The Liver as a Major Site of Immunological Elimination of Murine Trypanosome Infection, Demonstrated with the Liver Perfusion Model

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The isolated liver perfusion model has been used to investigate immunological elimination of bacteria and yeasts but not for analysis of mechanisms of immunological destruction of extracellular parasitic protozoa. Extracellular trypanosomes are eliminated primarily through antibody (and complement?)-promoted hepatic (Kupffer cell) uptake and destruction. We studied the suitability of the isolated liver model system for analyzing the mechanism of immune elimination of mouse-specific *Trypanosoma musculi* and identified several factors which can complicate such analyses: (i) mechanical trapping of trypanosomes that are quite large (for example, reproducing forms or epimastigotes) or are nonviable and, therefore, nondeformable; (ii) variable species and concentrations of cytadhesive molecules; and (iii) the integrity and composition of the trypanosomal surface coat. There was a substantial difference between hepatic retention of infused *T. musculi* organisms coated with a specific antibody and those devoid of antibody when both were suspended in normal mouse serum. The difference appeared sufficient to allow accurate quantitative studies of immune destruction in the liver. Studies of whole mice indicated that quantitative investigations of immunological elimination of trypanosomes from the bloodstream are likely to be complicated by problems such as cytadherence of parasites to host endothelial cells and mechanical trapping. Uptake by the liver and spleen appeared more reliable. Thus, the isolated liver perfusion model should significantly benefit studies to elucidate the mechanisms of immune elimination of extracellular trypanosomes.

Studies on immunological elimination of extracellular trypanosomes (African and rodent trypanosomes in particular) have demonstrated that the liver is a major organ involved in clearing the parasites from the bloodstream (2, 7, 11, 14). The liver is also a major site for immunological elimination of microorganisms such as bacteria (10, 12, 15), yeasts (21), and probably plasmodia (5) and *Leishmania* spp. (6). Elimination of bacteria has been well investigated by the method of liver perfusion and shown to be promoted by antibodies and complement components (10, 12, 15) and to involve Kupffer cells (KC).

Presumably, antibodies, complement components, and KC are involved in immunological clearance of trypanosomes from the blood. However, there are factors that may complicate accurate assessment of uptake and retention by the liver that are not taken into account in studies with whole animals (generally, mice). For example, the large size of trypanosomes relative to the diameter of capillaries and the dimensions of hepatic sinusoids could result in mechanical trapping of parasites, especially in the presence of antibodies. Moreover, many parasites have the tendency to adhere to cells (e.g., platelets) and to endothelium (3, 17, 20, 22), and thus, retention by the liver may reflect sequestration rather than immunological elimination.

A reliable liver perfusion procedure for analysis of uptake and destruction of parasites by liver KC would greatly facilitate analysis of immunological elimination of trypanosomes. Therefore, we investigated some of the parameters that influence the retention of *Trypanosoma musculi* perfused through the isolated livers of mice. We conclude that this technique can be used to study the mechanisms of

immunological elimination of trypanosomes if attention is paid to certain complicating factors.

MATERIALS AND METHODS

Animals. Mice of the C3H/Anf Cum strain (Cumberland View Farms, Clinton, Tenn.) were used in all experiments when they were 4 to 6 months old. Rats of the Holtzman strain were purchased from Holtzman (Madison, Wis.) and were used for passage and maintenance of *Trypanosoma lewisi*. All animals were housed and maintained in the vivarium of the George Washington University School of Medicine. All experiments with animals were conducted in strict compliance with the guidelines for the care and use of laboratory animals promulgated by the National Institutes of Health.

Trypanosomes. The origin and maintenance of the line of *T. musculi* used in our laboratory have already been reported (1). The line of trypanosomes has been maintained by weekly passage in normal mice and replaced at irregular intervals from stock kept frozen in liquid nitrogen. Trypanosomes were isolated from the blood of infected mice by drawing the blood into citrate-saline anticoagulant and separating the trypanosomes by repeated differential centrifugation. The parasites were counted by use of hemacytometers under phase-contrast microscopy. In some cases, the parasites were collected from immunosuppressed mice which had been treated with cyclophosphamide (250 mg/kg, intraperitoneally) 8 to 24 h before *T. musculi* inoculation.

