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Alpha 2 macroglobulin activity in rats infected with *Trypanosoma lewisi* and treated with cyclophosphamide and its effect on the malignancy of the disease

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Abstract

Background & objectives: Trypanosoma lewisi is a common, flagellated parasite of the rat. Our previous study showed that rabbits injected with serum collected from rats infected with *Trypanosoma lewisi* and treated with cyclophosphamide (CyI) produced high levels of antibodies against a new protein in the CyI rat serum.

Results: In the present study, this protein was characterised as α2 macroglobulin (α2M) and the kinetics of its production and its influence on the malignancy of the disease were determined. In rats infected with *T. lewisi*, α2M was first demonstrated and peaked on the second day post-infection (972 μg/ml) and then reduced gradually, reaching a level of 32 μg/ml on the eighth day post-infection. However, in the CyI rats the level of α2M was gradually increased as the disease progressed, reaching a level of 890 μg/ml on the eighth day post-infection. Injection of both crude and purified α2M into rats infected with *T. lewisi* led to increased parasitaemia.

Interpretation & conclusion: The present study suggests that increased levels of α2M in the CyI rats contribute to the malignancy of the disease.

Key words Alpha 2 macroglobulin – cyclophosphamide – parasitaemia – *Trypanosoma lewisi*

Introduction

Trypanosoma lewisi is an obligatory, flagellated parasite of the rat with a world wide distribution. Similar to African trypanosomes, *T. lewisi* has two biological forms: epimastigote, in the insect's intestine and trypomastigote, in the rat blood[1]. The parasite is naturally transmitted to the rats by fleas by either the inoculation or the contamination pathways[1]. Unlike the African trypanosomes, *T. lewisi* has no antigenic variation and therefore, the disease in the rat is self-terminated, leading to a total elimination of the parasites and protection of the rats against re-infection[2]. The cured rats are resistant to re-infection with *T. lewisi*. Despite the fact that the rats naturally overcome the disease, a lethal infection can be mediated by the administration of an immunosuppressive agent like cyclophosphamide (Cy) or by splenectomy. In both cases, a severe malignant disease with a very high (50–60%) parasitaemia develops that kills the rats within 10–15 days post-infection[3].

Previous studies performed in our laboratory showed that injection of rabbits with sera collected from heavily infected rats that were treated with Cy (CyI), led to the production of antibodies against most of the

serum components, and against a new protein that was not detected in normal rat serum (N)[4]. The aim of the present study was, therefore, to isolate this protein from CyI rat serum, to characterise it, to determine the nature of its production, and to examine its influence on the malignancy of *T. lewisi* infection.

Material & Methods

Animals: Male rabbits supplied by Yokneam Company (Yokneam, Israel), weighing 2.5–3 kg were immunised against rat's sera and α2M and their sera were used in immunological tests. Male and female Lewis rats (Specific pathogen free), supplied by Harlan Company (Rehovot, Israel) weighing 150–450 g were used for *T. lewisi* growth and as a source of sera from treated infected rats.

Parasites: T. lewisi parasites were obtained from the American Type Culture Collection (strain number 30022, Manassas, USA) and kept in rats and as a stabilate at –70ºC. Rats were inoculated intra-peritoneally (ip) with 1 ml saline containing 2×10^6 trypanosomes. At various times post-infection, blood samples were collected from either the tail or directly from the heart of ether-anesthetised animals. The parasitaemia was determined by counting the live parasites by haemocytometer or by calculating the number of trypanosomes *vs* 1×10^4 erythrocytes, in Giemsa's stained preparation.

Statistical analysis: Results in each experiment are summarised as the mean parasitaemia values and the standards of deviation (SD).

Parasites' homogenate: The blood was first collected from infected rats, using heparin as anticoagulant. The blood was centrifuged at 2000 rpm for 10 min (Beckman centrifuge) at room temperature, and the parasites that were concentrated in the upper part were collected, washed ×3 with saline and centrifuged again at 3000 rpm for 10 min at room temperature. The pellet was then resuspended in saline and brought to a concentration of 2×10^7 parasites/ml. A homogenate was prepared by five cycles of freezing and thawing, followed by a sonication at medium power five (Sonifier cell disruptor B-30, Branson Sonic Power, Danbury, CT, USA) using three pulses, each lasting 30 sec. The homogenate obtained was centrifuged at 14000 rpm for 10 min at 4ºC. The supernatant was collected and the pellet was washed three times with saline and finally resuspended in 50% of the original volume. Both the supernatant and the pellet were kept at –20ºC until used.

