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INCREASED RESISTANCE TO *AEROMONAS HYDROPHILA* IN MICE EXPERIMENTALLY INFECTED WITH *TRYPANOSOMA CRUZI*

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ABSTRACT: Mice that differ in susceptibility to *Trypanosoma cruzi* were shown to differ in susceptibility to *Aeromonas hydrophila* as well when infection with *A. hydrophila* succeeded an established infection with *T. cruzi*. Challenge with *A. hydrophila* had no effect on longevity in *T. cruzi*-infected mice. Mice which are highly susceptible to *T. cruzi* (C3H(He)) exhibited increased resistance to an intravenous challenge with *A. hydrophila* on the 7th day of infection; then resistance waned, and by the 21st day of infection, C3H(He) mice were more susceptible than uninfected mice to challenge with *A. hydrophila*. Mice that are relatively resistant to *T. cruzi* (C57BL/6) expressed increased resistance to an intravenous challenge with *A. hydrophila* from the 7th through the 28th day of infection, although no increase in resistance occurred from the 7th day forward. Neither C3H(He) nor C57BL/6 mice infected with *T. cruzi* developed altered susceptibility to an intraperitoneal challenge with *A. hydrophila*. While the growth of *A. hydrophila* in the blood, liver, and spleen increased during the first 36 hr in normal mice, marked retardation of growth occurred at the times of increased resistance in both strains of mice. In C3H(He) mice, bacterial growth returned to normal as resistance was lost. The degree of resistance to *A. hydrophila* in mice increased as the *T. cruzi* infection inoculum increased from 10^2 to 10^4 ; however, at the highest dose tested (5×10^4), both strains of mice showed lowered resistance, yet were still more resistant than uninfected mice. Even though resistance to *A. hydrophila* increased and decreased in C3H(He) mice, and increased and plateaued in C57BL/6 mice, titers of agglutinating antibody to *A. hydrophila* continued to increase throughout the course of infection with *T. cruzi* in both strains of mice. Passive suppression of mice with the *T. cruzi*-induced suppressor substance prior to challenge with *A. hydrophila* rendered C57BL/6 mice more resistant than normal mice, had no effect on the susceptibility of C3H(He) mice, but lowered the anti-*A. hydrophila* agglutinating antibody titers in both strains of mice.

The cellular and antibody-mediated immune responses both are important in immunity against *Trypanosoma cruzi* (e.g., Kierszenbaum and Howard, 1976; Trischmann et al., 1978). In other studies it has been demonstrated clearly that infection with *T. cruzi* profoundly alters the mammalian host's immune responses to a variety of antigens. These immune alterations are manifested as immunosuppression of antibody responses to T-dependent and -independent antigens (Clinton et al., 1975; Cunningham et al., 1978; Cunningham and Kuhn, 1980a-c; Ramos et al., 1978) and of cell-mediated responses to homologous (Rowland and Kuhn, 1978a) and heterologous antigens (Reed et al., 1977, 1978; Cunningham et al., 1981a) and mitogens

(Rowland and Kuhn, 1978b; Cunningham and Kuhn, 1980d; Ramos et al., 1979). Although immunosuppression develops early in experimental Chagas' disease and is generally complete, the significance of the suppression of immune responses on development of protective immunity against *T. cruzi* is not known. Likewise, it is perplexing that mice which survive acute infection, enter and survive long-term chronic stages of the disease (yet remain unresponsive to challenge with heterologous antigens), and do not succumb to bacterial or other microbial infections.

Herein we present the results of experiments on the effect of infection of mice with *T. cruzi* on the ability of these mice to mount protective immune responses to a virulent strain of *Aeromonas hydrophila* (Fliermans and Hazen, 1980), a bacterium implicated in fatal infection of compromised hosts (Davis et al., 1978).

MATERIALS AND METHODS

Animals

Female C3H(He) (Flow Laboratories, Dublin, Virginia) and C57BL/6 mice (Jackson Laboratories,

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Bar Harbor, Maine), 8 to 14 wk old, were used in all experiments. The mice were housed in a temperature-controlled animal room in bonnet-covered plastic cages with eight mice per cage. Purina laboratory chow and water were supplied ad lib.

Infections

Mice were infected i.p. with 10^8 blood-form trypomastigotes (Brazil strain) as described (Kuhn and Murnane, 1977), unless specified otherwise. With this infection inoculum, C3H(He) mice live approximately 24 days and C57BL/6 mice live 45+ days and often survive (Marr and Pike, 1967; Trischmann et al., 1978). Mice were infected with the LN1 strain of *A. hydrophila* i.p. or i.v. A lethal dose killed mice 16 to 20 hr following inoculation, and a sublethal dose was cleared systemically within 8 to 13 days. The LD₅₀ values for *A. hydrophila* were determined by probit analysis.

Counting bacteria

The number of *A. hydrophila* in the blood, liver, and spleen of mice was estimated according to the procedure of Blanden et al. (1969) except tissue samples were plated on R-S media and incubated at 35 C for 16 to 18 hr prior to counting of colonies.

Antibody titers

Anti-*A. hydrophila* agglutinating antibody titers were determined using a modification of the technique of Kuhn and Vaughn (1976). *Aeromonas hydrophila* which had been grown on 3% TSB media for 24 hr at 35 C were thrice-washed with cold Locke's solution and resuspended in the same at 10^{10} organisms/ml. The bacterial suspension was then radiolabeled with ^{51}Cr at 500 $\mu\text{Ci/ml}$ and placed in a water bath-shaker at 35 C for 2 hr. The labeled bacteria were washed three times in an equal volume of Locke's solution and resuspended in 0.85% saline at a final concentration of 10^{10} organisms/ml. Mouse serum was diluted serially (2-fold) in $13 \times 100\text{-mm}$ test tubes in 0.85% saline in a final volume of 0.25 ml, to which was added 0.25 ml of the labeled bacteria. Following a 1-hr incubation at 35 C, 0.2 ml of the supernatant was carefully removed from each tube and radioactivity determined in a Beckman automated gamma-counter. The agglutination titer is presented as the reciprocal of the dilution of immune mouse serum in the tube containing significantly fewer counts in the supernate than the matched tube of normal mouse serum. Serum from mice infected with *T. cruzi* alone did not show increased levels of agglutinating antibody against *A. hydrophila* when compared to normal serum.

