml. Every hour the survival of the parasites was calculated by counting the remaining parasites in the presence and absence of TNF- $\alpha$ .

To examine temperature-dependent, TNF- $\alpha$ -mediated lysis, incubations were performed at different indicated temperatures in the presence of  $10^4$  U/ml TNF- $\alpha$ , up until the moment that the percentage of lysis reached its maximal level.

To analyze the relation between TNF- $\alpha$  uptake and TNF- $\alpha$ -mediated lysis, trypanosomes were incubated for 1 h at 17° or 21°C in the presence of  $10^4$  U/ml TNF- $\alpha$ , washed, and incubated for another 4 h at 30°C. In the reverse experimental setting, trypanosomes were incubated with TNF- $\alpha$  for 1 h at 30°C, washed, and further incubated at 17° or 21°C. Control parasites were incubated for 1 h with TNF- $\alpha$  at 17°, 21°, or 30°C, washed, and subsequently further incubated at the initial incubation temperature.

Inhibition lysis experiments were performed for up to 5 h at 30°C in the presence of  $10^4$  U/ml TNF- $\alpha$  and different concentrations of NH<sub>4</sub>Cl (0.1–mM). Concentrations of NH<sub>4</sub>Cl >5 mM were toxic during long periods of incubation.

The neutralizing capacity of anti-TIP/TNF antibodies on TNF- $\alpha$ -mediated trypanolysis was analyzed by preincubation of TNF- $\alpha$  for 1 h with 10  $\mu$ g/ml of the tested monoclonal and polyclonal antibodies.

For transmission electron microscopy (TEM) analysis of TNF- $\alpha$ -mediated lysis, monomorphic trypanosomes, isolated at the peak of the parasitaemia, were washed and resuspended in PSG (pH 8.0) supplemented with  $10^5$  U/ml TNF- $\alpha$ , to a final cell density of  $5 \times 10^7$ /ml. After various periods of incubation at 30°C, parasites were pelleted by centrifugation at 1,000 g for 10 min and washed two times with cold PSG followed by one wash in cold cacodylate buffer (pH 7.2). Subsequently, parasites were fixed overnight at 4°C in cacodylate buffer containing 2.5% glutaraldehyde. After two washes in cacodylate buffer, the cells were fixed in 2% OsO<sub>4</sub> in the same buffer, wrapped in agar, dehydrated in ethanol, and embedded in Epon. Ultrathin sections were floated on uranyl acetate and lead citrate solution before being observed in an AEI 6B electron microscope at 60 kV. Samples analyzed in TEM were analyzed in parallel in forward scatter (FSC), using a FACStar<sup>®</sup> (Becton Dickinson, San Jose, CA) with a forward amplification gain of 16. At regular time intervals, parasite samples were checked under a light microscope to calculate the percentage of trypanolysis. The obtained values were compared with the values obtained by subtracting the FACS<sup>®</sup> profile of control parasites from the

FACS® profile obtained after TNF- $\alpha$  incubations. Both calculation methods gave comparable results.

## L929 Lysis Assays

Lewis lung carcinoma L929 cells were cultured in RPMI 1640 medium supplemented with 10% heat inactivated FCS, L-glutamin (0.3  $\mu$ g/ml), penicillin (100 U/ml), and streptomycin (0.1 mg/ml), all obtained from GIBCO BRL/Life Technologies (Gaithersburg, MD). To analyze TNF- $\alpha$ -mediated lysis, cells were treated with actinomycin D (1  $\mu$ g/ml) at a concentration of  $2 \times 10^6$  cells/ml. 100  $\mu$ l of the suspension was mixed with 50  $\mu$ l of different TNF- $\alpha$ /medium dilutions and 50  $\mu$ l medium in 96-well flat bottom culture plates. After 18 h of culture in a humid incubator, at 37°C and a 5% CO<sub>2</sub> atmosphere, viability of the cells was assessed by dye uptake analysis. Medium was decanted from the plates and cells were stained for 10 min with 0.5% solution of crystal violet dissolved in a 1:5 mixture of methanol/water. Plates were rinsed extensively in distilled water, and dye uptake was assessed at 450 nm with a Titertek Multiscan MCC 340 ELISA Reader (Flow Laboratories, McLean, VA). The neutralizing capacity of the anti-TNF/TIP antibodies was analyzed by preincubation of TNF- $\alpha$  for 1 h at 37°C with 10  $\mu$ g/ml of the tested monoclonal or polyclonal antibodies.

## <sup>125</sup>I-TNF- $\alpha$ Binding Assays

Purified recombinant TNF- $\alpha$  (100  $\mu$ g) was labeled with <sup>125</sup>I by the chloramine-T method (17) and then filtered on Sephadex G-25. Bloodstream and procyclic forms of *T. brucei* (10<sup>8</sup> cells/ml) were incubated at 4°C with <sup>125</sup>I-TNF- $\alpha$  in 1 ml PSG equilibration buffer (pH 8.0) supplemented with 1% BSA. After 6 h of incubation with gentle shaking, the trypanosomes were washed five times with ice-cold PSG. The cell associated radioactivity was measured in a  $\gamma$ -counter. Nonspecific binding was determined as the amount of <sup>125</sup>I-TNF- $\alpha$  bound in the presence of 100-fold excess unlabeled TNF- $\alpha$ . It ranged from 15 to 30% of total binding. A similar level of aspecific labeling was observed when cells were incubated with rabbit <sup>125</sup>I-immunoglobulin (Amersham Corp., Arlington Heights, IL). The nonspecific binding on bloodstream forms equaled the total and nonspecific binding of <sup>125</sup>I-TNF- $\alpha$  on procyclic forms. The binding data were analyzed using the Prism program (GraphPad Software, San Diego, CA). Both estimations of dissociation constant and the number of binding sites/cell were obtained via a one site binding fit (hyperbola).

## Nonradioactive TNF- $\alpha$ Binding Assays and Affinity Measurements

Affinity measurements between TNF- $\alpha$  and total parasite lysate, as well as between TNF- $\alpha$  and anti-TNF/TIP monoclonal antibodies, were performed using the Iasys instrument with aminosilane cuvettes (Affinity Sensors, Cambridge, UK) according to the procedures recommended by the manufacturer. This type of measurement is based on the use of optical biosensor technology (12).

The immobilization of TNF- $\alpha$  to aminosilane was performed as follows. After initial preincubation of the aminosilane cuvettes with 10 mM phosphate buffer (pH 7.7), the homobifunctional crosslinker bis(sulfosuccinimidyl)suberate (BS3; Pierce, Rockford, IL) was added for 10 min to activate the surface. After reequilibration with phosphate buffer, TNF- $\alpha$  was immobilized for 10 min at 100  $\mu$ g/ml in phosphate buffer. Remaining active sites were blocked with 1 M ethanolamine (pH 9.0). Unbound TNF- $\alpha$  was removed by a single wash with 50 mM HCl. Specific binding resulted in a 300–500 arcseconds laser reflection shift. All binding reactions were performed at 27°C in PBS. Different concentrations of total lysate and antibodies were injected into the TNF- $\alpha$ -loaded cuvette. Association and dissociation (after removal of the excess of unbound ligand) were measured (Iasys software; Affinity Sensors). Regeneration of the cuvette was obtained by washing with 0.2% SDS in PBS.

The obtained data were analyzed by nonlinear regression, using the FASTfit® software (Affinity Sensors). The association constant  $k_{\text{ass}}$  was calculated for monoclonal anti-TNF/TIP antibodies using the slope of the  $k_{\text{on}}$  versus the antibody concentration. The dissociation constant  $k_{\text{diss}}$  was directly measured from dissociation experiments. Dissociation constant values equal  $k_{\text{diss}}/k_{\text{ass}}$ .

## Binding and Uptake Studies

TNF- $\alpha$ -gold complexes were prepared using 10-nm gold beads (Amersham Intl., Buckinghamshire, UK) following the prescribed protocol.

Briefly, the colloidal gold solution was equilibrated to pH 5.2, using 0.2 M H<sub>3</sub>PO<sub>4</sub>, as the pI of recombinant murine TNF- $\alpha$  is 4.7. A minimal protecting amount of TNF- $\alpha$  was added under continuous stirring for 2 min. Next, the pH was increased to 9.0, using 0.2 M K<sub>2</sub>CO<sub>3</sub>. 10% BSA (pH 9.0) was added to a final concentration of 1% after which the beads were pelleted for 30 min using an SW41 rotor at 45,000 g. The supernatant was discarded, and the pellet was washed once more at 50,000 g using a 10–30% glycerol gradient in 1% BSA-20 mM Tris/HCl buffer (pH 8.2). The pellet was collected and dialyzed overnight against the 1% BSA-Tris buffer. To estimate the amount of TNF- $\alpha$  bound to the gold beads, the concentration of nonbound TNF- $\alpha$  in all supernatant collected during the washings was determined using a specific ELISA. The TNF- $\alpha$ -gold complexes were stored in the 1% BSA-Tris buffer at an estimated concentration of 100  $\mu$ g/ml TNF- $\alpha$ . Control beads were prepared in parallel using the same condition, only without adding TNF- $\alpha$  at the start of the preparation.