Effect of various treatments on T. lewisi development in rats: Rats, weighing 150–220 g were inoculated ip with $1–2 \times 10^6$ trypanosomes. The infected rats were either further inoculated ip with a single dose of cyclophosphamide (Cy, 10 mg/100 g, Cytophosphan, Taro, Taro Pharm Indust Ltd, Haifa, Israel) (CyI) or were splenectomised (SpI)[4]. Normal uninfected rats inoculated with Cy only (Cy) were used as control. In addition, normal and infected rats, either treated or untreated with Cy, were inoculated ip with a single dose of 0.25 ml of turpentine oil (Tp) (Sigma)[5], or were injected daily ip, with 1.25–2.5 mg/100 g mercaptopurine (6-Mercaptopurine, Sigma)[6].

Rabbits immunisation: Rabbits (2.5–3 kg) were injected eight times, at a 10 days intervals, with 1 ml of either CyI or Tp rat serum mixed (vol/vol) with Complete Freund's Adjuvant (CFA, Sigma). Approximately 10 days after each injection, a blood sample was collected, the serum was separated and tested for anti-α2M antibody activity by immunodiffusion (ID) and immunoelectrophoresis (IEP), prior and after its absorbance to normal rat serum. Rabbits were also immunised against anti-α2M/α2M immune complexes and α2M fraction extracted from both, agar gel and SDS polyacrylamide gel.

Immunological tests: ID, IEP and IMB were performed as previously described[5]. ID and IEP were performed in 0.9% agar gel and the results were recorded in fresh preparations and after staining with

amido black. IMB was performed, using both native (without detergents) and SDS-PAGE gels as previously described[5]. The concentration of α2M in rats' sera was determined using a radial immunodiffusion made in 0.7% agarose solution in PBS buffer[6]. The test was performed on a 8 × 10 cm glass slide covered with 8 ml of (pre-heated to 55°C) rabbit anti-α2M serum (RαSgel) diluted 1:10 in agarose solution. The diameters of the precipitation circle lines obtained were measured and the level of α2M in each sample was calculated based on a standard curve (R^2, mm), obtained from samples with known α2M concentrations (N and Tp rat sera)[7].

α2M purification: α2M was purified from rat sera by column chromatography and by extraction from agar gel. Sera samples from rats were first fractionated on a Sephadex G-100 column (40–120 μm beads, Pharmacia fine chemicals), using borate saline buffer (BSB) as elution buffer. Before fractionation, the sera were dialysed overnight against 300–400 vol of BSB, at 4°C. The fraction containing α2M eluted from Sephadex G-100 column was further fractionated on a DEAE52-cellulose (DE32) (Whatman, 40–120 μm beads) column, using Tris-HCl buffer containing: 0.03M–0.2M NaCl as eluant gradient. Before fractionation, sera samples were dialysed overnight at 4°C against 250–300 vol of Tris-HCl buffer. In both cases, fractions of 3.5 ml were collected by a fraction collector (LKB, Bromma, Ultrarac 7000), and the optical density (OD) at 280 nm wavelength of each fraction was determined by a spectrophotometer (Genesys 5, Spectonic). The eluted fractions from each peak were concentrated to a 0.5–1 ml volume and examined for the presence of α2M by ID, IEP and IMB. For the α2M extraction from gel, sera samples (0.25–0.5 ml) were first separated by EP in 1.1% noble agar gel for 1.5–2 h, at a constant 80 V voltage. After separation, one gel was fixed for an hour using a fixative solution (40 vol %Ethanol; 2 vol %acetic acid) and stained with amido black in order to visualise the α2M band. Another gel was cut into 0.5 cm width slices and the slice containing α2M was removed and transferred to a tube containing 2 ml saline. After overnight incubation at 4°C, the tubes were centrifused at 14000 rpm for 10 min at 4°C and the soluble fraction was collected, concentrated to 1 ml volume and examined for the presence of α2M as previously described.

Effect of α2M on T. lewisi development: The effect of α2M on *T. lewisi* development *in vivo* in experimentally infected rats was determined using both purified (gel extraction, antigen-antibody complexes) and non-purified (CyI and Tp rat sera) α2M. SI rats were inoculated ip three times, on the day of infection and on the second and the fourth day post-infection with 0.5 ml of either CyI or Tp rat sera or purified α2M originated from 0.5 ml Tp rat serum.

