Pathobiochemical Alterations in Experimental Chronic and Acute Trypanosomal Infection in Mice\textsuperscript{1})

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Summary: During an experimental chronic infection of inbred mice with *Trypanosoma congolense* several physiological parameters become altered. Splenomegaly followed later by hepatomegaly are predominant. Lactate dehydrogenase and aminotransferase activities of the plasma are elevated, the number of erythrocytes and thrombocytes decreases, whereas monocyte cells are detected in higher concentrations. \(\gamma\)-Globulins and transferrin become elevated. Some of the pathobiochemical alterations depend directly on the parasitaemia and are reversed to normal values after chemotherapy with diminazene aceturate (Berenil\textsuperscript{®}). The curative effect of this drug depends largely on when it is administered.

In acute *T. congolense* infections, leading to the death of the animals in 3 – 4 days, pathobiochemical alterations are found only shortly before the exitus.

Introduction

African trypanosomes are the causative agents of severe epidemic infections in man and animals. The infections are characterized by alternating parasitae-mic waves, each wave representing a population of parasites carrying a new antigenic surface coat. By this “antigenic variation” the trypanosomes escape the host’s specific immune response. The clinical manifestations under chronic infection are diverse and depend both on the host species as well as on environmental factors like nutritional and hygienic state, physical stress etc. Correspondingly, various and often contradictory findings are reported on pathological alterations during infections. The mechanisms eventually leading to the patient’s death are largely unknown; direct toxicity of trypanosomal products as well as immunopathological processes induced by antigen/antibody complexes have been suggested (1, 2).

In order to minimize heterogeneities due to outbred animals, pathobiochemical alterations were investigated in a rather reproducible experimental model, the infection of an inbred strain mice with a defined strain of *Trypanosoma congolense*. This paper presents and compares results of the alteration of blood analytes in chronic and acute *T. congolense* infections. Moreover, data are given on the relapse of the parasitic infection after chemotherapy with diminazene aceturate (Berenil\textsuperscript{®}).

Materials and Methods

Female mice of the inbred strain NMRI, 20 – 25 g body weight, were used. Acute infections were induced by *Trypanosoma congolense* clone BeTat 1 (Institut für Parasitologie, Freie Universität Berlin). Chronic infections were induced by *T. congolense* strain 4905, isolated 1981 from cattle in Togo; both strains were kept as stablate in liquid nitrogen.

*Acute infection:* 40 mice were infected intraperitoneally with 0.1 ml \((4 \times 10^6 \text{ trypanosomes})\) per animal of the stablate. After 24, 48, 72, 84 and 96 hours, 5 mice were decapitated, and blood and plasma were collected separately from each mouse.

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**Chronic infection:** Lethally irradiated mice (900 rad) were injected intraperitoneally with stabilite *T. congolense* 4905 (0.3 ml; 6.5 x 10^6 trypanosomes). Three to four days later the blood was collected in cold PS/G-citrate (44 mmol/l NaCl, 55 mmol/l glucose, 3 mmol/l NaH₂PO₄, 10 mmol/l sodium citrate, pH 8.0). Using this living trypanosome containing blood, 110 mice were infected with 1 x 10⁶ trypanosomes; 110 mice remained unaffected (controls). At intervals of 3—4 days, 4—5 mice were randomly selected from the groups. Body weight was determined and about 1.0 ml blood was taken from the vena cava posterior (pentobarbital 30 mg/kg as anaesthetic) to determine parasitaemia, plasma enzymes, plasma proteins and haematological parameters. Liver, spleen and kidneys were removed, weighed and examined histologically.

Samples were taken between 8 and 9 a.m., after the animals had been fasted for 12 hours. Parasitaemia was assayed in a Neubauer haemocytometer.

Activities of lactate dehydrogenase, aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase were assayed according to methods described (3), urea and glucose by test combinations (Boehringer, Mannheim, FRG), total bilirubin, total plasma protein by standard procedures (4, 5).

Plasma proteins were separated on agarose-coated slides (26 x 100 mm) at 10°C in an electrophoretic chamber (6). The amido black stained pherograms were evaluated by an analog computer program (7). Transferin was identified by a reference standard (8).

A modified rocket immunoelectrophoresis (9) was used for the determination of immunoglobulin M, with goat-anti-mouse IgM (Meloy Corporation) included in the agarose. After electrophoresis the precipitation bands were stained with l g/l Coomassie Blue R 250 (Sigma) in 395.5 g/l methanol, 104.9 g/l acetic acid.