To examine the binding and uptake of TNF- $\alpha$ , DE52-purified parasites were washed and resuspended in PSG (pH 8.0) at a final cell density of  $5 \times 10^7$ /ml. After a 1 h equilibration period at 30°C, 5  $\mu$ l TNF- $\alpha$ -gold corresponding to  $\sim 10^5$  U (0.5  $\mu$ g) of TNF- $\alpha$  was added, and the samples were incubated for various periods of time. Subsequently, the parasites were washed twice in PSG and fixed overnight in cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. Competition experiments were performed using a 50-fold excess of unlabeled TNF- $\alpha$ , anti-TNF/TIP antibodies (10  $\mu$ g/ml) or *N,N'*-diacetyl-chitobiose (1  $\mu$ g/ml), all added 1 h before incubation with TNF- $\alpha$  gold particles.

To analyze the influence of temperature on TNF- $\alpha$  uptake and TNF- $\alpha$ -mediated lysis, parasites were washed twice after 1 h of TNF- $\alpha$  gold incubation at 4°, 17°, or 30°C. Afterwards, the parasites were further incubated in fresh PSG equilibrated to the corresponding temperatures used before the washings. After a total of 4 h of incubation, samples were fixed overnight in cacodylate buffer (pH 7.2) containing 2.5% glutaraldehyde. Further preparation of the samples for TEM analysis was performed as described above.

## Staining of Trypanosomal Lysosome Compartment

Trypanosomes were stained with LysoTracker™ Red DN-99 (Molecular Probes Europe BV, Leiden, The Netherlands) for up to 3 h in the absence or presence of TNF- $\alpha$ . Cells were washed three times in PBS and fixed in 3% formaldehyde/0.5% glutaraldehyde. The LysoTracker™-stained parasites were observed on an Olympus BX50 microscope with an Uplanapo 100 $\times$  objective and a filter set for rhodamine fluorescence (excitation filter 510–550-nm long pass). The images were integrated during a fixed number of video cycles (64 for fluorescence; 16 for transmitted light) on an ImagePoint cooled CCD video camera (Photometrics Inc., Tucson, Arizona) and transferred to an IBAS image analyzer (Kontron, Munich, Germany). The region of interest was zoomed up twofold, and the gray level distribution was normalized. Finally, the video screen was photographed. The image treatment parameters (all exposure times and gray level scalings) were identical for the different experimental conditions.

## In Vivo Antibody Treatment

F1 mice infected with 2,000 pleomorphic AnTat 1.1 parasites were given one single intraperitoneal antibody injection 24 h before infection of 50  $\mu$ g purified antibody. During this treatment, the neutralizing monoclonal anti-TNF- $\alpha$  antibody 1F3F3 (23) was used, as well as monoclonal and polyclonal anti-TNF/TIP antibodies, directed against amino acid sequence 99–115 of mouse TNF- $\alpha$ . Control mice were treated with a monoclonal antibody 15D3 against *Bandeiraea simplicifolia* BS-I isolectin B4 or with a polyclonal anti-Con A antibody.

## Generation of Anti-TNF/TIP Antibodies

**Monoclonal anti-TNF/TIP antibodies.** F1 mice were immunized intraperitoneally a first time at day 0 with 20  $\mu$ g biotinylated mTIP peptide complexed to avidin (25) in complete Freund's adjuvant. The peptide used encompasses the 99–115 amino acid sequence (CGPKDTPEGAELKPWYC) of mouse TNF- $\alpha$ . At day 21, a first intraperitoneal boost with 20  $\mu$ g mTIP peptide-avidin complex was given in incomplete Freund's adjuvant, and at day 42 a final intraperitoneal boost of 20  $\mu$ g mTIP peptide-avidin complex was given in PBS. At day 45 the animals were killed, and the spleen was used to produce monoclonal antibodies. Mouse hybridomas were prepared by fusion of immune mouse splenocytes to the HAT-sensitive murine myeloma cell line NSO with polyethylene glycol 4000 at a splenocyte-

lymph node cell/NSO ratio of 10:1. Anti-mTIP monoclonal antibody-producing hybrids were cloned twice by limiting dilution (0.6 cell/well) in HAT medium. Screening of the anti-TIP monoclonal antibodies was done on an mTIP peptide–streptavidin coating using casein–streptavidin as control, and purification of positive antibodies was done on a protein G column.

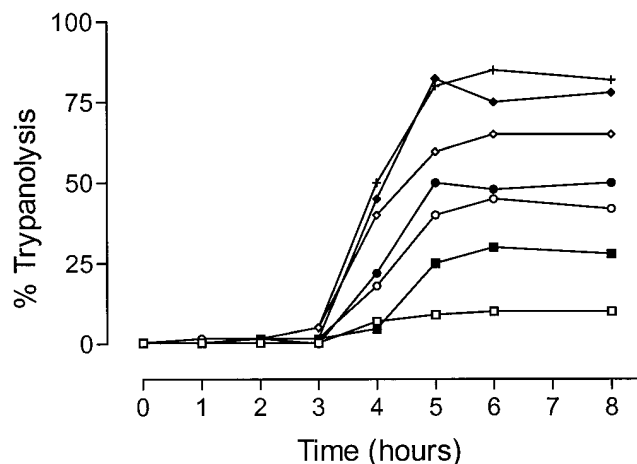
**Polyclonal anti-TNF/TIP antibodies.** The generation of the polyclonal anti-TNF/TIP antibody has previously been described (25). In short, the following procedure was followed: a rabbit was immunized subcutaneously at day 0 with a mixture of 1  $\mu$ g/ml mTIP peptide–avidin complex in 1 ml PBS and complete Freund's adjuvant in a ratio of 1:1. At day 14 and 28, a boost with 0.5 mg mTIP peptide–avidin complex in 1 ml PBS was given. At day 77, a final boost was given of a mixture of 1 mg mTIP peptide–avidin complex in 1 ml PBS and incomplete Freund's adjuvant in a ratio of 1:1. One week later the rabbit was bled to obtain antipeptide serum. Polyclonal anti-mTIP antibodies were purified on a protein G affinity column.

## Results

### Ultrastructural Analysis of TNF- $\alpha$ Mediated Lysis of *T. brucei*

Monomorphic AnTat 1.1 trypanosomes, isolated from the blood of infected mice, were incubated up to 8 h at 30°C in PSG containing different concentrations of recombinant murine TNF- $\alpha$  to follow trypanolysis as a function of time. As shown in Fig. 1, a dose-dependent lysis of the parasites was recorded, starting between 3 to 4 h of incubation and reaching maximal levels after 5 h of incubation. No significant lysis was recorded in the absence of TNF- $\alpha$ .

To study the morphological alterations that may occur during trypanolysis, TNF- $\alpha$ -treated parasites were analyzed by TEM and flow cytometry (FSC analysis). Concordant with the lysis experiments, TNF- $\alpha$ -induced morphological alterations occurred suddenly and progressed very fast (Fig. 2). Indeed, after 1 to 3 h of incubation with TNF- $\alpha$ , TEM analysis of the parasites revealed no major morphological changes (Fig. 2, *Aa* and *Ab*) and only a minor shift in



**Figure 1.** Lysis of bloodstream forms of *T. brucei* in function of time (hours) and concentration of TNF- $\alpha$ : 1 U/ml (□), 10 U/ml (■), 10<sup>2</sup> U/ml (○), 10<sup>3</sup> U/ml (●), 10<sup>4</sup> U/ml (◇), 10<sup>5</sup> U/ml (◆), and 10<sup>6</sup> U/ml (+). Trypanosomes were isolated from the blood of an infected mouse and incubated for up to 8 h in PSG (pH 8.0) at 30°C. Lysis assays were performed, and the percentage of lysis was calculated as described in Materials and Methods. Each concentration of TNF- $\alpha$  was tested in at least three independent experiments, and a representative experiment is shown.