Results

T. lewisi development in rats: The development of *T. lewisi* in SI, SpI and CyI rats is summarised in Fig. 1. In the SI rats the parasites were first observed on the second day post-infection. The parasitaemia peaked (8.4%) on the fifth day and then gradually decreased, reaching 2–3% ± SD on the eighth day. During the first week of infection small, big and dividing and non-dividing forms were observed that were transformed into elongated non-dividing parasites during the late stage of the disease. In the CyI and the SpI rats the parasitaemia was gradually increased, reaching maximal values of 40–50% ± SD on the eighth day of infection (Fig. 1). Until the fifth day, the parasitaemia in the SpI rats was lower than that in the CyI group, after which it was almost similar in both the groups. During all the experiments, trypanosomes of various size as well as diving and non-dividing forms were present (Fig. 1).

Rabbits immunisation: As expected, rabbits immunised against either CyI or Tp rat sera produced antibodies against α2M and against most of the rat sera components. Absorption of the immune rabbit sera to normal rat sera removed most of the contaminating

normal serum components (Fig. 2). Immunisation of rabbits against α2M/anti-α2M immune complexes showed the production of antibodies against α2M and against several contaminating serum components, probably due to non-specific absorption. Also, rabbits immunised against α2M extracted from agar gel produced antibodies against α2M and against other contaminating components of the rat serum. The most purified anti-α2M antibodies were obtained in rabbits immunised against α2M extracted from SDS gel, showing antibodies against α2M and also against an additional component, probably α1M.

α2M purification and characterisation: α2M that was demonstrated in the first peak eluted from the Sephadex G-100 column was contaminated with five additional proteins. Further, fractionation of this fraction on a DE32 column removed four of the contaminating proteins. α2M was eluted from the DE32 column at 0.09M NaCl. Extraction of α2M from CyI rat serum by agar gel electrophoresis, followed by a fractionation on the Sephadex G-100 column eliminated most of the rat serum proteins, but two that contaminated the α2M preparation.

α2M was characterised in rats sera by affinity chromatography, ID, IEP, EP and IMB. The results obtained showed total homology between α2M from CyI and Tp-treated rats (Fig. 2). Also the location of α2M demonstrated by EP, IEP and IMB confirmed the α2M characteristic of this protein. Native α2M is a slightly negatively charged molecule of a 720 kDa (native PAGE+IMB), that is separated into four identical subunits of 180 kDa by SDS-PAGE (Fig. 3). α2M was neither detected in normal and Cy-treated rats sera nor in homogenate of parasites. The level of α2M produced in rats was in the following order: Tp-treated rat (2nd day post-treatment) >CyI (eighth day post-treatment)> SpI (eighth day post-treatment).

Effect of α2M on T. lewisi development: The immunosuppressive effect of α2M is well demonstrated in