The origin and maintenance of the *T. lewisi* used in our laboratory have also already been described (8). These trypanosomes were maintained by weekly passage in rats. For some experiments, trypanosomes were treated with

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trypsin to remove the surface coat material by a published procedure (8, 9).

Media and reagents. Phosphate-buffered saline–glucose (PBSG; 9) was used for routine suspensions and washing of trypanosomes. Hanks salt solution with Ca^{2+} and Mg^{2+} was prepared and sterilized. The reagents mannan, hydralazine, and cyclophosphamide were purchased from Sigma Chemical Co., St. Louis, Mo. These substances were dissolved in Hanks salt solution or PBSG. ^{75}Se-labeled methionine was purchased (Amersham Corp., Arlington Heights, Ill.) and used to radiolabel *T. musculi* by a published procedure (2, 11).

Liver perfusion. Liver perfusion performed on mice was patterned after that of other investigators (10, 12). One cannula was placed in the portal vein, and a second cannula was introduced through the right atrium into the thoracic portion of the vena cava. The abdominal segment of the inferior vena cava was clamped, and the flow of fluid into the liver was started. Perfusion of the liver was achieved by use of a peristaltic pump adjusted to a constant flow rate (about 2 ml/min) that avoided causing any swelling of the liver. The effluent leaving the liver was collected in approximately 1-ml fractions until 15 to 20 ml of fluid had been pumped through the liver, and then larger fractions (5 to 10 ml) were collected until at least 50 ml of fluid had passed through the liver. The number of parasites in each fraction was determined, and the data were plotted as cumulative recovery of trypanosomes as a function of perfusate volume.

In some experiments, the washed liver was first infused with a solution of mannan (10 mg/ml) or with fresh, whole normal mouse serum (NMS). This was followed by a suspension of *T. musculi* in PBSG, PBSG containing mannan (10 mg/ml), or whole, fresh NMS. In other experiments, the trypanosomes were first incubated in hyperimmune mouse serum (from mice inoculated repeatedly with *T. musculi* after they had recovered from an initial infection), washed, and suspended in whole NMS.

Pretreatment with hydralazine was performed to induce dilatation of the small blood vessels. The drug was administered intraperitoneally 15 min before the mice were anesthetized or about 20 min before surgery commenced.

Distribution and clearance of ^{75}Se-labeled *T. musculi*. ^{75}Se-labeled *T. musculi* organisms were obtained from cyclophosphamide-treated mice and from mice on days 8 to 9 of *T. musculi* infection. These trypanosomes were carefully washed and injected into normal mice. The latter were anesthetized 2 h later, blood samples were collected, the mice were sacrificed by euthanasia, and the livers and spleens were removed. Radioactivity remaining in the blood or taken up by the livers and spleens was estimated with a gamma counter (Beckman LS 1800). Liver and spleen radioactivity levels were corrected for contaminating blood present in the organs.