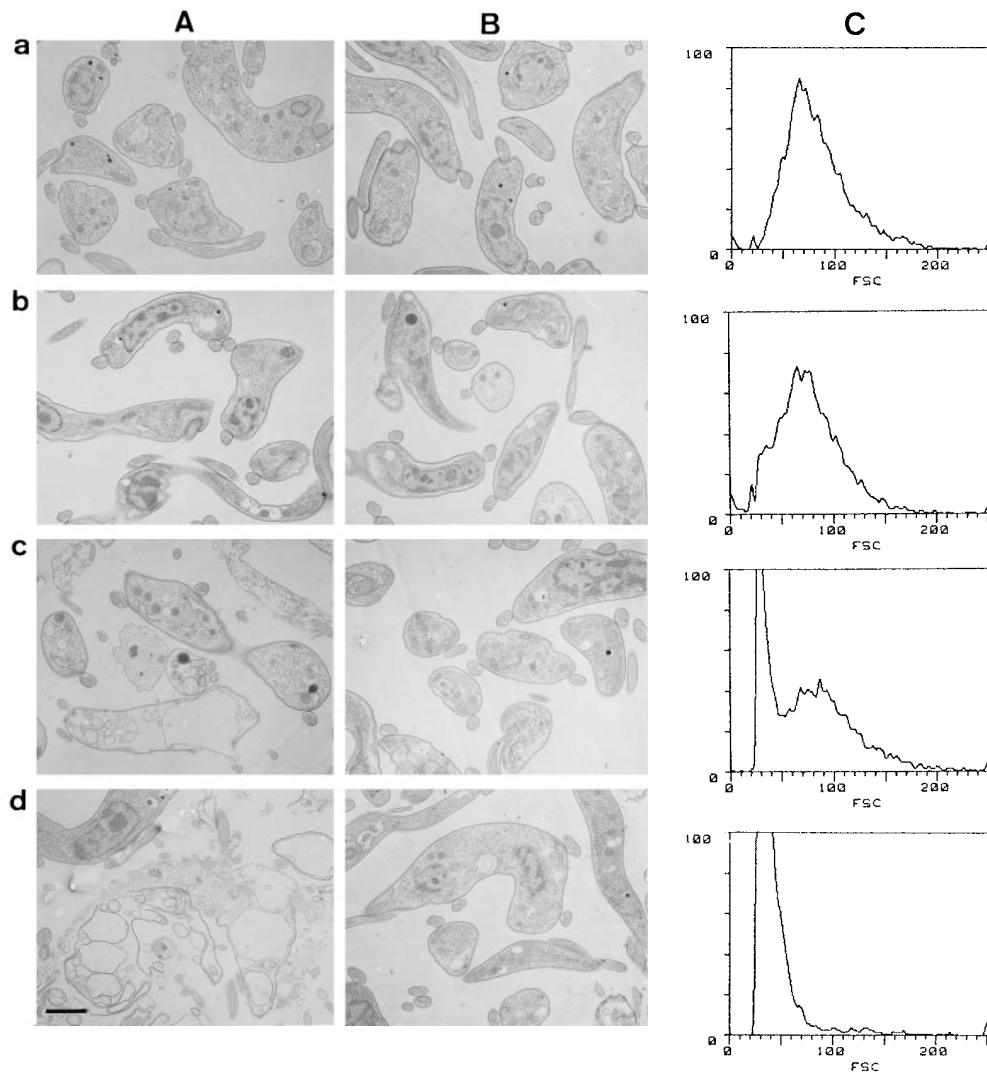
FSC signals (~5%) was recorded by flow cytometric analysis after 3 h of incubation (Fig. 2 *Cb*). At this time point, light microscopy analysis only indicated that a minor population became nonmotile. After 4 h, an important part of the trypanosome population analyzed in TEM manifested gross morphological alterations in the presence of TNF- $\alpha$ . As shown in Fig. 2 *Ac*, normal cells were observed in the vicinity of cells with swollen organelles and ruptured plasma membranes, reminiscent of an osmotic shock treatment. Concomitantly, the FSC signal of a major part of the parasites shifted to the left (Fig. 2 *Cc*) and indicated a trypanolysis of ~40%. After 5 h of incubation, TEM analysis showed a far progressed lysis, although some completely intact cells were still found to be present (Fig. 2 *Ad*). FSC analysis indicated a trypanolysis in the range of 85% (Fig. 2 *Cd*). Control parasites that were incubated in the absence of TNF- $\alpha$  and analyzed in TEM showed no changes in morphology during the course of this experiment (Fig. 2 *B, a-d*). During trypanolysis experiments, we never recorded a TNF- $\alpha$  specific lysis >85%, not even when incubations were performed for periods up to 8 h. This observation indicates that within an apparently morphologically homogenous population of monomorphic trypanosomes, there is a heterogeneity with regard to TNF- $\alpha$  sensitivity.

### Quantitative Analysis of TNF- $\alpha$ Binding on Live Trypanosomes and Trypanosome Lysate

To perform a quantitative analysis of the binding of TNF- $\alpha$  to live monomorphic AnTat 1.1 trypanosomes, parasites were incubated for 6 h at 4°C with different molar concentrations of <sup>125</sup>I-TNF- $\alpha$ . The results shown in Fig. 3 *a* confirm a specific binding of <sup>125</sup>I-TNF- $\alpha$  on bloodstream form trypanosomes. Competition binding experiments with <sup>125</sup>I-TNF- $\alpha$  in the presence of a 100-fold molar excess of cold TNF- $\alpha$  showed a significant reduction of the parasite labeling. No specific TNF- $\alpha$  binding could be recorded on procyclic parasites. Analysis of the specific binding data obtained with bloodstream form trypanosomes (Fig. 3 *b*) revealed an affinity constant of 37.2  $\pm$  14.8 nM and 1,558  $\pm$  302 TNF- $\alpha$ -specific binding sites. To confirm the binding of TNF- $\alpha$  to trypanosomes, a different method was adopted. Using an optical biosensor, the direct binding of TNF- $\alpha$  on total trypanosome lysate was measured (Fig. 4). To test whether this binding reflects an interaction between the lectin-like activity of TNF- $\alpha$  (25) and glycosylated trypanosome components, the trypanosome lysate was treated with N-glycosidase F. This treatment strongly reduced the binding of TNF- $\alpha$  to trypanosome lysate, indicating that indeed a glycoprotein of bloodstream-form trypanosomes binds TNF- $\alpha$  with high specificity via its N-linked carbohydrate moiety.

### Ultrastructural Analysis of TNF- $\alpha$ Binding and Uptake

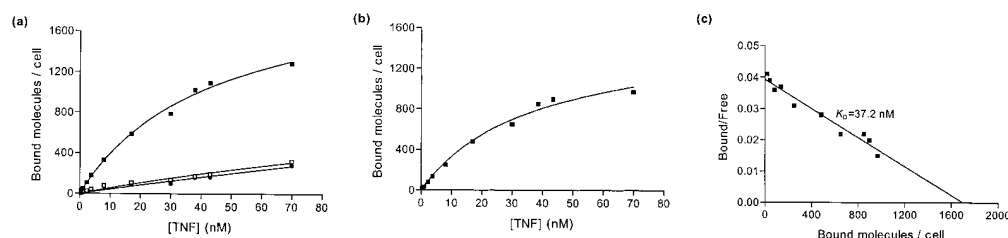
To localize TNF- $\alpha$  binding sites on intact parasites and to follow intracellular uptake of TNF- $\alpha$ , the cytokine was conjugated to 10-nm colloidal gold particles and subsequently incubated with monomorphic AnTat 1.1 trypanosomes at 30°C. The bulk of the TNF- $\alpha$ -gold labeling was localized in the flagellar pocket, where beads concentrated in coated pits (Fig. 5 *a, arrowhead*). Sporadically, TNF-



**Figure 2.** Morphological analysis by TEM and FSC of TNF- $\alpha$ -induced trypanolysis in function of time (*a*, 1 h, *b*, 3 h, *c*, 4 h, *d*, 5 h). Purified trypanosomes were incubated for various periods of time in PSG (pH 8.0) in the presence or absence of  $10^5$  U/ml TNF- $\alpha$ . (*A*) TEM analysis of TNF- $\alpha$ -treated *T. brucei* parasites. Swelling of organelles and lysis of parasites was recorded from 4 h of incubation onwards (*Ac*). (*B*) TEM analysis of non-treated *T. brucei* parasites. No lysis was observed. (*C*) FSC of TNF- $\alpha$ -treated *T. brucei* parasites. A major shift to the left of the FSC spectrum was observed from 4 h of incubation onwards (*Cc*). Bar, 1  $\mu$ m.

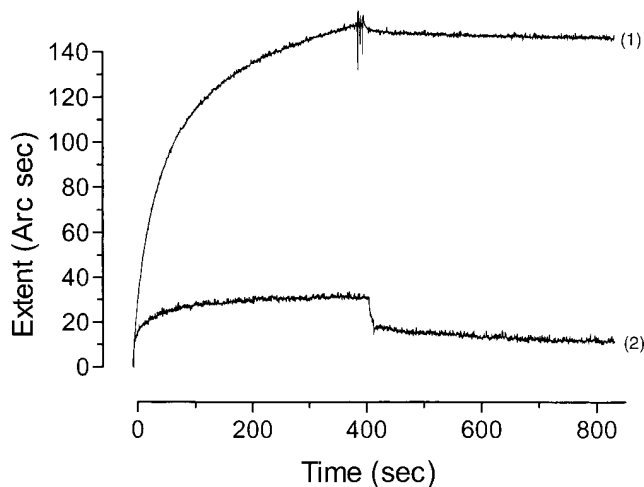
$\alpha$ -gold particles were found in association with the flagellum in the flagellar adhesion zone (Fig. 5, *b* and *c*), at the entrance of the flagellar pocket, or in association with tiny filamentous material at more distant regions of the flagellum (Fig. 5 *d*). After binding, gold labeled TNF- $\alpha$  was endocytosed through coated vesicles (Fig. 5 *e*). Coated vesicles containing TNF- $\alpha$ -gold particles were often seen fusing with larger electrolucent vacuoles wherein a cytoplasmic

area was invaginated, assuming cup-like shapes (Fig. 5 *f*) which might look like rings in TEM (Fig. 5 *g*). Gold particles were also observed in tubular vesicular structures (Fig. 5, *h* and *i*, *top*) but more frequently in dilatations of the collecting membrane system described by Langreth and Balber (21) which contain electron-dense material (Fig. 5 *i*, *lower part*, *j* and *k*, *middle*). In parasites incubated for longer periods (1 or 2 h), gold particles were also local-



**Figure 3.** Binding of  $^{125}$ I-TNF- $\alpha$  to bloodstream-form and procyclic trypanosomes. (*a*) Bloodstream-form trypanosomes (■) and procyclic trypanosomes (□) were incubated in the presence of different molar concentrations of  $^{125}$ I-TNF- $\alpha$ . Bloodstream forms were also incubated

with  $^{125}$ I-TNF- $\alpha$  in the presence of a 100-fold molar excess of cold TNF- $\alpha$  (●). The number of bound molecules per cell was plotted in function of the dose of  $^{125}$ I-TNF- $\alpha$  added. (*b*) Specific  $^{125}$ I-TNF- $\alpha$  binding to bloodstream-form trypanosomes. (*c*) Scatchard plot presentation of the same results. All the results shown are from one representative experiment.