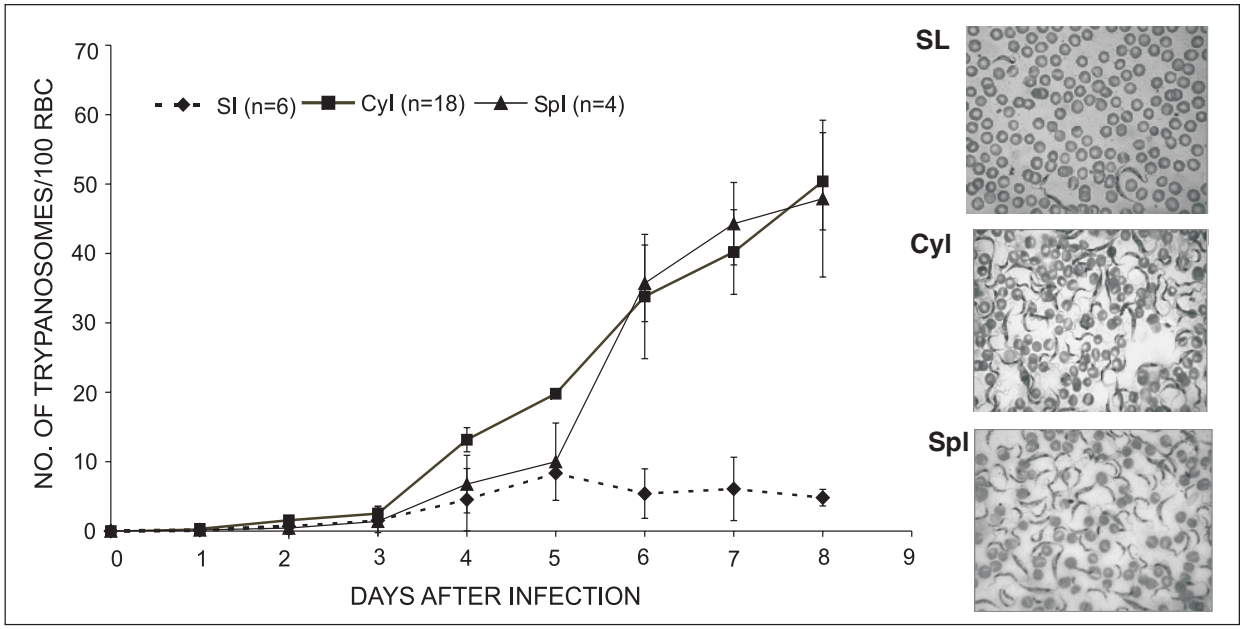

Fig.1: *Trypanosoma lewisi* development in saline treated (SI), cyclophosphamide treated (CyI) and spenectomised (SpI) rats. The rats, weighing 150–200 g, were ip injected with 1×10^6 parasites and the parasitaemia was determined as the ratio of the *T. lewisi* parasites to 1×10^4 erythrocytes. Inside the graph from top to bottom-Giemsa stained blood slides taken from the SI, CyI and SpI rats on the eighth day post-treatment (X 1000 magnification)

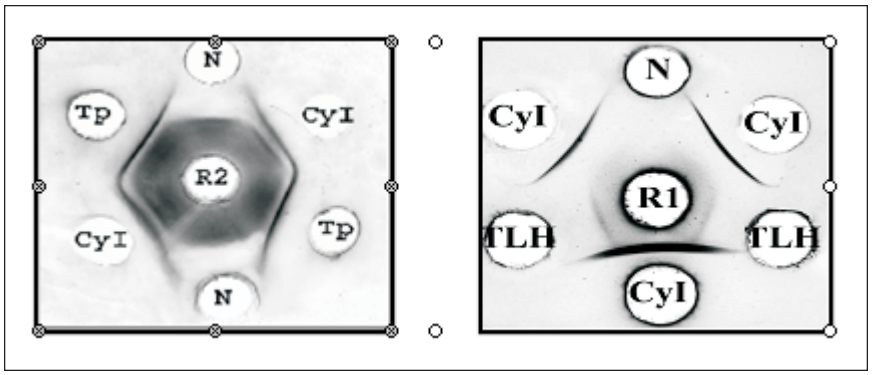

Fig. 2: Anti-α2M antibody activity produced in rabbits immunised against sera from cyclophosphamide-treated infected rat (R1) and turpentine-treated normal rat (R2). The rabbit sera (R1, R2) were adsorbed to normal rat serum before testing (N–Normal rat serum; CyI–Cyclophosphamide treated infected rat serum; SpI– Spelenctomised infected rat serum; TLH–*T.lewisi* homogenate; and Tp–Turpentine treated uninfected rat serum)

