Immune and Nonimmune Regulation of the Population of
Trypanosoma musculi in Infected Host Mice

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A clear understanding of the population dynamics of trypanosome infections is lacking. In the case of murine Trypanosoma musculi infections, there are no answers to questions concerning (i) the nature of the prolonged plateau phase during which the number of parasites present in the host remains nearly constant (is it a static or dynamic steady state?); (ii) the origin of new parasites, if the plateau is a dynamic steady state, given the relatively early disappearance of generative forms from the bloodstream; and (iii) the role, if any, of a putative ablastin (reproduction-restricting antibody) in regulating the population dynamics of T. musculi infections. We describe here the results of studies of the number and distribution of mature and reproductive forms (RF) in the bloodstream and peritoneal space of both immunocompetent and cyclophosphamide-treated mice throughout the course of infection. While the RF disappeared from the blood within a few days after parasite inoculation, a high fraction (20 to 30%) of the parasites in the peritoneal space were RF throughout the course of infection and, thus, represented a source of new parasites. If an ablastin is responsible for inhibiting RF in the blood, it appeared to have no effect on RF in the peritoneal space. The results of this investigation support the conclusion that the control of the dynamics of T. musculi infections is largely nonimmunological (until cure of the infection) and probably is exercised by the supply of nutrients and reproduction-inhibiting (nonimmunological) and maturation-promoting factors that affect the generative fraction of the population.

Trypanosoma musculi is an extracellular, hemoprotozoan species of parasite that apparently infects only mice. The typical course of an infection extends for a period of approximately 3 weeks and ends when the host generates a vigorous immune response. Thereafter, the immune mouse is permanently resistant to reinfection. However, a few parasites may remain in the immune mouse, apparently cloistered in the vasa recta of the kidneys (25).

T. musculi infections are generally referred to as "nonpathogenic," presumably because the infections are generally not fatal in healthy young adult mice and leave no major residual pathological changes. However, during the course of infections there are gross pathological changes, the most apparent of which is the profound hyperplasia of lymphoid and reticular tissues (J. W. Albright, K. L. Holmes, and J. F. Albright, submitted for publication). Furthermore, in both very young and aged mice, and in young adult mice rendered moderately immunodeficient by splenectomy, T. musculi infections are frequently fatal (11). It is probably incorrect to conclude that T. musculi infections are nonpathogenic; rather, they induce pathological changes that are largely reversible once the infections are cured.

Most studies of T. musculi infections have been focused on the population of parasites located in the bloodstream. Very few studies have included the parasites located in the peritoneal space (PS). However, it has been pointed out that the trypanosomes in the PS proliferate throughout the course of infection, unlike those in the bloodstream (17, 24), and that infection persists until the parasites have been eliminated from the PS (11, 17).

In typical infections of young adult mice, dividing forms (DF) of T. musculi can be found in the blood for only a few days after inoculating the parasites (postinoculation [p.i.]). It has been assumed that the elimination of DF is attributable to the appearance of a DF-inhibiting antibody termed ablas-
tin (e.g., see references 8 and 22), a type of antibody that has been studied in considerable detail in rats infected with T. lewisi (9, 10, 13). Several investigators have presented evidence which was purported to show the involvement of ablastin in the course of T. musculi infections (16, 20, 21; D. G. Dusanic, J. Protozool. 21:422, 1974). However, there are difficulties with the conclusion that ablastin is responsible for elimination of the bloodstream DF of T. musculi, a major difficulty being the persistence of DF in the PS throughout the course of infection, as has been pointed out (23, 24). In addition to the problems associated with a clear direct demonstration of the presence of ablastin in infected murine serum, there is a conceptual difficulty in assuming that the production of new organisms ceases with the disappearance of DF from the bloodstream. The difficulty lies in explaining the nearly constant number of mature trypanosomes that persist in the blood between the disappearance of DF and the phase of rapid immune elimination, a period of approximately 1 week. Is it possible that the same parasites are present at the beginning and end of this week interval?