The isoenzyme pattern of plasma enzymes was studied by electrophoresis (3). Staining for lactate dehydrogenase followed the method described in 1. c. (10). Staining for aspartate aminotransferase was performed with a modified sandwich method (11).

First, substrate gels (1.4 mm thick) were prepared on microscope slides by mixing equal volumes of 30 g/l agar (OXOID, No. 3) and Substrate buffer (100 mmol/l Tris, 200 mmol/l aspartate, 24 mmol/l 2-oxoglutarate, 10 mmol/l ethylenediamine tetraacetic acid disodium salt, 30 g/l polyvinylpyrrolidone-40 and 0.2 mmol/l pyridoxal phosphate, pH 7.2). After electrophoresis the Substrate gel was layered over the electrophoresis gel, the sandwich placed into a wet chamber and incubated for 10 minutes at 37°C. The substrate gel was removed and the electrophoresis gel covered with a filter paper soaked with 0.5 ml of a 40 g/l Fast-Violet B-solution (Sigma Corp., München, FRG). After 10 minutes at 37°C the paper was removed, the stained gel fixed for 30 minutes in 52.5 g/l acetic acid and washed in distilled water for 90 minutes. The pherograms were evaluated by a Zeiss-Chromatogram spectrophotometer. In order to associate certain isoenzymes with their organs of origin, some tissues (heart, liver, skeletal muscle) were extracted as described in 1. c. (12). The enzymes in these extracts served as references for the plasma enzymes.

Enzymes:

- Acid Phosphatase, Orthophosphoric monoester phosphohydrolase EC 3.1.3.2
- Alkaline Phosphatase, Orthophosphoric monoester phosphohydrolase EC 3.1.3.1
- Aspartate aminotransferase, L-aspartate: 2-oxoglutarate-amino transferase EC 2.6.1.1
- Alanine aminotransferase, L-alanine: 2-oxoglutarate-amino transferase EC 2.6.1.2
- Lactate dehydrogenase, L-lactate: NAD oxidoreductase EC 1.1.1.27
- Determinations of haemoglobin, haematocrit, thrombocyte count, erythrocytes, leukocytes and the differential white count were performed by standard methods (13).

**Chemotherapy:** *T. congolense* 4905-infected mice were treated with 7 mg/kg dianimazene aceturate (Berenil®) 30 days after infection, followed by a second dose of 35 mg/kg 2 days later. Another group was given 35 mg/kg 5 days after infection. Controls were non-infected animals, with or without dianimazene aceturate (Berenil®) injection. Berenil® was purchased from Hoechst AG (Frankfurt, FRG).

Standard deviations were calculated and Student's t-test used to judge significance of differences between means: *: P < 0.05, **: P < 0.01, ***: P < 0.001.

**Results**

**Chronic infection**

Figure 1 shows the course of the parasitaemia. On the third day after infection the concentration of trypanosomes was 7 x 10^⁶/ml. The concentration decreased on day seven to 3.3 x 10^⁶/ml and, a second maximum with 3.5 x 10^⁶/ml occurred on days 14—17. The waves of parasitaemia continued, showing the typical pattern of antigenic variation. Individual mice (not separately shown) were not exactly synchronous, but exhibited small deviations in the appearance of the peaks. On reaching the second peak of parasitaemia, 26 of the 110 mice died with very high parasitaemia.

**Haematology**

Three days after infection and onwards, the haemoglobin content and haematocrit (fig. 2) were 10—30% lower in the infected than in the control animals, indicating a persistent mild to moderate anaemia. The erythrocytes in the infected animals exhibited increased osmotic fragility as compared to controls. At day 17 after infection (second peak of parasitaemia) 50% of the erythrocytes became lysed in 6 g/l NaCl (room temperature, 3 hours); in the control mice, haemolysis under these conditions was about 13%. Although not shown in figure 1 some mice were infected with parasites for up to 4 months. The blood count values after 120 days are given in table 1. Erythrocyte numbers were generally lowered by approximately 18%. A marked decrease in thrombocytes was observed, and the whole blood clotting time was prolonged. The dramatic rise in the leukocyte number was primarily caused by the increase of monocytes (tab. 2), and to a lesser extent by increased neutrophilic and eosinophilic cells. Lymphocytes were lowered to about 75% relative to the controls. Considerable individual differences were obtained among single infected mice. At this late stage of infection...
the parasitaemia was very heterogenous among the mice. Only in 2 of the 5 mice parasites were detectable in the blood. These animals also possessed the highest numbers of monocytes. In contrast, maximal numbers of eosinophilic cells were found in animals with no detectable parasites in the blood.