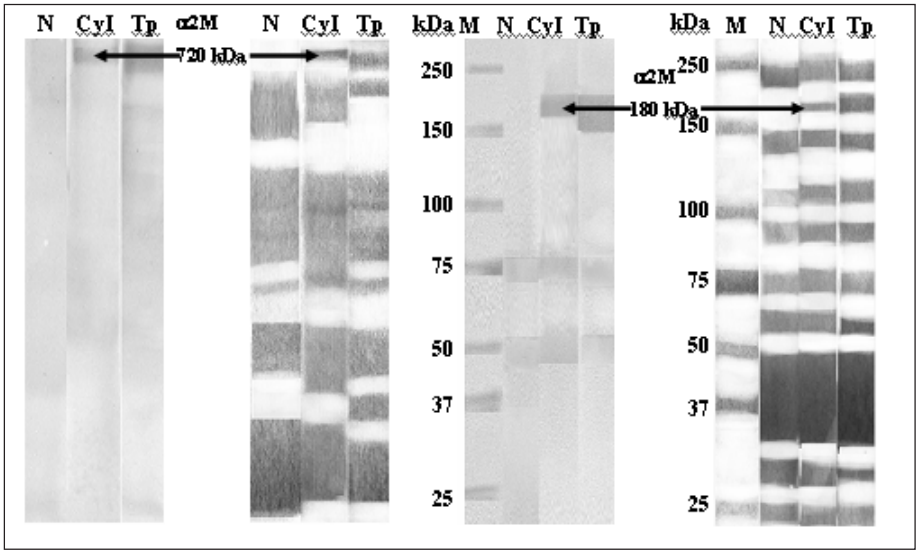

Fig. 3: Immunoblot analysis of rat sera in 7.5% SDS-PAGE (middle) and 5% native PAGE (left), using serum from rabbit immunised against cyclophosphamide-treated infected rat serum, as an antibody. The rabbit serum was absorbed with normal rat serum before applying to the test (N–Normal rat serum; CyI–Cyclophosphamide treated infected rat serum; Tp–Turpentine treated rat serum; and M–Molecular weight marker)

rats infected with *T. lewisi*. Inoculation of infected rats with sera from either CyI or Tp-treated (α2M inducer) rat increased the peak parasitaemia (sixth day post-treatment) by 4 and 7%, compared to that of SI rats, respectively (Fig. 4). The injection of purified α2M of agar gel origin also led to the increased peak parasitaemia compared to the SI control (12% ± SD compared to 7% ± SD). The most significant effect of α2M was observed in rats inoculated with anti-α2M/α2M immune complexes showing as high as two times higher parasitaemia compared to that of the control group (15% ± SD compared to 7% ± SD) (Fig. 4). At the end of the experiment (eighth day post-treatment) the parasitaemia in all treated groups reduced

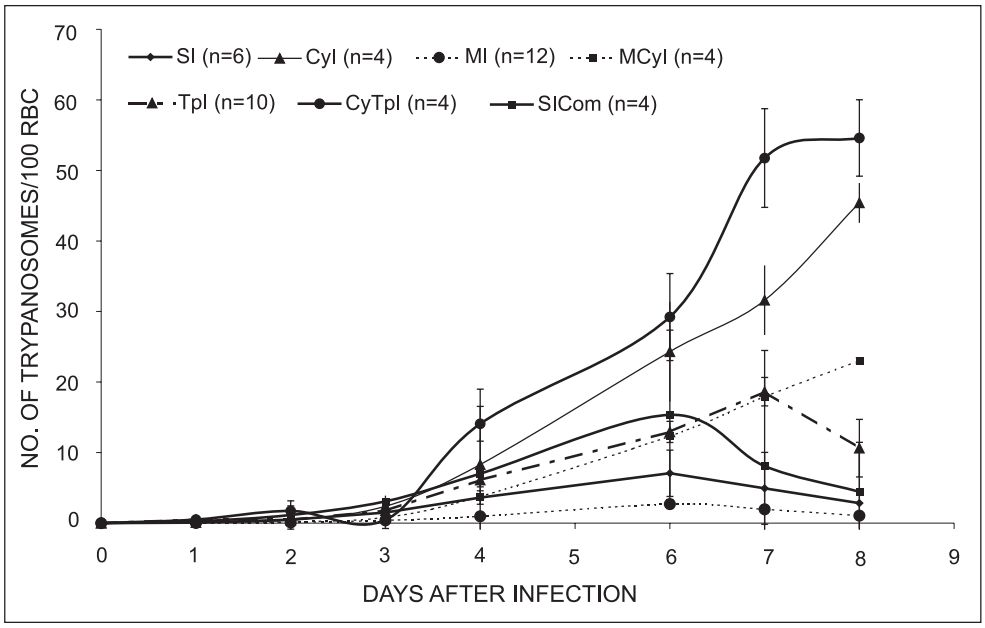

Fig. 4: Effect of α2M on *Trypanosoma lewisi* development in rats. Rats, weighing 150–170 g, were first infected ip with 1×10^6 trypanosomes and then were either left untreated (SI) or were treated with cyclophosphamide (CyI), anti-α2M/α2M immune complexes (SICom), turpentine (TpI), cyclophosphamide + turpentine (CyTpI), mercaptopurine (MI), or mercaptopurine + cyclophosphamide (MCyI)

to a level similar to that of the control rats. In addition, the injection of Tp (inducer of α2M production) into infected rats led to an increased parasitaemia that reached its peak on the seventh day post-treatment (19% ± SD), while inhibition of α2M production by mercaptopurine reduced the parasitaemia to 2–3% ± SD in the SI rats and to 23% ± SD in the CyI rats (Fig. 4). A combined treatment of rats with Tp and Cy led to the highest parasitaemia values on the eighth day post-treatment that were even higher than those of the CyI rats (55% ± SD compared to 45% ± SD).