To gain insight concerning the dynamics of the population of T. musculi in the blood and PS, the steady-state number of parasites in the host during the plateau phase of infection, and the possible influence of factors such as ablastin, the investigations reported here were conducted.

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 of age. All animals were housed and maintained in the vivarium of the George Washington University School of Medicine.

Trypanosomes. The origin and maintenance of the line of T. musculi used in this laboratory have been reported (5). The line of trypanosomes has been maintained by weekly passage in normal C3H/Anf Cum mice and replaced at irregular intervals from stock kept frozen in liquid nitrogen.
Trypanosomes were collected from the blood of infected mice by drawing the blood into citrate-saline anticoagulant and separating the trypanosomes by repeated differential centrifugation. Phosphate-buffered saline-glucose (12) was utilized for routine suspensions and washing of the trypanosomes. The parasites were counted under phase microscopy with the aid of hemacytometers. In some cases, the parasites were collected from immunosuppressed mice which had been treated with cyclophosphamide (CY; 250 mg/kg, intraperitoneally [i.p.]) 8 to 24 h prior to T. musculi inoculation.

**Course of murine infections with T. musculi.** Infections were initiated with a known number of T. musculi inoculated into recipient mice by either the intravenous (i.v.) or the i.p. route. At intervals, infected mice were weighed and blood samples were collected either from the tail vein or by intracardiac puncture if the animals were sacrificed (by euthanasia). In the latter case, the peritoneal cavity immediately was flushed with a known volume of warm phosphate-buffered saline-glucose, and the peritoneal contents were harvested. The body weight was used to estimate blood volume (6.7% of body weight). The numbers of trypanosomes were determined from counts by phase microscopy.

**In vitro cultivation of trypanosomes.** Isolated trypanosomes were suspended in conditioned medium (1) and adjusted to 10^6/ml. Aliquots, 12 ml, were delivered to each petri dish (100 by 15 mm; VWR Scientific Inc., Baltimore, Md.). At various time intervals, parasites were collected and the total number of trypanosomes was determined. Slides were prepared and the trypanosomes were stained by use of buffered differential Wright-Giemsa stain.

**Analysis of DF and non-DF of trypanosomes.** Those samples for staining were prepared by making thin films on clean slides, air drying overnight, and fixin g in 95% methonal for 10 min. Each fixed and dried slide was dipped in stain for 10 s followed by 20-s washing in distilled water (or longer for desired color balance). The percentages of DF and non-DF were determined by well-established criteria (15).

**RESULTS**

The number and persistence of DF of T. musculi might be regulated, in part, by the total number of parasites present in the blood of infected mice. Furthermore, the DF population could reflect the available generative parasites (epimastigotes) present in the inoculum introduced into the host. The results shown in Fig. 1 came from studies of the influence of such parameters on the course of T. musculi infections in normal, young adult mice. Several important points are indicated by the data: (i) regardless of the number of T. musculi injected (10^6 or 10^7 per mouse, i.v.), the level of parasitemia reached the same steady-state number of parasites and the infections followed an identical course thereafter.
ter; (ii) regardless of the total number of parasites inoculated, the number of DF in the blood reached identical levels, the difference between inocula of $10^5$ and $10^6$ being only the time required to generate the same level of DF; (iii) although it took about 2 days longer for the inoculum of $10^5$ to generate the same number of DF generated by the inoculum of $10^6$, the disappearance of the DF from the blood followed identical courses; (iv) while one inoculum of $10^8$ parasites taken from CY-treated donors contained many more epimastigotes than the other inoculum (at least 280-fold more), the proportion of DF by 1 day pi was the same for both inocula, suggesting rapid conversion of non-DF to DF during the first day p.i.; (v) in the case of the inocula of $10^8$ T. musculi, the doubling times of the populations of parasites between days 1 and 4 p.i. were approximately 21 and 19 h for the entire population and the population of DF, respectively; (vi) in the case of the inoculum of $10^9$ T. musculi, the doubling time for the entire population between days 3 and 5 p.i. was approximately 8 h and that of the DF appeared to be about the same (based on two data points). A striking feature of these results is the well-regulated constancy of the proportion of total parasites present as DF until the time they are cleared from the blood. A second striking feature is the establishment of the DF subset as a precise fraction (ranging from about 5 to 9%) of the total parasites during the exponential and early plateau phases.