**Figure 4.** The binding between TNF- $\alpha$  and total trypanosome lysate (1) or N-glycosidase F-treated trypanosome lysate (2) was analyzed using a biosensor. Sample injection was done at  $t = 0$ , and lysate was allowed to bind to a TNF- $\alpha$  coating on aminosilane. Free lysate was removed by a PBS wash after 400 s, and dissociation of the bound lysate was recorded. Both binding and dissociation are measured as a shift in laser reflection angle (Arc sec) as function of time (sec).

ized in larger lysosome-like digestive vacuoles with more (Fig. 5 *k*, *top*, and *l*) or less (Fig. 5 *m*) electron-lucent contents. These vacuoles often sequestered areas of cytoplasm remaining probably in connection with the surrounding ground cytoplasm. As shown in Fig. 5 *n*, the vacuole from a partially lysed parasite contains some gold particles (*arrow*) in addition to several such cytoplasmic areas.

When procyclic parasites were incubated with TNF- $\alpha$ -gold particles, no binding of TNF- $\alpha$  was seen in the flagellar pocket lumen, although rare formation of coated pits by the membrane of the flagellar pocket was observed (Fig. 5 *o*). Consequently, no internalization of gold particles was seen. As expected, no electron-dense inner layer, corresponding to the VSGs in the bloodstream forms (compare Fig. 5, *o* with *e*), was observed in procyclic coated pits.

When BSA-gold was used as a control, gold particles were only found in some intracellular vacuoles but not in coated pits or vacuoles. Competition experiments with an excess of unconjugated TNF- $\alpha$ , reduced substantially the cellular labeling, demonstrating that the binding of labeled gold particles was TNF- $\alpha$  specific. Similar inhibition of binding of TNF- $\alpha$ -gold particles was observed when the particles were preincubated with anti-TNF/TIP antibodies or *N,N'*-diacetyl-chitobiose before incubation with the trypanosomes (data not shown). These data are concordant with our previous report that the lytic activity of TNF- $\alpha$  is mediated via the lectin-like TIP-domain (25).

### **Influence of Temperature and Intracellular pH on TNF- $\alpha$ -mediated Lysis**

Incubations of parasites at 4°C with TNF- $\alpha$ -gold particles yielded a very rare labeling of the lumen of the flagellar pocket, and at this temperature no lysis was recorded, not even after 24 h of incubation (results not shown). To

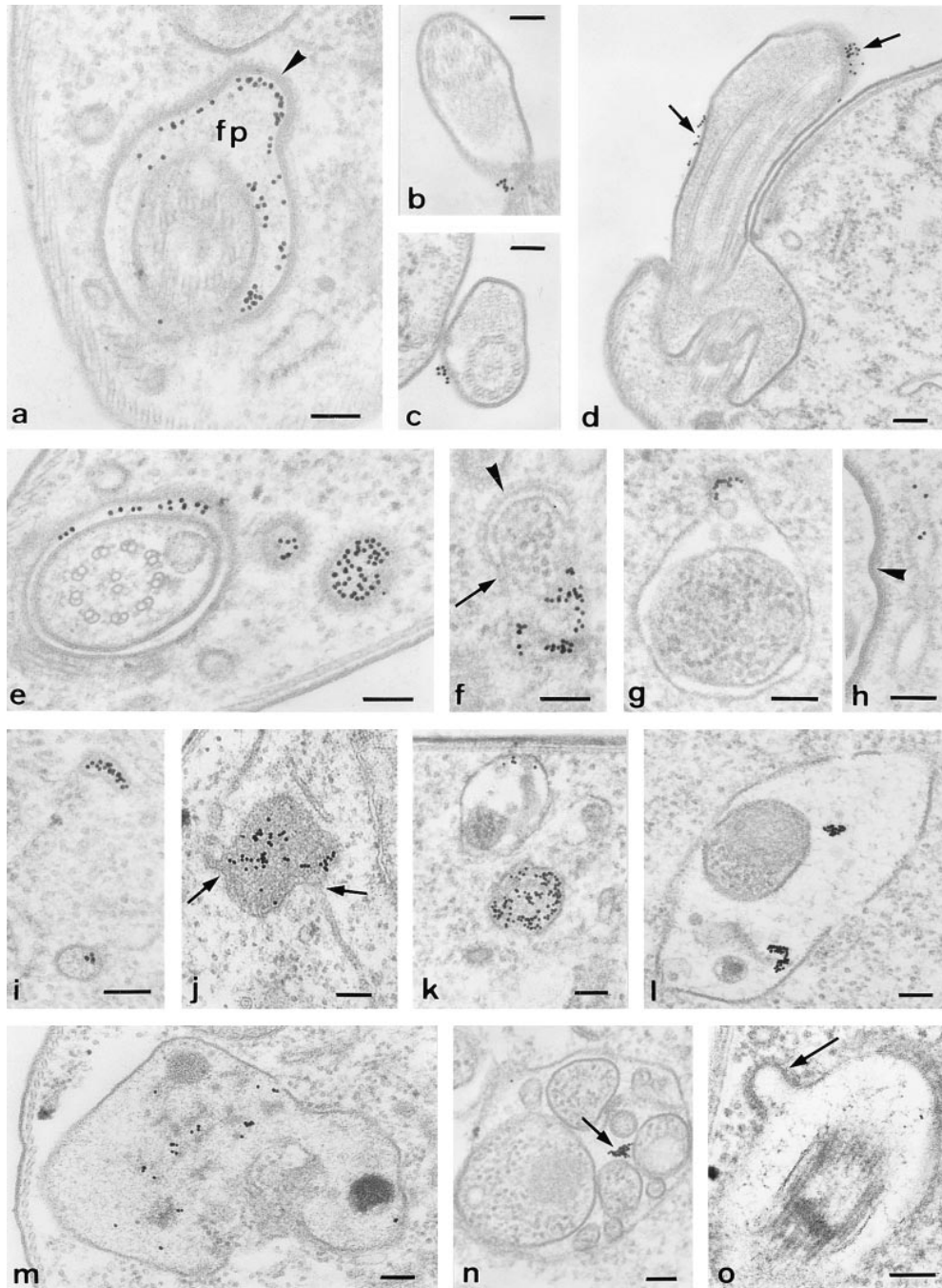
evaluate to which extent TNF- $\alpha$ -mediated trypanolysis is temperature dependent, experiments with monomorphic AnTat 1.1 trypanosomes were performed in parallel at different temperatures ranging from 37° to 17°C. According to the results shown in Fig. 6, the same maximal lysis was obtained at 37°, 32°, or 29°C, although the incubation time required to reach the lytic plateau value increased slightly with lower temperatures. At 26°C, ~50% of the maximal TNF- $\alpha$ -mediated lysis was recorded, and practically no lysis was observed at lower temperatures, not even when samples were incubated up to 18 h.

TNF- $\alpha$ -induced morphological alterations which preceded lysis could only be observed by light microscopy when incubation temperatures above 26°C were used. At 30°C in the absence of TNF- $\alpha$ , virtually no morphological changes were observed after a 5 h incubation period (Fig. 7 *a*). However, in the presence of TNF- $\alpha$ , a large number of cells were lysed, and most of the remaining cells showed a clearly altered swollen morphology (Fig. 7 *b*). When incubations were performed at 21°C, no morphological differences were observed between the cells incubated in the absence and presence of TNF- $\alpha$ , as shown in Fig. 7, *c* and *d*, respectively.

To analyze whether the insensitivity of the parasites to TNF- $\alpha$ -mediated lysis recorded at lower temperatures reflects inefficient TNF- $\alpha$  binding and/or uptake, monomorphic trypanosomes were incubated during 1 h at 17° or 21°C with TNF- $\alpha$  and subsequently washed and transferred to 30°C for another 4 h of incubation. In a reversed experimental setting, parasites were incubated with TNF- $\alpha$  for 1 h at 30°C and subsequently washed and transferred to 17° or 21°C. Control parasite populations were incubated with TNF- $\alpha$  during 1 h at 17°, 21°, or 30°C, washed, and further incubated at their initial temperatures. The results, shown in Table I, clearly indicate that during the first hour of incubation at 17° or 21°C, enough TNF- $\alpha$  was bound and internalized to yield a similar lysis as the lysis recorded during continuous incubation at 30°C. However, lysis was blocked at 17° and 21°C, even when the initial incubation was performed at 30°C.

Similar experiments were performed with TNF- $\alpha$ -gold particles, i.e., trypanosomes were incubated for 1 h with TNF- $\alpha$ -gold at 17° or 30°C. Subsequently, the samples were washed and re-incubated during 4 h at the respective temperatures. TEM analysis shown in Fig. 8 indicates that parasites preincubated with TNF- $\alpha$ -gold particles during 1 h at 30°C, started lysing after a total incubation time of 4 h at this temperature. (Fig. 8 *a*). In contrast, parasites preincubated at 17°C with TNF- $\alpha$ -gold particles, and kept at this temperature for another 3 h, did not exhibit any sign of lysis, although TNF- $\alpha$ -gold complexes were clearly internalized (Fig. 8 *b*). At 30°C, lysis was found to be preceded by swelling of the vesicles containing TNF- $\alpha$ -gold particles and of the mitochondria. Often, vesicles containing TNF- $\alpha$ -gold particles exhibit large disruptions of their membranes (Fig. 8 *a*, *arrow*). Such events preceded prompt lysis. At 17°C, gold conjugates were found to be endocytosed, yet swelling of TNF- $\alpha$ -collecting organelles or lysis of cells was not recorded (Fig. 8 *b*).