Plasma, biochemical determinations

Total plasma protein content increased significantly from approx. 50 g/l in the controls to 65 g/l in the infected animals (fig. 3). Plasma electrophoresis revealed clear qualitative and quantitative differences (fig. 4). Infected animals showed an increase in the β- and γ-globulin fraction, but a decrease of albumin, as reported by other groups (14), was not seen. By comparison of the elevated β-fraction with transferrin standard from mouse in SDS-PAGE and agarose-electrophoresis, it was shown that the increment in the β-globulin fraction in infected mice can be attributed to transferrin.

The elevated γ-globulin is mainly caused by an increase of the IgM levels as indicated in figure 5. Because of lack of mouse IgM standard only the increase of IgM in the infected animals relative to that of the control mice can be shown. As in the parasitaemia plasma, IgM shows increases and decreases. Roughly, peaks of parasitaemia correspond to troughs of IgM and vice versa.

The plasma levels of urea, glucose and total bilirubin during the chronic infection did not vary significantly; individual variations of glucose and bilirubin were too high to demonstrate significant infection-dependent differences. As expected from the well known glucose-dependency of trypanosomes, relatively low glucose values were found in mice with high parasitaemia, but even here individual variations were considerable.

Total lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase activities (figs. 6—8) increased in the plasma of the infected animals.
after day 3: lactate dehydrogenase 7–10-fold, aspartate aminotransferase and alanine aminotransferase 2–3-fold, compared with the controls. Alkaline phosphatase activity did not change (fig. 8). The increments of lactate dehydrogenase and aspartate aminotransferase are caused by individual isoenzymes. Quantitative evaluation of lactate dehydrogenase isoenzymes established that lactate dehydrogenase...
Tab. 3. Absolute (units) and relative activities (total area = 100 percent) of individual lactate dehydrogenase (LDH) isoenzymes in plasma of control and infected (24, 28, days after infection) mice.

<table>
<thead>
<tr>
<th>Absolute activities (units)</th>
<th>Relative activities (percent)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>LDH₁</td>
<td>65.4 ± 25.2</td>
</tr>
<tr>
<td>LDH₂</td>
<td>52.7 ± 21.0</td>
</tr>
<tr>
<td>LDH₃</td>
<td>115.2 ± 19.2</td>
</tr>
<tr>
<td>LDH₄</td>
<td>34.2 ± 1.8</td>
</tr>
<tr>
<td>LDH₅</td>
<td>68.4 ± 0.6</td>
</tr>
</tbody>
</table>

Nase isoenzyme 5, the dominant isoenzyme of skeletal muscle and liver in mice, was elevated 28-fold over the controls, whereas the isoenzymes 2—4 showed a 6—8-fold increase, and isoenzyme 1 increased only twofold (tab. 3).

Unlike lactate dehydrogenase, which trypanosomes do not contain, aspartate aminotransferase isoenzymes in the blood could also be derived from degraded trypanosomes (15). In a sonicated T. congoense sample (3 × 10⁹ cells per ml) total aspartate aminotransferase activity was 1.3 × 10⁶ U/l. Aspartate aminotransferase electropherograms distinguished between trypanosomal and host aspartate aminotransferase. Aspartate aminotransferase from trypanosomes migrated solely as a cathodic peak (fig. 9, bottom), slower than the enzymes from heart and liver mitochondria. The electropherogram of plasma from infected mice contains, in its cathodic area (II), a mixture of trypanosomal and host aspartate aminotransferase isoenzymes (fig. 9, top). The increase in the anodic fraction, however, clearly shows augmented host-specific aspartate aminotransferase isoenzyme activity, quantitatively equivalent to the increment in the total aspartate aminotransferase activities.

Inspection of organs

The body weight of the infected animals increased during the period of the experiment by 48%, in the controls only by 12%. This increment could be attributed exclusively to spleno- and hepatomegaly (fig. 10). Phagocytic cells were dramatically increased in both organs, as shown by histochemical assay of the lysosomal enzyme acid phosphatase (not shown) (16). In the liver, clusters of acid phosphatase positive cells with the size and the shape of Kupffer Stern cells were found. The red pulp of the spleen also contained large numbers of acid phosphatase positive macrophages, which were enlarged and filled with lysosomes. The total volume of the red pulp increased remarkably and steadily. No alterations were found in the kidneys.
Influence of diminazene aceturate (Berenil®)

Animals treated by day 5 after infection remained free of parasites for more than 5 months and were therefore considered to be cured. The mice that received two injections of Berenil® 30 days after infection also contained no detectable parasites in the periphery. Thirty four days after the first Berenil® administration, however, trypanosomes recurred in 2 of the 4 mice.