An additional set of experiments was performed with ^{75}Se-labeled *T. musculi* obtained from mice on day 11 of infection. In this type of experiment, the trypanosomes were collected from the blood after centrifugation and immediately suspended in fresh serum collected from other mice infected for 11 days (not injected with a radioisotope). These labeled *T. musculi* organisms, suspended in serum from donors infected for 11 days, were then injected into other mice that were at day 11 of infection. These mice were sacrificed 2 h after parasite injection, and the distribution of the labeled parasites in blood, liver, and spleen was evaluated.
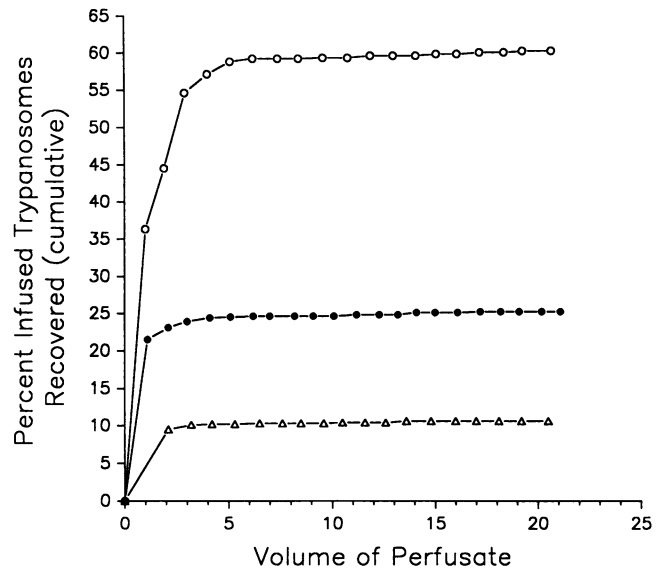

FIG. 1. Dynamics of recovery of *T. musculi* infused into the liver as a suspension in 100% NMS (O) or PBSG (●) or as antibody-coated *T. musculi* suspended in 100% NMS (△). Cumulative recovery (ordinate) is plotted against the volume of Hanks salt solution perfused through the liver after infusion of the parasites.

RESULTS

Dynamic and quantitative aspects of retention of infused trypanosomes by an isolated liver. There was marked retention of *T. musculi* introduced into the liver as a suspension in PBSG (Fig. 1; Table 1). Nearly all of the parasites that were not retained were collected in the first 5 ml (approximately) of perfusate. Thereafter, there appeared to be no release of parasites initially retained (Fig. 1 shows the cumulative number collected in 20 ml of perfusate, but there were very few, if any, parasites collected in the additional volume of perfusate up to 50 ml). About 75% of the trypanosomes in PBSG were retained by the liver. In contrast, when the trypanosomes were introduced into the liver as a suspension in whole NMS, only about 40% of them were retained (Fig. 1; Table 1). However, merely perfusing the liver with whole NMS before infusing the parasites in PBSG altered the retention of parasites very little in comparison with the PBSG used throughout the procedure. Thus, for NMS to have an effect, the trypanosomes had to be suspended in it. Results of tests of the statistical significance of the differences between the means of the various treatment groups shown in Fig. 1 and Table 1 are given in Table 1, footnote *a*. As shown, the effect of suspending *T. musculi* in NMS on liver retention was highly significant in comparison with *T. musculi* in PBSG.

When the trypanosomes were exposed to the antibodies present in hyperimmune serum before being suspended in NMS, the liver retention was much greater than that of parasites not exposed to antibodies (Table 1, compare treatment groups 3 and 6). Although the baseline retention of parasites in NMS (about 40%) was rather high, the effect of the antibodies on liver retention of the parasites was evident.

The isolated liver preparations displayed an impressive ability to retain large numbers of infused trypanosomes (Fig. 2). The proportion of the dose of parasites suspended in PBSG that was retained in the liver was nearly constant over a 43-fold range in the number of parasites infused. This

TABLE 1. Effects of various treatments and conditions on retention of trypanosomes by perfused liver

Group (species)	Condition of trypanosomes infused (medium)	Liver pretreatment	Mean (± SD) % of trypanosomes retained[a]
1 (T. musculi)	Intact (PBSG)	None	75.3 (12.6)
2 (T. musculi)	Intact (PBSG)	NMS infusion	64.8 (9.5)
3 (T. musculi)	Intact (NMS)	None	40.3 (11.7)
4 (T. musculi)	Intact with RF[b] (PBSG)	None	91.9 (3.3)
5 (T. musculi)	Intact with RF[b] (PBSG)	Hydralazine[c]	83.5 (7.9)
6 (T. musculi)	Antibody treated[d] (NMS)	None	89.5 (8.5)
7 (T. musculi)	Intact (PBSG)	Mannan[e] infusion	61.4 (11.9)
8 (T. musculi)	Intact (PBSG-mannan)[e]	Mannan infusion	56.3 (10.6)
9 (T. musculi)	Nude[f] (PBSG)	None	16.1 (9.6)
10 (T. lewisi)	Intact (PBSG)	None	89.7 (5.7)
11 (T. lewisi)	Nude (PBSG)	None	27.3 (4.6)
12 (T. musculi and T. lewisi)	Fixed[g] (PBSG)	None	98.7 (2.2)