The TEM analysis with TNF- $\alpha$ -gold particles strongly suggests that the particles reach a lysosome-like compartment as a final destination. Hence, the influence of pH ele-



**Figure 5.** Localization by TEM of TNF- $\alpha$  binding and internalization at 30°C by *T. brucei*. TNF- $\alpha$  was conjugated to 10-nm gold particles as described in Materials and Methods. Cells were incubated with TNF- $\alpha$ -gold particles at a concentration of  $\sim 10^5$  U/ml. (a) TNF- $\alpha$ -gold particles binding in the flagellar pocket (fp) and concentrated in a coated pit formed by its limiting membrane (arrowhead). (b and c) TNF- $\alpha$ -gold particles visible at and near the contact zone between the flagellum and the cell body. (d) TNF- $\alpha$ -gold particles associated with the flagellum. (e) TNF- $\alpha$ -gold particles present in the lumen of the flagellar pocket and two vesicles in the cytoplasm. (f) A coated vesicle (arrowhead) in continuity (arrow) with a vacuole containing TNF- $\alpha$ -gold particles. (g) A vesicle containing TNF- $\alpha$ -gold particles fusing with a vacuole surrounding a cytoplasmic area. (h) TNF- $\alpha$ -gold particles present in tubular vesicular structures in close proximity to the coated region of the flagellar pocket membrane (arrowhead). (i) A flattened vesicle (top), and another one with a more electron-dense content, containing TNF- $\alpha$ -gold particles. (j) A dilatation of the collecting membrane system with electron-opaque lumen, containing TNF- $\alpha$ -gold particles, seen in continuity (arrows) with tubular structures. (k) An electron-lucent vacuole containing a few gold particles and an electron-opaque vacuole filled with TNF- $\alpha$ -gold particles are visible near the parasite surface. (l) Two groups of TNF- $\alpha$ -gold particles present in a large electron-lucent vacuole surrounding a cytoplasmic area. (m) A large digestive vacuole containing dispersed gold particles and containing a group of gold particles (arrow). (n) A vacuole surrounding several cytoplasmic areas and containing a group of gold particles (arrow). (o) Flagellar pocket of a procyclic form of *T. brucei*, whose limiting membrane forms a coated pit (arrow). Observations a–j were done within the first of our TNF- $\alpha$  incubations. Observations k–o were made 2 h after the start of the TNF- $\alpha$  incubation. Bars, 0.1  $\mu$ m.

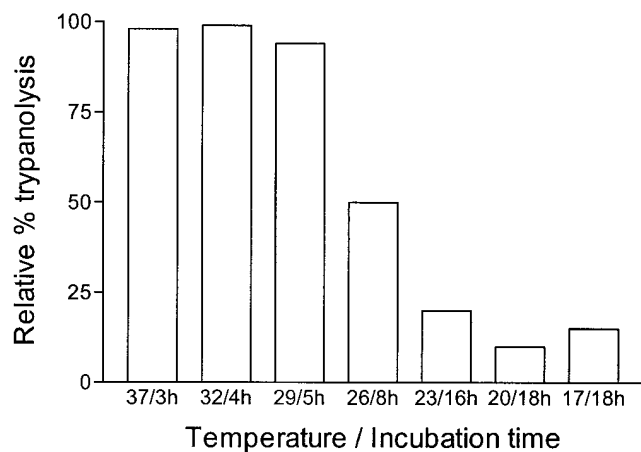
ible near the parasite surface. (l) Two groups of TNF- $\alpha$ -gold particles present in a large electron-lucent vacuole surrounding a cytoplasmic area. (m) A large digestive vacuole containing dispersed gold particles and containing a group of gold particles (arrow). (n) A vacuole surrounding several cytoplasmic areas and containing a group of gold particles (arrow). (o) Flagellar pocket of a procyclic form of *T. brucei*, whose limiting membrane forms a coated pit (arrow). Observations a–j were done within the first of our TNF- $\alpha$  incubations. Observations k–o were made 2 h after the start of the TNF- $\alpha$  incubation. Bars, 0.1  $\mu$ m.

vation on trypanolysis was analyzed. As shown in Fig. 9 a, the presence of NH<sub>4</sub>Cl during TNF- $\alpha$  incubations resulted clearly in a dose-dependent inhibition of lysis. Maximal inhibitions of  $\sim 90\%$  were recorded using concentrations of 1 mM of NH<sub>4</sub>Cl or higher. Inhibition of TNF- $\alpha$ -mediated lysis by 1 mM of NH<sub>4</sub>Cl was even recorded when the compound was added 2 h after incubation with TNF- $\alpha$  (Fig. 9 b). Addition of NH<sub>4</sub>Cl after 3 h of TNF- $\alpha$  treatment did

not result in a substantial inhibition of lysis. Hence, an acidic environment at the destination site of internalized TNF- $\alpha$  appears to be required for TNF- $\alpha$ -mediated lysis.

#### ***TNF- $\alpha$ -mediated Lysis of *T. brucei* Is Developmentally Regulated***

To analyze whether different developmental stages of *T.*



**Figure 6.** TNF- $\alpha$ -mediated lysis of bloodstream forms of *T. brucei* as function of temperature and time. A lysis assay was performed using a TNF- $\alpha$  concentration of  $10^4$  U/ml. Samples were kept at the indicated temperatures for various periods of time, up till the moment that a plateau of TNF- $\alpha$ -specific lysis was reached. The percentage of lysis was calculated as described in Materials and Methods.

*brucei* differ in sensitivity towards TNF- $\alpha$ , lysis experiments were performed at 30°C on both monomorphic and pleomorphic AnTat 1.1 trypanosomes isolated at the initial phase of the parasitaemia and at the peak of the parasitaemia. As shown in Fig. 10, *a* and *b*, TNF- $\alpha$ -mediated lysis of both monomorphic and pleomorphic trypanosomes occurred only when these bloodstream parasites were isolated at the peak of the parasitaemia, while no lytic effect was observed when parasites were isolated at the beginning of the infection. These results point again to a heterogeneity of trypanosomes with respect to their susceptibility to TNF- $\alpha$ .

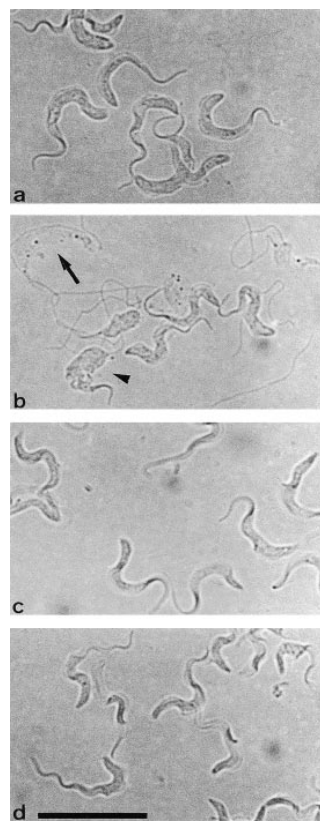
Taking into account the possible role of lysosome damage as an early event in TNF- $\alpha$ -mediated trypanolysis, both early (Fig. 11, *a* and *b*) and late stage trypanosomes (Fig. 11, *c* and *d*) were stained with the fluorescent lysosome marker LysoTracker™ in the absence or presence of TNF- $\alpha$ . Both the morphology of the parasites and the intracellular localization of the marker were analyzed after 3 h

**Table I.** TNF-mediated Trypanolysis in Function of Temperature

| Trypanosome treatment* |          | Percentage of Trypanolysis <sup>‡</sup> |          |
|------------------------|----------|---|----------|
| 0 to 1 h               | 1 to 5 h | TNF                                     | Controls |
| 17°C                   | 17°C     | 0 ± 1                                   | 0 ± 2    |
| 17°C                   | 30°C     | 69 ± 10                                 | 2 ± 1    |
| 21°C                   | 21°C     | 2 ± 3                                   | 4 ± 2    |
| 21°C                   | 30°C     | 72 ± 8                                  | 3 ± 3    |
| 30°C                   | 17°C     | 6 ± 5                                   | 4 ± 2    |
| 30°C                   | 21°C     | 15 ± 5                                  | 8 ± 3    |
| 30°C                   | 30°C     | 64 ± 8                                  | 3 ± 2    |

\*Purified bloodstream form trypanosomes were preincubated for 1 h at 17°, 21°, or 30°C with  $10^4$  U/ml TNF- $\alpha$ , washed, and further incubated for 4 h as indicated. Control parasites were incubated under the same temperature conditions in the absence of TNF- $\alpha$ .

<sup>‡</sup>The percentage of lysis was calculated as described in Materials and Methods after a total incubation time of 5 h.