One other significant feature of the results shown in Fig. 1 is the loss of trypanosomes from the circulation within hours after i.v. injection. Comparison of the concentrations (number per milliliter) of the parasites immediately after injection and at 12 or 24 h p.i. indicated that >50% of the total parasites and of the generative forms had disappeared from the circulating blood.

If T. musculi were present only in the blood of the host, the data presented in Fig. 1 would suggest that all or most of the mature parasites present beyond approximately day 8 p.i. constituted a stable population from which organisms were gradually lost by death or attrition. It would be a static population, rather than a dynamic one, not influenced by turnover (loss and replacement) of organisms.

To obtain a clear picture of the influence of the trypanosomes in the PS on the population in the bloodstream, we monitored total organisms and DF in both the bloodstream and PS of mice through the course of T. musculi infections. Figure 2 depicts the infection profiles of normal young adult mice inoculated i.v. with $1.4 \times 10^8$ T. musculi. The results are presented as total organisms present in the blood (blood volume assumed to be 6.7% of body weight) or in the PS.

FIG. 2. Profiles of infection of young adult C3H mice: total trypanosomes (○) and total DF (△) present in the blood and total parasites (▲) and DF (▲) present in the PS following inoculation of $1.4 \times 10^8$ T. musculi i.v. Each point is the mean ± 1 standard deviation of samples from five to eight mice (two complete experiments). T. musculi was collected from donor mice on days 10 to 12 of infection.
The most interesting feature of the data shown in Fig. 2 is the population of DF present in the PS. It required a period of about 4 days for the development of this subset of DF in the PS following i.v. inoculation of \(10^8\) organisms into the host mice. The inverse relationship between numbers of DF in the blood and in the PS is striking. As the DF were cleared from the bloodstream, the DF in the PS increased steadily until the onset of immune elimination of all parasites. It is also noteworthy that the DF constituted a moderately large but nearly constant fraction of the total trypanosomes in the PS (ranging from 22 to 33% between days 4 and 13 p.i.).

The maximum number of bloodstream parasites was reached on day 5 p.i. (Fig. 2). There was a gradual decline in numbers of parasites in the blood thereafter; at the same time, there was a gradual increase in the total organisms in the PS. The net result was a near-constant total number of trypanosomes per mouse from days 7 through 13 p.i. (range, \(6.4 \times 10^7\) to \(7.5 \times 10^8\)).

We and other investigators (24) have noted that CY-treated mice can control \(T.\) \textit{musculi} infections, although they soon die from the infections (at least the mice of relatively susceptible strains such as C3H), presumably as a result of inability to generate appropriate antibodies. Therefore, to minimize the influence of antibodies (including putative ablastin) on the dynamics of \(T.\) \textit{musculi} infections, CY-treated hosts were used. These immunodeficient animals were inoculated either i.v. or i.p. with \(10^8\) \(T.\) \textit{musculi} derived from infected, CY-treated donors on day 6 of infection (containing 5.8% generative forms). The results are displayed in Fig. 3. The course of infection in the hosts inoculated i.v., up until death of the hosts, was quite similar to that observed in immunocompetent hosts (Fig. 1). However, there was no indication that the DF would be eliminated from the blood. It is noteworthy that the DF remained a nearly constant proportion of the total parasites (range, 5 to 9%, approximately) throughout the 6-day course of infection preceding death of the hosts. One day following inoculation of \(10^8\) \(T.\) \textit{musculi} i.p., the parasites could be estimated in the blood. Both mature forms and DF accumulated in the blood to reach concentrations equal to those obtained from i.v. injection of the parasites. The proportion of DF ranged from approximately 6 to 12% of the total population.