[a] Means of results from 4 to 7 mice per treatment or condition are shown. Statistical analyses of differences between means ($P < 0.05$ indicated a significant difference) were performed. Groups: 1 and 2, $P = 0.22$; 1 and 3, $P = 0.008$; 1 and 4, $P = 0.01$; 4 and 5, $P = 0.053$; 3 and 6, $P = 0.005$; 1 and 7, $P = 0.11$; 1 and 8, $P = 0.04$; 1 and 9, $P = 0.001$; 10 and 11, $P = 0.001$.

[b] T. musculi suspensions containing 10 to 15% reproductive forms (RF [epimastigotes]) were collected from cyclophosphamide-treated donor mice.

[c] Mice were injected with hydralazine (250 µg/kg of body weight) 15 min before surgery.

[d] See Materials and Methods.

[e] Mannan (10 mg/ml) was in PBSG or Hanks salt solution.

[f] Surface glycoprotein was removed by trypsin treatment.

[g] Trypanosomes were fixed in 0.01% glutaraldehyde.

constancy in the proportion of parasites retained, independent of the number infused, was also true of the trypanosomes suspended in NMS (Fig. 2). As a result, the difference in parasite retention between suspensions in PBSG and those in NMS was also constant over at least a 32-fold range.

Treatment group 4 (Table 1) revealed that infusion of trypanosomes collected from donor mice on days 4 to 5 after inoculation, in which there were 10 to 15% reproductive forms (epimastigotes), resulted in very high retention of the parasites (92%). This was significantly higher than the retention of trypanosomes collected in the mid-plateau of infection (8 to 10 days), which include few, if any, dividing forms (i.e., they were virtually all nonreproducing trypomastigote forms). Pretreatment of mice with hydralazine before infusion of the suspension containing epimastigotes suggested that the high level of retention in the liver of the parasites in similar suspensions infused into the livers of normal mice (not pretreated with the drug) was largely mechanical; i.e., capillary dilatation induced by the drug resulted in a degree of hepatic retention similar to that in normal mice infused with preparations of trypomastigotes (compare groups 1 and 4 with 1 and 5).

We lacked insight into the possible ligand-receptor interactions that might account for retention of the trypanosomes suspended in PBSG; therefore, we chose one combination based on the known presence in the liver of the mannose-fucose receptor (4). Either isolated liver preparations were preperfused with a solution of mannan in PBSG and then infused with trypanosomes suspended in PBSG or the trypanosomes were suspended in PBSG containing mannan.

The presence of mannan in the medium of the trypanosomes significantly reduced the proportion of parasites retained (Table 1). Preperfusion with mannan solution alone did not significantly affect parasite retention.

The contribution of the surface coat material to retention of trypanosomes in the liver was clearly demonstrated for both T. musculi and T. lewisi (Table 1, groups 9 and 11). Only 16% of the infused, trypsin-treated (nude, i.e., lacking the surface glycoprotein coat) T. musculi organisms and 27% of nude T. lewisi organisms remained in the liver. Clearly, the surface coat material bears important interactive molecules (ligands and/or receptors). Finally, as shown by group 12, destroying the plasticity (deformability) of the trypanosomes by fixation in glutaraldehyde resulted in nearly all of them being trapped in the liver.