**Figure 7.** Morphological characteristics of temperature dependent TNF- $\alpha$ -mediated lysis of bloodstream forms of *T. brucei*. Lysis assays were performed for 5 h at 30° and 21°C as described in Materials and Methods, using a TNF- $\alpha$  concentration of  $10^4$  U/ml. (*a*) At 30°C no background lysis was observed in the absence of TNF- $\alpha$ . (*b*) In the presence of the cytokine, most parasites were lysed after the 5-h incubation period. Remaining cells had a ghost-like appearance (*arrow*) or showed an abnormal swollen morphology (*arrowhead*). At 21°C, no signs of altered morphology or lysis were observed in the absence (*c*) or presence (*d*) of TNF- $\alpha$ . Bar, 20  $\mu$ m.

of incubation at 30°C. First, Fig. 11 shows that lysed parasites (*arrow*) and parasites with altered morphology (*arrowhead*) were only observed when late stage parasites were treated with TNF- $\alpha$  (Fig. 11 *Ad*). No cell damage was observed in the other samples (Fig. 11 *A, a-c*). Furthermore, Fig. 11 *Aa* (early stage) and *Ac* (late stage) show that in the absence of TNF- $\alpha$  the lysosome marker was localized essentially in a single spot. In the presence of TNF- $\alpha$ , a similar localization was still observed in the early stage parasites (Fig. 11 *Ab*), while a diffuse staining was observed in the late stage parasites (Fig. 11 *Ad*). These results suggest that TNF- $\alpha$ -mediated damage of the trypanosomal lysosome-like organelles precedes total lysis of the

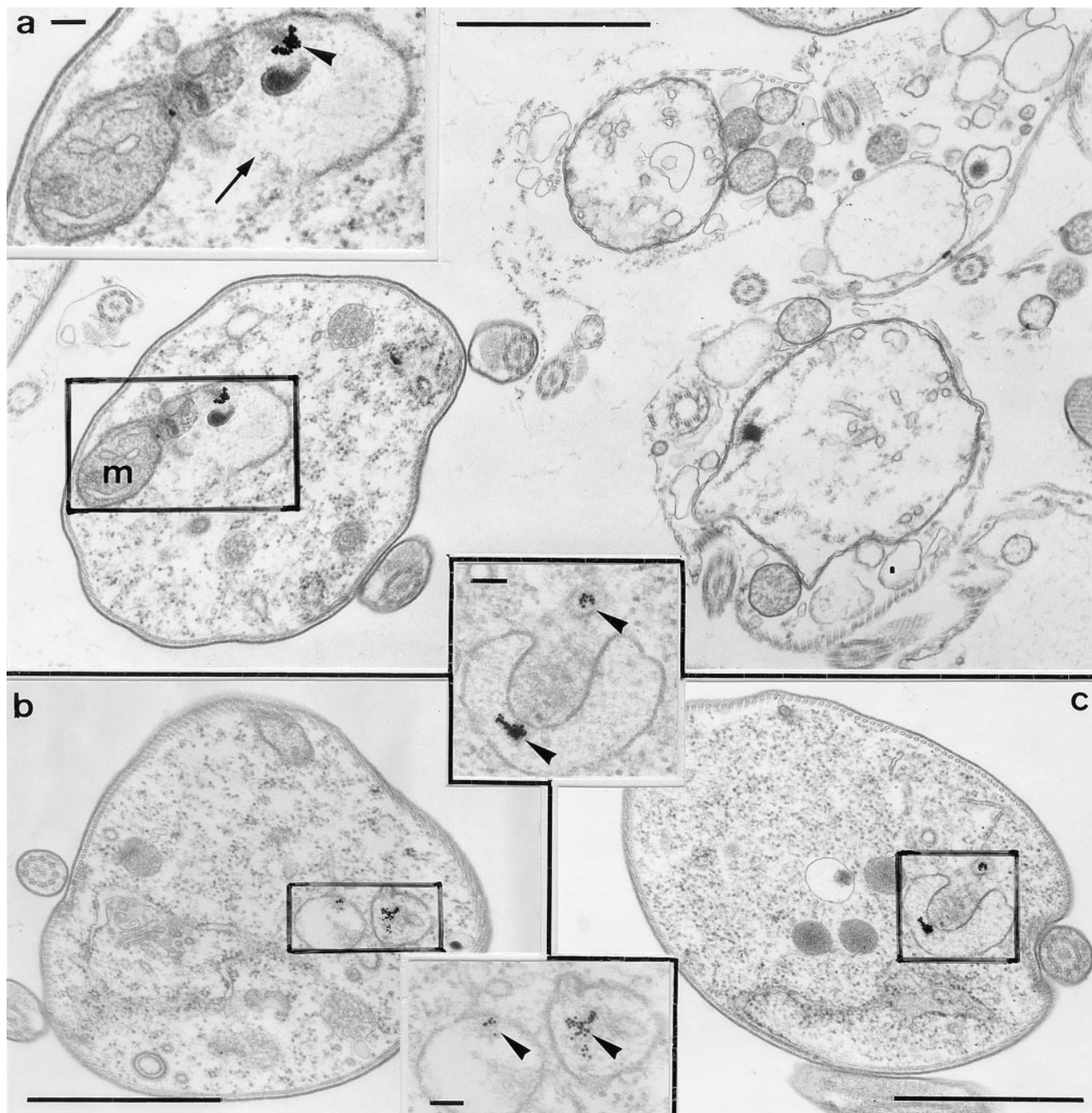
**Table II.** Influence of Anti-TNF Antibodies on Parasite Development in Trypanosoma-infected Mice

| Antibody tested* | Percentage increase in parasitaemia versus control <sup>‡</sup> |        |             |            |
|------------------|---|--------|-------------|------------|
|                  | Blood   | Spleen | Lymph nodes | Peritoneum |
| 1 E 12           | 174 ± 74  | 28 ± 4 | 34 ± 8      | 87 ± 32    |
| 24 C 11          | 219 ± 69  | 34 ± 2 | 35 ± 4      | 105 ± 31   |
| anti-TIP (poly)  | 155 ± 33  | 53 ± 4 | 241 ± 7     | 58 ± 2     |
| 1F3F3            | 158 ± 47  | 44 ± 6 | 130 ± 13    | 242 ± 82   |

\*Mice were infected intraperitoneally with pleomorphic *T. brucei* 24 h after intraperitoneal pretreatment with 50  $\mu$ g of purified anti-TNF- $\alpha$  antibodies (10 mice/group). 1E12 and 24C11 are monoclonal antibodies directed against the TIP domain of TNF- $\alpha$ . Anti-TIP (poly) is a polyclonal rabbit anti-TNF/TIP antibody. 1F3F3 is a neutralizing monoclonal anti-TNF antibody.

<sup>‡</sup>Results are expressed as average percentage increase of parasitaemia (peak stage) as compared to controls (animals treated with irrelevant monoclonal or polyclonal antibodies).

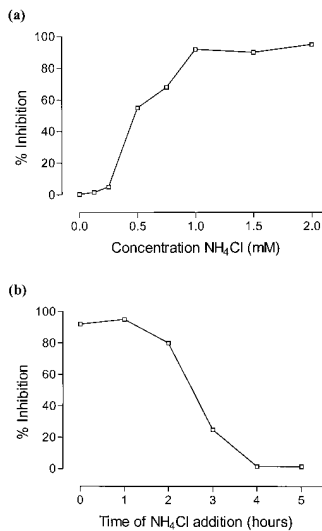




**Figure 8.** Transmission electron microscopy analysis of intracellular uptake of TNF- $\alpha$ -gold particles and lysis of bloodstream forms of *T. brucei*, isolated at the early stage and the peak of the parasitaemia. Cells were incubated with TNF- $\alpha$ -gold particles at a concentration of  $\sim 10^5$  U/ml. (a) Peak stage parasites were incubated at 30°C with TNF- $\alpha$ -gold particles for a total period of 4 h as described in Materials and Methods. TNF- $\alpha$ -gold particles are observed in a vacuole of an apparent intact parasite (*inset*, *arrowhead*), while other cells are completely lysed. A large disruption in the membrane of the TNF- $\alpha$ -gold containing vesicle is indicated in the *inset* (*arrow*). *m*, mitochondria. (b) Peak stage parasites were incubated at 17°C with TNF- $\alpha$ -gold particles for a total period of 4 h. TNF- $\alpha$ -gold particles are observed in two vacuoles (*center inset*, *arrowhead*). No lysed cells were observed under these experimental conditions. (c) Early-stage *T. brucei* bloodstream forms were incubated with TNF- $\alpha$ -gold particles for a total period of 4 h. TNF- $\alpha$ -gold particles are observed in a vacuole surrounding a cytoplasmic area and in a smaller vesicle (*bottom inset*, *arrowheads*). No lysis was observed under these experimental conditions. Bars: (a–c) 1  $\mu$ m, (*inset*) 0.1  $\mu$ m.

cells and is developmentally regulated. To exclude the possibility that the lack of lysosomal damage in early stage trypanosomes was due to a lack of TNF- $\alpha$  uptake, these parasites were also incubated with TNF- $\alpha$ -gold particles

for a total of 4 h at 30°C. Again, none of the events preceding trypanolysis were observed. As shown in Fig. 8 c, in such parasites, TNF- $\alpha$ -gold particles were endocytosed and subsequently collected in vesicles in a similar way as



**Figure 9.** Inhibition of TNF- $\alpha$ -mediated trypanolysis by NH<sub>4</sub>Cl. Lysis assays were carried out as described in Materials and Methods. (a) NH<sub>4</sub>Cl effects on lysis of *T. brucei* as function of concentration. Trypanosomes were incubated at 30°C in the presence of 10<sup>4</sup> U/ml TNF- $\alpha$  and different concentrations NH<sub>4</sub>Cl. (b) Effects of NH<sub>4</sub>Cl on trypanolysis as function of addition after preincubation of parasites with TNF- $\alpha$ . Trypanosomes were incubated for 5 h at 30°C in the presence of 10<sup>4</sup> U/ml TNF- $\alpha$ . Every hour NH<sub>4</sub>Cl was added to one sample to a final concentration of 1 mM.