α2M production in rats: Determining the kinetics of α2M production by RID showed no change in α2M level (11–32 μg/ml) in the sera of both normal uninfected and Cy-treated rats during the experiment (Fig. 5). In the normal infected (SI), splenectomised infected (SpI) and Tp-treated infected (TpI) rats a maximal level of α2M (972 μg/ml, 1869 μg/ml, 3023 μg/ml, respectively) was demonstrated on the second day post-infection. These levels were then reduced gradually reaching a level of 300–600 μg/ml, on the eighth day of infection (Fig. 5). A different pattern of α2M production was observed in the CyI-treated rats. In this group the α2M level gradually increased during experiment reaching a maximal level of 890 μg/ml on the eighth day post-infection (Fig. 5). Combined treatment of infected rats with Cy and Tp induced an additive α2M activity, reaching a value of 1781 μg/ml on the eighth day of infection. As expected, a mercaptopurine treatment of infected rats either untreated or treated with Cy significantly inhibited α2M synthesis, showing a maximal α2M level of 182 μg/ml (Cy untreated) and 231 μg/ml (Cy treated), on the second and the fourth day post-infection, respectively (Fig. 5).

Discussion

The present study confirms again our previous study[4] indicating the immunosuppressive action of both splenectomy and Cy treatment on *T. lewisi* development in rats. Both treatments were associated with the production of a new component, that was shown to

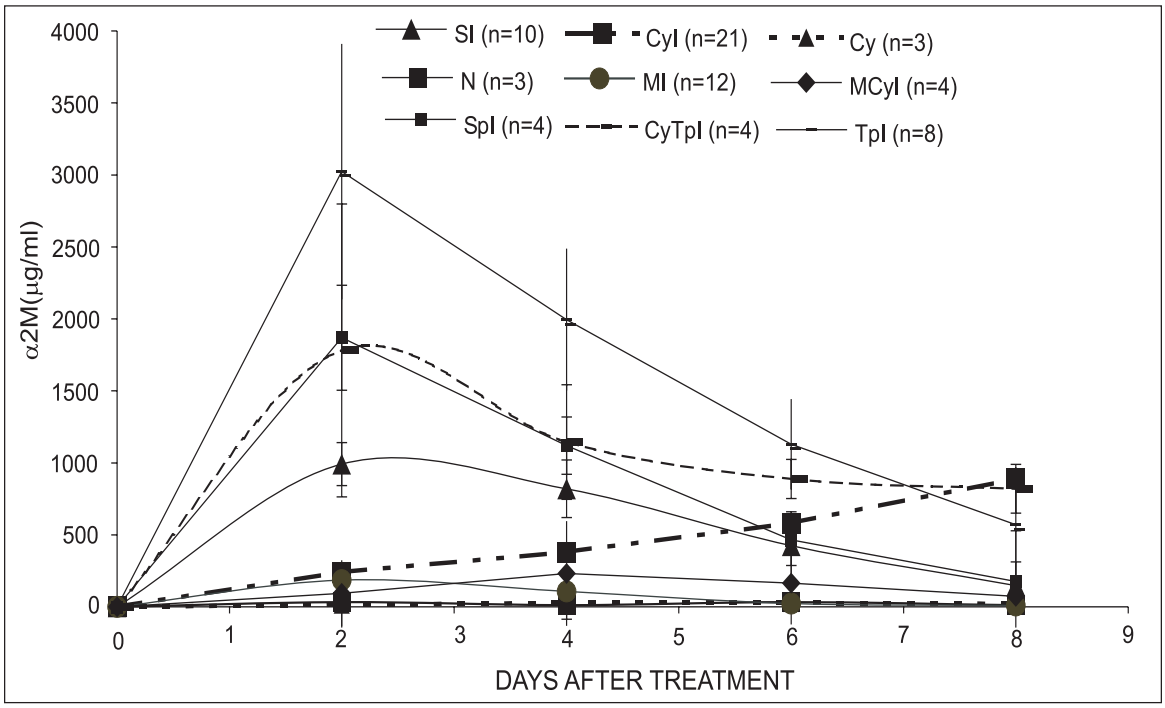

Fig. 5: Kinetics of α2M production in rats (150–200 g) infected with 1×10^6 *Trypanosoma lewisi*. The rats were either left untreated (SI), or were splenectomised (SpI), treated with cyclophosphamide (CyI), turpentine (TpI), cyclophosphamide + turpentine (CyTpI), mercaptopurine (MI), or mercaptopurine+ cyclophosphamide (MCyI)