Complexities of intact-animal studies that illustrate the utility of the isolated perfusion model. The results obtained from studies of retention of trypanosomes by isolated livers indicated that T. musculi has a tendency to adhere to host cells, presumably endothelial cells, even in the presence of whole serum. To gain insight into the possibly confusing effects of this adherence on analyses of immunological elimination of parasites, two types of experiments were performed. One set of experiments was designed simply to assess the removal of intravenously injected parasites from the blood within a few hours after inoculation. The time chosen (12 h) was too soon either for reproduction of the injected T. musculi or for any immune response to have occurred. The results are shown in Table 2. The trypanosomes were collected from donors at the plateau of infection and injected in two different doses into normal recipients (calculated mean blood volume, 1.8 ml). The total number circulating in the blood of recipients 12 h after injection of 10^7 parasites was 1.4×10^6 or about 14% of the number injected. Therefore, 86% of the number injected (8.6×10^6 organisms) had, presumably, adhered to the vascular endothelium. The number of trypanosomes present in the circulating blood 12 h after intravenous injection of 10^8 parasites was 5.4×10^7. Thus, about 4.6×10^7 organisms had
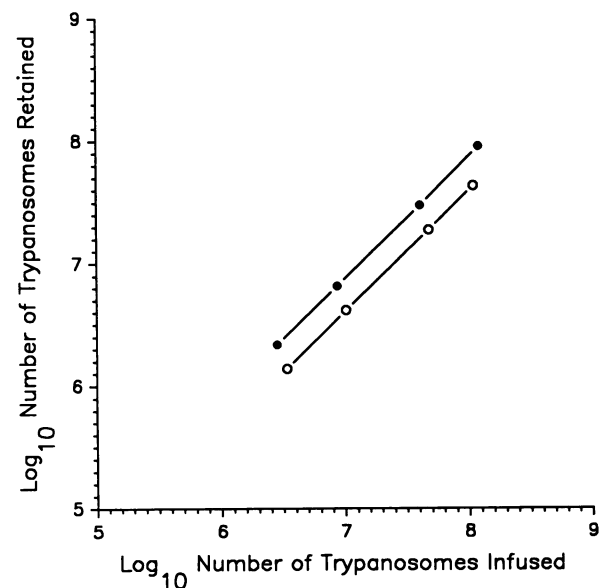

FIG. 2. Retention of T. musculi organisms infused into the liver as suspensions in PBSG (●) or in 100% NMS (○) plotted against the number of organisms infused.

TABLE 2. *T. musculi* present in circulating blood 12 h after intravenous injection into normal mice[a]

No. of trypanosomes injected[b]	Mean no. (±SD) of trypanosomes circulating in the blood 12 h after injection[c]	
	Total	% of those injected
10^7	1.4×10^6 (0.3×10^6)	14.0 (2.8)
10^8	5.4×10^7 (0.7×10^7)	54.0 (6.9)

[a] Normal recipient mice weighed 26 to 28 g; the calculated blood volume (6.7% of body weight) was 1.7 to 1.9 ml.

[b] Trypanosome donors had been infected for 8 to 10 days.

[c] There were five or seven mice per condition.

presumably become attached to the vascular endothelia of the recipient mice.

The second set of experiments provided results that indicated the complexities that can arise in intact-animal experiments designed to evaluate immunological elimination of trypanosomes and highlighted the potential utility of the liver perfusion model. The data presented in Table 3 represent one experiment from a set of five, performed with minor modifications, that yielded very similar results. Several factors can influence the apparent clearance of trypanosomes from the blood. *T. musculi* collected from cyclophosphamide-treated donors (including 10 to 15% epimastigotes [Table 1]) appeared to be cleared efficiently from the blood of normal mice in the absence of antibodies. By comparison, *T. musculi* organisms collected from donor mice on days 8 to 9 of infection (a time when no antibodies capable of facilitating immunological elimination are present; 2) were cleared with significantly lower efficiency from the blood of normal recipient mice. Radiolabeled *T. musculi* organisms collected from donor mice on day 11 of infection (a time when clearance-promoting antibodies begin to appear; 2) were suspended in serum also collected from mice on day 11 of infection and then injected intravenously into either normal mice or other infected mice at day 11 of infection. In both cases, elimination of parasites from the blood of recipients was unimpressive—substantially less than would be expected from antibody-facilitated elimination. In contrast to the results of assessment of bloodstream clearance of trypanosomes, uptake of the parasites by the liver and spleen more accurately reflected immunological elimination (Table 3). However, antibody-promoted uptake was evident only in mice that were themselves infected. In normal C3H mice, there was no uptake by organs, even of *T. musculi* organisms that were suspended in serum obtained from mice infected for 11 days.