The percentage of lysis inhibition was calculated compared to a control lysis of 10<sup>4</sup> U/ml in the absence of NH<sub>4</sub>Cl.

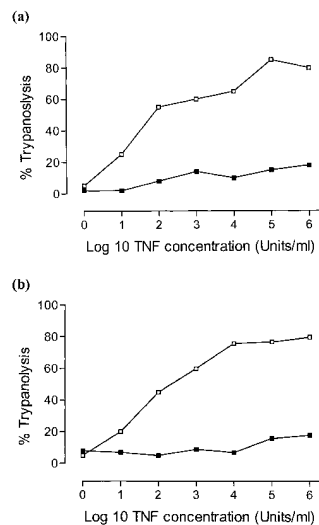
observed with peak stage parasites (Fig. 8 a), but no subsequent swelling or lysis of the cells was observed. These results stress again that the uptake of TNF- $\alpha$  is a necessary but not sufficient step to culminate in trypanolysis. Together with the earlier observed lack of TNF- $\alpha$  endocytosis by procyclic trypanosomes (Fig. 5 o) and the lack of TNF- $\alpha$ -mediated trypanolysis of these forms (data not shown), all results indicate that only late stage bloodstream-form trypanosomes are TNF- $\alpha$  susceptible while other forms are completely refractory to the trypanolytic activity of TNF- $\alpha$ .

#### **Influence of Monoclonal Anti-TNF- $\alpha$ Antibodies on TNF- $\alpha$ -mediated Trypanolysis In Vitro**

To confirm the involvement of the previously described lectin-like TNF/TIP domain in TNF- $\alpha$ -mediated trypanolysis (25), new monoclonal anti-TNF/TIP antibodies were elicited against TNF/TIP peptides encompassing the lectin-like activity of TNF- $\alpha$ . Antibodies were initially selected based on their affinity in ELISA for the TIP peptides used for immunization. For the selected monoclonal antibodies 1E12 and 24C11, the affinity for native TNF- $\alpha$  was further checked using the biosensor technique previously used to measure the affinity between trypanosome lysate and TNF- $\alpha$ . The dissociation constant values recorded were  $32.7 \pm 4.1$  nM and  $160.4 \pm 67.9$  nM for 1E12 and 24C11, respectively. As shown in Fig. 12, both antibodies and a previously described polyclonal rabbit anti-TIP antibody (25) were capable of neutralizing the trypanolytic effect of TNF- $\alpha$  in vitro (Fig. 12 a), while no inhibition activity was observed in a classical L929 TNF- $\alpha$  tumor lysis assay (Fig. 12 b).

#### **Influence of Anti-TNF- $\alpha$ Antibodies on *T. brucei* Development In Vivo**

To evaluate the in vivo relevance of the in vitro TNF- $\alpha$ -mediated trypanolytic activity, *T. brucei*-infected mice were treated with the above described anti-TNF/TIP antibodies.



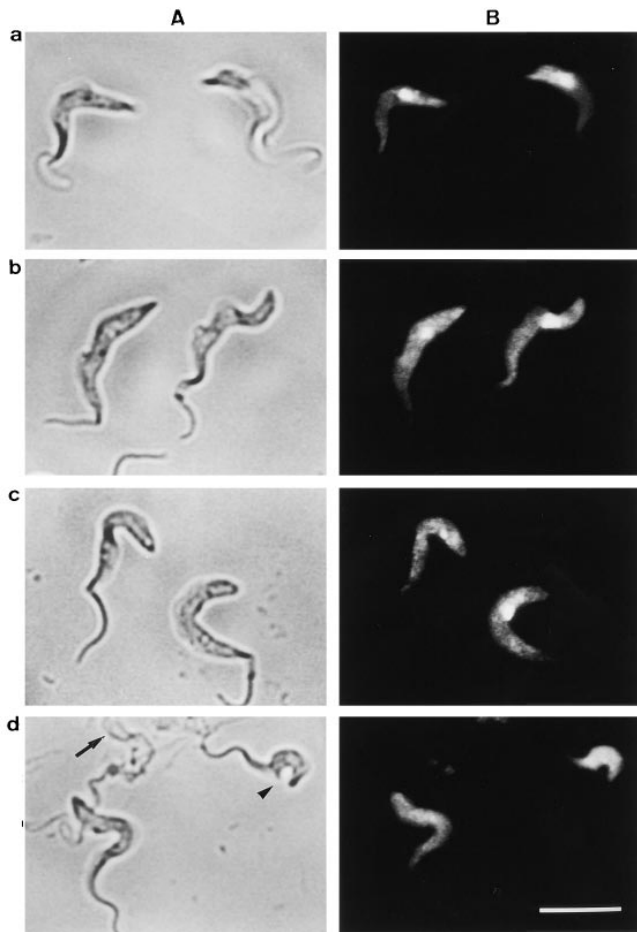
**Figure 10.** Trypanolytic activity of TNF- $\alpha$  on both monomorphic and pleomorphic bloodstream forms of *T. brucei*. Lysis assays were carried out at 30°C in the presence of different concentrations of TNF- $\alpha$  as described in Materials and Methods. (a) Monomorphic *T. brucei* parasites, isolated at the early stage (■) and the peak (□) of the parasitaemia, were incubated with TNF- $\alpha$ . The percentage of TNF- $\alpha$ -specific lysis was calculated as described in Materials and Methods. (b) Pleomorphic *T. brucei* parasites, isolated at the early stage (■) and peak (□) of the parasitaemia, were incubated with TNF- $\alpha$ , the same way as the monomorphic parasites.

We have reported earlier that the neutralizing monoclonal anti-TNF- $\alpha$  antibody 1F3F3 increases the number of parasites present in the blood of infected mice, indicating that TNF- $\alpha$  plays a controlling role during the normal course of a *T. brucei* parasitaemia (24). These experiments have now been repeated with the above described monoclonal and polyclonal anti-TNF/TIP antibodies. The results presented in Table II indicate that the in vivo treatment of *T. brucei* infected mice with the TIP-specific monoclonal antibodies 1E12 and 24C11 or the TIP-specific polyclonal antibody, resulted in a dramatic increase in the number of parasites during the first peak of the parasitaemia, as did the treatment with the 1F3F3 monoclonal anti-TNF- $\alpha$  antibody. The number of parasites, compared to control-treated animals, increased in the bloodstream, the spleen, the lymph nodes, and the peritoneal cavity. These results suggest that in vivo, TNF- $\alpha$  exerts a growth limiting effect on *T. brucei* via its lectin-like TIP domain and thus that the herein described trypanolytic activity of TNF- $\alpha$  is physiologically relevant.

#### **Discussion**

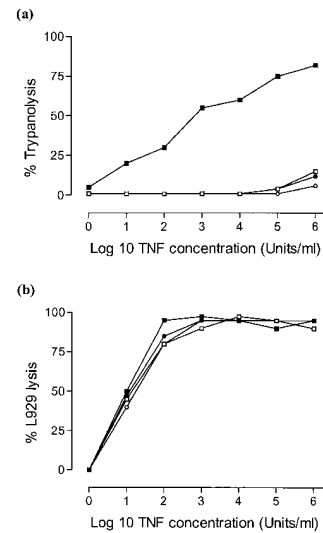
The herein described data extends and corroborates our previous findings on the trypanolytic activity of the cytokine TNF- $\alpha$  on African trypanosomes (24–26). Furthermore, evidence is provided for a direct involvement of TNF- $\alpha$  in the growth regulation of *T. brucei* in its mammalian host.

TNF- $\alpha$  induces the lysis of trypanosomes via a process that occurs suddenly and proceeds quickly after about 4 h of incubation at 30°C. This process of lysis is preceded by cellular swelling as if the cells are subjected to an osmotic shock. Hereby it should be emphasized that even when high concentrations of TNF- $\alpha$  (up to 10<sup>6</sup> U/ml) were used along with long incubation times (up to 8 h), a variable proportion of trypanosomes (15–30%) was found to be refractory towards TNF- $\alpha$ -mediated lysis. TNF- $\alpha$  lysis assays performed on monomorphic and pleomorphic AnTat



**Figure 11.** TNF- $\alpha$ -mediated lysis of bloodstream forms of *T. brucei* is preceded by the destruction of their lysosome-like organelles. Both early and late stage trypanosomes were incubated for 3 h at 30°C with the lysosomal marker LysoTracker™ in the absence or presence of 10<sup>4</sup> U/ml TNF- $\alpha$ . Early stage parasites showed a normal morphology (Aa) and a localized lysosomal staining (Ba). In the presence of TNF- $\alpha$ , both the morphology (Ab) and lysosome staining (Bb) are unaltered. Late stage trypanosomes also show a normal morphology (Ac) and lysosome coloration (Bc) in the absence of TNF- $\alpha$ . In the presence of TNF- $\alpha$  (Ad), some parasites are lysed (arrow) or have an abnormal morphology (arrowhead) and show intracellular diffusion of the lysosome marker (Bd). Bar, 10  $\mu$ m.