be, by various procedures, an α2M globulin. Fractionation of α2M, its travelling in gels and its cross reactivity with α2M from Tp-treated rats confirmed the α2M nature of this protein. Also, the most purified α2M preparation was obtained by its extraction from SDS gel, as was shown by antibodies produced in rabbits. However, this fraction was still contaminated with an additional protein, probably alpha 1 macroglobulin (α1M), which has similar physicochemical properties to α2M[8,9]. α2M was further shown to be of host origin since it was not detected in neither soluble nor particulate fractions of *T. lewisi* homogenate. α2M was neither demonstrated in normal nor Cy treated rat sera, indicating that α2M is either produced only in response to inflammation (Tp injection) or to infection with various pathogens.

The immunosuppressive effect mediated by α2M, leading to a malignant *T. lewisi* infection was further confirmed in this study. α2M accelerated the infection with *T. lewisi* in both splenectomised and Cy-treated rats, while inhibition of its production by mercaptopurine led to the development of a moderate disease. It is of interest to mention that while in the normal-infected (SI), in Tp-treated infected (TpI) and in the splenectomised-infected (SpI) rats, the maximal level of α2M was observed on the second day post-infection, in the Cy-treated infected rats (CyI) α2M was gradually accumulated during infection. These results clearly indicate the correlation between the α2M levels and the inflammatory response mediated by the parasites. Furthermore, the removal of α2M from circulation was severely impaired in the CyI rats. Cy is an alkylating agent that destroys almost every dividing cell, including those of the reticuloendothelial system[10]. The depletion of these cells from the circulation caused by Cy treatment together with the inflammation induced by the parasites, led to the accumulation of α2M in the CyI rats[11,12]. A combined treatment of infected rats with

Cy+Tp caused a higher accumulation of α2M due to the Tp effect on α2M production and the depletion of macrophages caused by Cy.

In humans, α2M is a constitutive component of the serum (8–10% protein content) while in rats, it is an acute phase protein induced by inflammation[13], and its synthesis is induced by IL-6, hepatocyte growth factor (HGF), IL-1, TGF-β and steroid dexamethasone[14]. α2M is composed of four identical subunits (180 kDa) and acts as a proteinase inhibitor[14]. The binding of proteinases to α2M leads to a conformational change and activation of the α2M (activated α2M- Fα2M)[15]. Fα2M also binds peptides and cytokines[13]. The Fα2M-substrate complexes are cleared from circulation through binding to the α2M receptor (α2MR) expressed by hepatocytes, fibroblasts and macrophages[15,16]. α2M is a part of innate immune system and acts against different pathogens including parasites—*Leishmania* and *T. cruzi*, by virulence restriction via inhibition of their proteinases[17,18]. It has many physiological functions besides being inhibitor and scavenger of proteinases. α2M binds numerous cytokines and growth factors and also activates cellular signaling pathways[13]. It can act as an immunostimulator or an immunosuppressor depending on its surroundings. Most of the cytokines that bind to the Fα2M (covalent binding) are usually removed from the circulation while the cytokines that bind native α2M (non-covalent binding), like TGF-β are very stable and can disengage freely from the complex acting on their cellular targets[19].

The results described in this study showed that the α2M accumulated in the CyI rats further increased the immunosuppressive effect mediated by Cy. These results are in contrast to those observed with American trypanosomiasis and leishmaniasis, in which a decrease in parasites development was found to be caused by α2M, due to inhibition of parasite proteases mediated by α2M[17,18]. It is, therefore, possible that the immunosuppressive action of α2M in the CyI rats may be the result of modulation of the αM-oxidative status during inflammation[20]. The low affinity binding of oxidative α2M to the immunosuppressive cytokines (IL-4, TGF-β, IL-10) and its high affinity binding to the pro-inflammatory ones (IL-6, TNF-α, IL-2) may allow the activity of the first group only, leading to severe immunosuppression and the development of a malignant disease[21].

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