Haemoglobin and haematocrit depressions in the infected animals became normalized 3 days after the second Berenil® treatment (fig. 11), but after recurrence of the trypanosomes the anaemia also recurred.

Electrophoresis of the plasma of a Berenil®-treated control together with a Berenil®-treated infected animal showed only slight differences in the relative distribution of plasma proteins, but for a higher level of immunoglobulin in the infected mouse (not shown).

After the second application of Berenil®, the activities of lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase in the plasma decreased significantly. In the Berenil®-treated controls, however, a steep rise in the plasma enzymes took place, probably due to a toxic effect of the drug (fig. 12). Fifteen days later the enzyme levels in the infected mice and in the treated controls were practically indistinguishable. The levels of each of the lactate dehydrogenase isoenzymes in the treated infected animals dropped to the values found in the treated non-infected controls. No clear evidence was found for an immediate increase of enzyme activities after the recurrence of trypanosomes, but the effect probably was masked by the stimulation induced by Berenil® alone. A clear coincidence, however, was found between spleno- and hepatomegaly and the success of the Berenil® therapy (fig. 13). Immediately after the treatment, the weight of the spleen decreased and 15 days later reached approximately 2.8 times the values of the control. Similarly the weight of the liver also dropped. In those animals with a parasitaemic relapse, both spleen and liver rapidly returned to the weights typical of the infected animals. Histochromically, the acid phosphatase active cells in liver and spleen became reduced after Berenil® treatment. The Berenil® therapy itself, however, produced some alterations like the augmentation of acid phosphatase active granula in the liver cells (not shown).
Tab. 4. Haematological, biochemical parameters, and tissue-weights in mice on day 3 of acute infection.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/l)</td>
<td>163.2 ± 9.4</td>
<td>155.3 ± 10.1</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>0.594 ± 0.032</td>
<td>0.494 ± 0.017**</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/l)</td>
<td>246.4 ± 21.6</td>
<td>2106.8 ± 296.3***</td>
</tr>
<tr>
<td>Aspartate aminotransferase (U/l)</td>
<td>77.4 ± 9.6</td>
<td>268.1 ± 58.8***</td>
</tr>
<tr>
<td>Alanine aminotransferase (U/l)</td>
<td>60.6 ± 3.6</td>
<td>110.4 ± 12.6***</td>
</tr>
<tr>
<td>Alkaline phosphatase (U/l)</td>
<td>35.4 ± 4.8</td>
<td>62.4 ± 4.2**</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>8.05 ± 2.05</td>
<td>6.16 ± 1.47</td>
</tr>
<tr>
<td>Glucose (mmol/l)</td>
<td>6.31 ± 1.18</td>
<td>6.06 ± 1.92</td>
</tr>
<tr>
<td>Protein (g/l)</td>
<td>46.1 ± 2.0</td>
<td>50.8 ± 2.1</td>
</tr>
<tr>
<td>Spleen/Body weight × 100</td>
<td>0.57 ± 0.09</td>
<td>0.73 ± 0.10***</td>
</tr>
<tr>
<td>Liver/Body weight × 100</td>
<td>5.04 ± 0.42</td>
<td>5.28 ± 0.42</td>
</tr>
<tr>
<td>Kidneys/Body weight × 100</td>
<td>1.25 ± 0.09</td>
<td>1.21 ± 0.10</td>
</tr>
</tbody>
</table>

**: P < 0.01
***: P < 0.001

Acute infection

Using the "acute strain" BeTatl, the same assays were carried out, as described with the chronic infection. The basic difference between the chronic and the acute infection was the level of parasitaemia. Eighty-four hours after the infection more than 1 × 10⁸ trypanosomes per ml blood were detected, and after 96 hours all animals were dead. Table 4 summarizes the alterations found with the different parameters on day 3 of the infection. As in the chronic infection, aspartate aminotransferase and lactate dehydrogenase activities increased with high parasitaemia, whereas only slight differences could be found with alanine aminotransferase and alkaline phosphatase. Determination of the lactate dehydrogenase isoenzymes demonstrated some increase in fractions 5 and 4 of the lactate dehydrogenase (not shown), but these differences were not as clear as in the chronic infection, although the clinical condition of acutely infected animals was worse. The haematocrit values were lowered, but no difference could be found in the haemoglobin content. Glucose, urea and total plasma protein did not show any alteration. Splenomegaly was detectable, but no enlargement of liver or kidneys could be found.