1.1 *T. brucei* parasites, isolated at the early parasitaemia phase or at the peak of the parasitaemia, revealed that the sensitivity of trypanosomes towards the lytic activity of TNF- $\alpha$  is marked by a destruction of the trypanosomal lysosome-like organelles and is developmentally regulated. As TNF- $\alpha$  sensitivity is only acquired at the peak of the parasitaemia, the minor population of nonsensitive trypanosomes still present during the peak, might represent a population that did not yet reach this sensitive stage. Since both monomorphic and pleomorphic trypanosomes, isolated at the peak of the parasitaemia, were found to be equally TNF- $\alpha$  sensitive, a differential TNF- $\alpha$  susceptibility of long slender versus short stumps is excluded. Furthermore, procyclic trypanosomes were found to be completely resistant towards TNF- $\alpha$ . This lack of TNF- $\alpha$  sensi-



**Figure 12.** Specific inhibition of TNF- $\alpha$ -mediated lysis of bloodstream forms of *T. brucei* by anti-TIP antibodies. (a) Freshly isolated bloodstream-form trypanosomes were incubated for up to 8 h in PSG (pH 8.0) at 30°C in the presence of different concentrations of TNF- $\alpha$ . Trypanolysis was calculated as described in Materials and Methods (■). Lysis was inhibited by preincubation of TNF- $\alpha$  with three different TIP-specific antibodies. The polyclonal antibody (□) as well as both monoclonal antibodies 1E12 (●) and 24C11 (○), all inhibited the TNF- $\alpha$ -specific trypanolysis to approximately the same extent. (b) None of the anti-TIP antibodies inhibited TNF- $\alpha$ -mediated lysis of the TNF- $\alpha$ -sensitive L929 cell line. The same symbols are used as in panel (a).

tivity of the procyclic form is due to the lack of TNF- $\alpha$  binding. Scatchard analysis of <sup>125</sup>I-TNF- $\alpha$  binding on bloodstream form trypanosomes revealed the presence of ~1,600 TNF- $\alpha$ -binding molecules, while no binding could be observed on procyclic parasites. Analyzing the binding between crude parasite lysates and TNF- $\alpha$  with an optical biosensor confirmed the presence of a bloodstream stage specific glycoprotein that is capable of binding TNF- $\alpha$ . Removal of N-linked carbohydrate groups by N-glucosidase F treatment strongly reduced the TNF- $\alpha$  binding. This observation confirms our previous finding that the lectin-like domain of TNF- $\alpha$  is involved in the interaction with trypanosomes (25).

Using TNF- $\alpha$ -gold particles, binding of TNF- $\alpha$  was localized mainly in the flagellar pocket. This binding could be inhibited by cold TNF- $\alpha$ , anti-TNF/TIP antibodies, and *N,N'*-diacetyl-chitobiose, indicating that the binding of gold particles is TNF- $\alpha$  specific and that the lectin-like domain of TNF- $\alpha$  is implicated in the binding process (25). Hence, TNF- $\alpha$  interacts most probably with specific carbohydrate components of the glycoprotein matrix enclosed in the flagellar pocket. In fact, WGA, which displays a similar carbohydrate specificity as TNF- $\alpha$  (35), was reported to bind selectively to the flagellar pocket of *T. brucei* (6), and we have shown that preincubation of *T. brucei* with WGA inhibits the trypanolytic activity of TNF- $\alpha$  (25). We found that TNF- $\alpha$  binds to the flagellar pocket and the flagellar adhesion zone of bloodstream forms but not of procyclic forms. Since the flagellar pocket glycoprotein composition was reported to differ between these two forms (13), it may be that TNF- $\alpha$  binding oligosaccharides are either absent or inaccessible in the flagellar membrane microdomains of procyclic trypanosomes.

After binding, TNF- $\alpha$ -gold particles were found to be endocytosed through coated pits and vesicles. In bloodstream-form trypanosomes, this pathway is used for instance for the specific uptake of transferrin via its heterodimeric glycoprotein receptor (30). Coated vesicles

containing TNF- $\alpha$ -gold were found to fuse with endosomes, and subsequently the particles were localized in tubular vesicular extensions and more electron-dense cisternae of the collecting membrane system, all structures that were extensively described in trypanosome bloodstream forms (21). Finally, TNF- $\alpha$ -gold complexes were found in digestive vacuoles connected to the collecting membrane system. Globally, the intracellular pathway of endocytosed TNF- $\alpha$ -gold complexes seems to involve similar structures as those ones associated with the specific uptake of transferrin (30, 37), ferritin (21), high density lipoprotein (HDL; 14), and low density lipoprotein (LDL; 7), suggesting that the final destination of the particles is lysosome-like digestive vacuoles. Disruption of these collecting vacuoles was frequently observed independent of whether native ligand (TNF- $\alpha$ ) or ligand conjugates (TNF- $\alpha$ -gold) were tested in the trypanolysis assay.

The exact mechanism underlying TNF- $\alpha$ -mediated trypanolysis is so far not defined. It is however clear that binding and endocytosis of TNF- $\alpha$  is necessary but not sufficient to induce trypanolysis. Indeed, endocytosis of TNF- $\alpha$  is comparable in early and peak stage bloodstream forms, yet only the peak-stage trypanosomes can be lysed by the cytokine. Furthermore, while TNF- $\alpha$  uptake occurs at 30° as well as at 21° and 17°C, its lytic activity appears to be strongly temperature dependent and requires a temperature >25°C. Hence an intracellular process that is developmentally regulated and temperature sensitive determines whether TNF- $\alpha$ -mediated lysis will or will not occur. Interestingly the threshold temperature required to inhibit the lytic activity of TNF- $\alpha$  does not correspond to that one preventing the fusion of endosomes and lysosomes (17°C) as documented for high density lipoprotein-mediated trypanolysis (14). Rather, this 25°C threshold temperature corresponds exactly to the temperature at which a shift in the membrane fluidity of trypanosomes occurs (20). Our results further indicate that TNF- $\alpha$ -mediated trypanolysis is pH dependent, since incubations with ammonium chloride strongly inhibit the lytic activity of TNF- $\alpha$ . Apparently an acid pH is required to allow TNF- $\alpha$  to exert its intracellular lytic activity. This observation may be related to the documented pore-forming capacity of TNF- $\alpha$  (18). Indeed at low pH, as a result of a conformational shift, TNF- $\alpha$  is able to integrate into mammalian membranes resulting in pore formation (3). Furthermore, the pore-forming capacity of TNF- $\alpha$  is mediated by the TIP-domain that is implicated in the binding of TNF- $\alpha$  on trypanosomes (18). The formation of ion-permeable channels could account for the influx of cytosolic ions into TNF- $\alpha$ -collecting organelles, leading to the features of osmotic shock that appear to be the primary event in TNF- $\alpha$ -mediated trypanolysis. After organelle rupture, release of proteolytic enzymes may accelerate the lytic process resulting in prompt and complete lysis. Hereby mitochondria could be among the first affected organelles, since circular cristae were frequently observed in mitochondria of TNF- $\alpha$ -treated cells. It should be emphasized that the proposed mechanism of TNF- $\alpha$  is not confined to trypanosomes but could also occur in mammalian cells. First, bypassing the classical TNF- $\alpha$  pathway involving the p55 and p75 TNF receptors by microinjection of the cytokine leads to lysis of mammalian cell lines such as L929 cells (36). Remarkably,

intracellular administration of TNF- $\alpha$  causes lysis of L929 cells after 4 h (similar for lysis of trypanosomes), while when TNF- $\alpha$  is supplied in the medium, lysis of L929 cells requires ~20 h. Second, as already mentioned, TNF- $\alpha$  exerts a pH dependent pore-forming activity on mammalian membranes. Third, degeneration of mitochondrial structures is an early event in TNF- $\alpha$ -mediated lysis of mammalian cells (31). So intracellular TNF- $\alpha$  may exert similar activities in trypanosomes and mammalian cells. Till now, we cannot exclude the possibility that part of the intracellular TNF- $\alpha$  is recycled to the membrane and exocytosed. This possibility may account for our observation that clear TNF- $\alpha$  concentration dependent plateau levels of lysis were recorded, since partial recycling of intracellular TNF- $\alpha$  could result in a steady-state situation.

The *in vivo* experiments with anti-TNF/TIP antibodies finally demonstrate that TNF- $\alpha$  exerts a growth-controlling function on trypanosomes. This host cytokine, which can be induced by the parasite itself (26, 38), apparently binds specifically on trypanosomes and kills certain developmental stages of the parasite. Though we cannot yet explain why TNF- $\alpha$  sensitivity is developmentally regulated, it is clear that trypanosomes are only lysed by TNF- $\alpha$  during the peak stage of the parasitaemia. Important to mention here is the recent report that TNF- $\alpha$  was shown to abolish the growth promoting effect of IFN- $\gamma$  (2). Our findings show that in addition to counteracting growth promotion, TNF- $\alpha$  may contribute to limit the number of parasites in the bloodstream, peritoneal cavity, and lymphoid organs by actively lysing trypanosomes.

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