DISCUSSION

The purpose of this investigation was to obtain background information that would guide the design of experiments concerned with mechanisms of immunological elimination of extracellular trypanosomes. In particular, it was important to determine whether or not perfusion of trypanosomes through the liver might be used as a model system in which to investigate the influence on immunological elimination of trypanosomes of variable factors such as (i) the titers and isotypes of antibodies (23), (ii) the different types of Fc receptors, (iii) the complement components and complement receptors, (iv) the activation status of KC, (v) various cellular adhesion molecules and lectins, and (vi) the composition of the surface coats of the trypanosomes. It appeared at the outset of the investigation that studies of uptake of trypanosomes by an isolated liver could be complicated by problems such as the size and motility of the organisms, the elaborate surface coats of the organisms, and the panoply of molecules that can function as cytadhesins. Our findings show that such problems can, indeed, complicate the analyses of immunological elimination by an isolated liver.

Approximately 75% of *T. musculi* organisms suspended in PBSG were retained by the liver, and this proportion was independent of the number perfused. Presumably, there is a number of trypanosomes that would exceed the retention capacity of the liver, but it must be greater than 10^8. The constant proportion of parasites retained suggests that there are two subsets in the ordinary population of *T. musculi*: those that are retained (by adherence?) and the 25% or so that are not.

Suspension of trypanosomes in whole NMS markedly reduced their retention by the liver. This finding, together with the effect of mannan in the medium used to suspend the parasites, strongly suggests that *T. musculi* can adhere to endothelial cells in the capillaries and liver sinusoids. Apparently, substances in NMS can interfere with this adhesion. It appears that interactions of surface coat molecules with mannan-fucose receptors in the liver may also occur. The importance of the surface coat of trypanosomes in their retention by the liver was shown by the striking recovery of nude trypanosomes following trypsin treatment. This was obvious for both *T. musculi* and *T. lewisi*.

The complications that may be introduced by mechanical factors in the use of the liver perfusion model were illustrated in studies of retention of *T. musculi* that had been fixed or that included significant proportions of reproducing forms (epimastigotes). The results with fixed organisms

TABLE 3. Distribution in normal or infected recipient mice of radioactivity of ^{75}Se-labeled *T. musculi* from immunosuppressed or infected donors

Trypanosome donor mice	Trypanosome recipient mice	Serum used (no. of samples)	Mean (±1 SD) % of injected radioactivity[a]		
			Cleared from blood	Taken up	
				Liver	Spleen
Cyclophosphamide treated	Normal	No serum (3)	64.0 (2.8)	14.1 (0.2)	2.0 (1.5)
Day 8–9 of infection	Normal	No serum (4)	47.2 (6.9)	10.8 (3.1)	1.7 (0.3)
Day 11 of infection	Normal	Day 11 of infection[b] (3)	26.1 (11.3)	8.0 (1.6)	1.2 (0.3)
Day 11 of infection	Day 11 of infection	Day 11 of infection[b] (3)	34.4 (11.5)	32.1 (4.1)	6.1 (2.1)

[a] Clearance from blood and uptake by organs were assessed 2 h after injection of radiolabeled *T. musculi*. All tabulated data were corrected for the radioactivity present in the blood contained in the organs.