Biochemical and histological variations in the infected animals were remarkably mild.

Discussion

In *T. congolense* infections probably the long lasting chronic situation and a complex process of gradual impairment of organs and tissues of the host lead to the eventual generalized collapse.

Nearly 23% of the mice died after reaching the second peak of parasitaemia; thereafter most of the animals survived for several months. A sufficiently balanced situation in the host-parasite relationship thereafter allows the establishment of a chronic infection. Spleno- and hepatomegaly were predominant in the chronic infection, whereas in the acute disease only a mild splenomegaly and no hepatomegaly could be found. The increased phagocytic activities, as shown also by others (17), seem to promote the degradation of altered erythrocytes with increased fragility, resulting in anaemia, a typical primary symptom in trypanosomal infections (18). In mice with acute infection the anaemia was less pronounced, and a decrease of the haemoglobin could not be detected.

Various reasons have been suggested for the development of the anaemia, such as trypanosomal haemolysins (19), increased phagocytosis of erythrocytes (20) impaired by passively adsorbed trypanosomal antigens or immune complexes (2), and opsonisation by cross-reacting antibodies (21). In our experiments no correlation can be recognized between the anaemia and the prognosis of the disease.

The variations in the differential blood count, as determined late in the chronic infection show predominantly an increase of monocytes and a decrease of lymphocytes. These findings are in accordance with the observed increased phagocytic activities and the immunosuppression typical for chronic trypanosomiasis (22). We could not, however, find a neutropenia as described in ruminants (23).

The alterations in the total plasma proteins differ from those of earlier reports. Whereas in the chronically infected mice total protein was significantly increased during the infection, in cattle either no change or even decreasing protein concentrations are reported (24, 25). The observed quantitative differences are caused by higher levels of the γ-globulins, mainly IgM, and transferrin. The tendency of IgM concentrations to alternate with the parasitaemic waves

probably represents the sequential immune response against the individual antigen variants. A loss of albumin, as found by other authors (25), was not detected in our experiments.

In chronic and in the late state of acute infections, high activities of lactate dehydrogenase, mainly isoenzyme 5, and also increased activities of aspartate aminotransferase and alanine aminotransferase were detected. Lactate dehydrogenase 5 in mice can originate from skeletal muscle as well as from liver. Our attempt to assay creatine kinase isoenzymes as a specific marker for distinguishing skeletal from heart muscle defects, failed, because these enzyme activities increased strongly due to the bleeding shock (26).

By electrophoretic separation of host aspartate aminotransferase from trypanosomal aspartate aminotransferase it was established that host aspartate aminotransferase also became elevated during the infection. From the enzyme assay we cannot definitely deduce that the enhanced enzyme activities originate exclusively from the liver, although the histological findings, also from other groups (27), support this interpretation. Further investigations are necessary to differentiate the lesions in the mouse system.

As expected, no single predominant mechanism for the pathogenesis is reflected by the histological or biochemical parameters assayed. The observed effects are similar to those found in other species. Neither the lesions in liver and/or muscle nor the anaemic Symptoms of trypanosomes after Berenil® treatment and the number of known resistant trypanosome strains is low (28). The effects observed during chronic infection could be reversed partially by therapy with Berenil®. The success of the chemotherapy was hampered by the recurrence of trypanosomes in the blood after terminating the treatment. Complete eradication of the trypanosomes is only possible, if the treatment is performed early in the infection (29).

Trypanosoma brucei is able to settle transiently in the brain and to escape from the action of the drug (30). For T. congolense the retreat into the central nervous system was observed only in a mixed T. brucei/T. congolense infection (31), but not in T. congolense monoinfected animals. The authors suggest that there might be a "helper"-activity of one species in promoting cerebral colonization by a second species. Recently (Jenni, personal communication) evidence was presented for an intracellular retreat of trypanosomes. We could not demonstrate the presence of T. congolense, which might have escaped from drug action in brain tissue, after transferring brain-homogenate of infected and Berenil®-treated mice to uninfected recipients (details not given in this paper). Further investigations are necessary to show which tissues or organs could allow a cryptic survival of T. congolense during chemotherapy. Berenil® therapy rapidly reversed most of the pathological changes, including the hepato- and splenomegaly, changes of lactate dehydrogenase and aspartate aminotransferase activities in the plasma and haematological values. The reversions, however, were masked in part by the toxic effects of Berenil®, as described (32).

The immediate correlation between the recurrence of trypanosomes after Berenil® treatment and the concurrent increase of signs of disease suggests that trypanosome-derived toxic factors act directly on certain sensitive tissues.

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References


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