In all of the in vivo studies of \(T.\) \textit{musculi} infection dynamics, the DF were found consistently to remain as some carefully regulated proportion of the total trypanosome population. To determine whether or not this regulation of the generative fraction was a parameter of \(T.\) \textit{musculi} populations independent of the host, we studied the dynamics of \(T.\) \textit{musculi} production in vitro. The results are shown in Fig. 4. In vitro the generation of new \(T.\) \textit{musculi} is quite different from that which occurs in host mice. The population of parasites inoculated in culture converts within 24 h to \(>95\%\) generative forms (epimastigotes) (Fig. 4). This occurs in the absence of new parasite generation and therefore represents both a morphological and a functional change in the parasites. Thereafter, over the next several days the proportion of generative forms declines as new parasites are produced (data not shown; see reference 1).

**DISCUSSION**

Regardless of the number or route of injection, the number of \(T.\) \textit{musculi} eventually present in the blood and PS of normal host mice was the same. That was also true of host mice pretreated with CY. In normal mice, once that maximum number was reached, the total population remained essentially constant until the beginning of immunological elimination of the parasites. In the C3H mice used in this investigation, that number was approximately \(5 \times 10^8\) parasites per mouse between days 5 and 14 p.i. From studies reported elsewhere, it appears that there is little effective immunity against the trypanosomes prior to day 12 p.i. at the earliest (2, 6), although antibodies against \(T.\) \textit{musculi} antigens can be detected earlier (6). Thus, it is reasonable to conclude that the control of the size (magnitude) of the parasite population is nonimmunological. This conclusion is supported by the results of studies performed in CY-treated mice (Fig. 3). A clear example of nonimmunological control of the magnitude of \(T.\) \textit{musculi} infection may be seen in C57BL/6 mice that have been exposed to 400 rads of ionizing radiation (4).

Regardless of the number injected or the proportion of DF...
in the inoculum (over, at least, a 280-fold range), the number of DF reached in the blood was the same. The number of DF eventually reached in the PS was greater than that in the blood. In both the blood and PS, the proportion of DF of the total *T. musculi* population remained at a near-constant fraction throughout growth of the population. However, in the PS the proportion of DF was two- to threefold higher than the peak reached in the blood. The proportion of DF present in the blood of CY-treated recipients remained constant until the host mice died; furthermore, that proportion of the total (between 5 and 10% until just before the mice died) was about the same as the proportion in immunocompetent mice (2.5 to 5%) preceding disappearance of the DF.

Evaluation of all of these results leads to the conclusion that the regulation of the growth and population size of *T. musculi*, as well as the maintenance of the steady-state population during the plateau of infection, are reflections of nonimmunological regulatory processes. Furthermore, it is probable that the generative subpopulation, reflected by the DF, remains a relatively constant proportion of the total population owing to some aspect of population dynamics. There are several possibilities. For example, regulatory substances might be elaborated by the mature forms (trypanomastigotes) in the population. Evidence that this occurs in vitro has been reported (19). It seems probable that the magnitude of infection (i.e., the total *T. musculi* per mouse) is controlled by the capability of the generative subpopulation and that such capability is a reflection of the availability of essential nutrients (possibly growth-promoting factors) provided by the host. Strong candidates for limiting nutrients are the available purines and pyrimidines which the trypanosomes appear unable to synthesize but obtain from the host (3, 14). Depletion of purines and pyrimidines from the blood, but a continuous although limited supply in the PS, could explain the apparent shift in generative activity from the blood to the PS.