[b] Before injection into recipient mice, the *T. musculi* organisms to be injected were incubated in serum collected from other mice on day 11 of *T. musculi* infection.

showed that the deformability of the trypanosomes is essential for them to percolate through the liver sinusoids. Any preparation of *T. musculi* that contained a significant proportion of nonviable organisms gave spurious results in the liver perfusion analysis. The high retention of the parasites in preparations containing 10 to 15% epimastigotes was almost surely a mechanical problem reflecting the overall greater size of the trypanosomes. The effect of pretreatment of the mice with hydralazine to dilate capillaries strongly suggested a mechanical explanation; the effect of this drug also suggested that the tendencies of parasite preparations that were entirely trypomastigotes and those that contained many epimastigotes to adhere to the liver endothelium were about the same.

Notwithstanding the potential complications revealed by the results included in Table 1, it appeared that the isolated liver preparation will be useful for analyses of mechanisms of immunological elimination of extracellular trypanosomes. As indicated by the data in Fig. 1 and Table 1, there was a very substantial increase in the retention of parasites in the presence of specific antibodies over the background of parasites in NMS. This difference should be sufficient to permit analysis of the components and events involved in KC uptake and destruction of parasites, even if the high background (i.e., retention of parasites suspended in NMS) cannot be diminished. However, it should be possible to reduce the background by reducing intravascular cytadherence.

It is likely that spontaneous retention of trypanosomes infused in NMS is a reflection of cytadhesins. That adhesins probably become involved in the apparent removal of *T. musculi* from the bloodstream was indicated by the results presented in Tables 2 and 3. The apparent removal of the parasites indicated in Table 2 could be due in part to mechanical trapping, but because the injected parasites were all trypomastigotes, it is probably a reflection of adherence to host cells.

The results provided in Table 3 illustrate some of the difficulties of studying mechanisms of immunological elimination in intact mice and highlight the potential uses of the liver perfusion model for studying trypanosome elimination. First, there is the possibility of mechanical trapping of parasites as shown by the apparent efficient elimination of the relatively large, epimastigote-containing preparation of parasites from cyclophosphamide-treated donors. Then, as shown by the remaining data in Table 3, there are conditions under which there is obvious selective, antibody-promoted uptake of trypanosomes by the liver and spleen but no evidence of elimination of the parasites from the bloodstream. Only in infected mice inoculated with antibody-coated *T. musculi* did it appear that all of the parasites removed from the blood in 2 h were those taken up by the liver and spleen. The reason for the differential effects of antibodies present in serum at day 11 of infection, when uptake of parasites by the liver and spleen was stimulated but elimination of parasites from the bloodstream was not, is uncertain. However, two explanations can be suggested. (i) As has been reported (2), it appears that blocking substances appear in the blood of infected mice during the plateau of infection; such substances can be antibodies (blocking antibodies) that fail to promote immune elimination of parasites but could easily alter adhesion of the parasites to host cells. Such substances could account for the apparent difference in bloodstream clearance of trypanosomes exposed to serum collected from donors on days 8 to 9 of infection compared with serum from day 11 of infection (Table 3). (ii) Animals

infected with *T. musculi* probably generate stress proteins and other substances, such as fibronectin, which appear in the blood in abnormal amounts and adhere to the parasites. Examples of elevated levels of fibronectin in the blood in infections have been reported (19), and fibronectin has been shown to bind to bacteria (13), *Trypanosoma cruzi* (16, 18, 24), and probably leishmanias (25). The appearance of adherent acute-phase and stress proteins during the course of infection might easily change the distribution of parasites in the blood between circulating and adherent organisms.

We realize that the data presented in this report represent an analysis of only some of the combinations of conditions and factors that can influence immune elimination of trypanosomes and use of the isolated liver perfusion model to analyze immune elimination. We conclude that the model is satisfactory for such analyses if complicating problems, such as mechanical trapping, adherence to host tissues, reproductive stages, and the integrity of the surface coat material, are carefully controlled.

ACKNOWLEDGMENTS

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