Certain aspects of the results presented here suggest that ablastin might be involved in the disappearance of DF from the blood. For example, the rapid disappearance of DF from the bloodstream of immunocompetent mice between days 6 and 10 p.i. did not occur in CY-treated mice, suggesting failure of ablastin production (ablastin is an immunoglobulin G molecule [13]). However, if an ablastin is responsible for the disappearance of DF from the blood, it becomes necessary to explain why it is ineffective against DF in the PS. There are several possibilities, including failure of the ablastin to enter the PS (unlikely) and the absence or inactivity of effector cells in the PS that might collaborate with ablastin to eliminate DF. The latter possibility has been suggested by other investigators (24). At the moment, however, it is unnecessary to invoke ablastin to explain the disappearance of DF from the bloodstream. This disappearance does not mean that they have been destroyed. Rather, they may simply convert to mature forms (trypanomastigotes). This might result from the unavailability of purines and pyrimidines to sustain DNA synthesis, or it might reflect a significant concentration in the blood of either reproduction-inhibiting factors or maturation-inducing factors or both. It has been postulated that host growth factors regulate reproduction of African trypanosomes and that exhaustion of such factors results in cessation of reproduction (7). However, one test of the hypothesis provided no support for the idea (18).

The results presented here and by other investigators (17) are particularly satisfying because they reveal that the prolonged plateau of *T. musculi* infection is probably a dynamic steady state, a balance between elimination of effete mature trypanosomes and their replacement by a generative subset of the parasite population. It is quite clear that trypanosomes are eliminated from the population during the plateau (6) and, therefore, must be replaced to sustain a relatively constant number. The relatively high fraction of DF in the PS throughout the plateau phase must generate the new parasites.

A substantial proportion (>50%) of the inoculated *T. musculi* disappeared from the circulation within hours after injection (Fig. 1 to 3). In part, this might have resulted from spontaneous elimination of the parasites by the liver (6). It is also likely to have resulted from adherence of trypanosomes to host cells, the vascular endothelium, in particular (Albright et al., submitted). Given the common observation that *T. musculi* reproduction appears to occur most efficiently in aggregates of parasites associated with supporting host cells (seen clearly in in vitro cultures), it seems possible that attachment of parasites to endothelial cells might significantly facilitate reproduction.

The results and interpretation presented here are at odds with the conclusions of other investigators (15, 18; Dusanic, J. Protozool. 21:422, 1974) concerning the presence and function of ablastin in *T. musculi*-infected mice. The earliest publications (20, 21) were provocative but open to alternative interpretation, in particular, because it was uncertain whether the effects of putative ablastin-containing antisera were due to inhibition of reproduction or preferential lysis or to technical uncertainty arising from the very large volumes of serum administered (relative to the normal mouse blood volume) and the route of serum administration (i.p.). Those investigators were unaware of the sizable population of *T. musculi* in the PS of infected mice. Their work, together with a later investigation (16), added a fascinating complication, viz., the existence of an early acting, nonimmunological, trypanocidal mechanism which appears to regulate the population of DF. That mechanism is damaged by the combina-
tion of splenectomy and reticuloendothelial blockade (Indian ink) (21) or by ionizing radiation (16). Further insight into that mechanism may be seen from the differential effects of ionizing radiation on the control of *T. musculi* parasitemia in different inbred strains of mice (4).

To conclude, the studies reported here, and the work of other investigators (16), lead to the hypothesis that the regulation of population dynamics of *T. musculi* in their natural host is largely a nonimmunological process up until the time of immunological cure of the infection. The control of population size ("parasite burden") is probably exercised through control of the size and activity of the DF generative fraction of the population. Although ablastin may affect the generative fraction in the blood, it has no apparent effect on the generative fraction in the PS. It is clear (Fig. 4) that a population of *T. musculi* maintained in vitro can convert to 95% DF. This does not usually happen in vivo when the restrictions on number or proportion of DF are probably imposed by limitations of nutrients (e.g., purines and pyrimidines), reproduction-inhibiting factors, and maturation-promoting factors.

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LITERATURE CITED