Trypanosoma cruzi-induced suppressor substance (SSS)

Serum was collected and pooled from 10 C3H(He) or 25 C57BL/6 mice infected with *T. cruzi* for 20 and 30 days, respectively, and used as a source of SSS (Cunningham et al., 1978; Cunning-

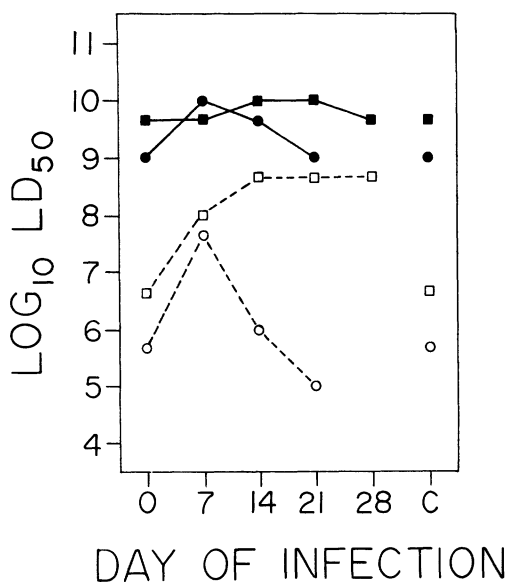


FIGURE 1. LD₅₀ for *A. hydrophila* in C3H(He) (circles) and C57BL/6 mice (squares) during infection with *T. cruzi*. Mice were infected via the intraperitoneal (closed symbols) or intravenous route (open symbols). The Dunnett's multiple comparison standard errors were 0.2660 and 0.3040 (C3H(He), intraperitoneal and intravenous, respectively) and 0.3230 and 0.3610 (C57BL/6, intraperitoneal and intravenous, respectively). C = uninfected control mice.

ham and Kuhn, 1980b, c). Suppression was induced in normal mice by transferring 0.2 ml of SSS i.v. to mice syngeneic to the SSS-donor. The SSS-recipients were challenged with a sublethal dose of *A. hydrophila* at times thereafter as indicated.

Statistics

Five mice were used in each experiment. Data were normalized with a log₁₀ or log₂ transformation when appropriate prior to statistical evaluation by an analysis of variance and Dunnett's multiple comparison test or a two-tailed, paired-sample *t*-test, as indicated (Zar, 1974). All results were considered statistically significant at a $P < 0.05$ level.

RESULTS

Mice that were infected with *T. cruzi*, and therefore immunosuppressed to test antigens and mitogens, showed no evidence of increased susceptibility to challenge with a highly virulent strain of *A. hydrophila* (Fig. 1). However, C3H(He) mice were more resistant to *A. hydrophila* on the 7th day of infection with *T. cruzi*, although the increased resistance was transient and declined thereafter.

TABLE I. Effect of *A. hydrophila* challenge on longevity in C3H(He) mice infected with *T. cruzi*.*

Day of infection	Day of death†
0	24.6
7	24.8
14	23.9
21	23.1

* Mice were inoculated i.v. with 2.5×10^8 *A. hydrophila*.

† The Dunnett's multiple comparison standard error was 0.4500.

Increased resistance to *A. hydrophila* also developed by the 7th day of infection with *T. cruzi* in C57BL/6 mice and this heightened level of resistance was maintained for at least 21 days, at which time the experiments were terminated. The altered susceptibility to challenge with *A. hydrophila* in *T. cruzi*-infected mice resulted only with intravenous administration of the bacteria; intraperitoneal challenge was without significant effect. Although C3H(He) mice which received *A. hydrophila* on the 21st day of infection with *T. cruzi* had a somewhat reduced longevity, this was not statistically significant, and, in fact, may reflect the moribund condition of the mice (Table I). In both C3H(He) and C57BL/6 mice the resistance to *A. hydrophila* increased as the infection inoculum of *T. cruzi* was increased from 10^2 to 10^4 (Table II). At the highest dose tested (5×10^4 trypomastigotes), the LD₅₀ dropped, yet remained substantially greater than the LD₅₀ for uninfected, control mice.

The growth of *A. hydrophila* in the blood, liver, and spleen of mice was monitored 12, 24, and 36 hr following intravenous inoculation (Fig. 2). The number of viable *A. hydrophila* increased rapidly in the tissues of normal C3H(He) and C57BL/6 mice (Fig. 2a, e). At the times of increased resistance in C3H(He) (Fig. 2b) and C57BL/6 mice (Fig. 2f-i) the growth of *A. hydrophila* was less pronounced than in uninfected controls, but in no case were the bacteria cleared during the 36-hr period. As resistance waned in C3H(He) mice, the growth of *A. hydrophila* in the blood, liver, and spleen returned to the normal rate (Fig. 2c, d).

To ascertain whether or not the *T. cruzi*-induced immunosuppression alone accounted for the increased resistance to *A. hydrophila*, SSS was transferred to normal mice which

TABLE II. Effect of dose of *T. cruzi* on resistance to *A. hydrophila* in mice.*

<i>T. cruzi</i> dose	LD ₅₀	
	C3H(He)	C57BL/6
Uninfected control	5×10^8	5×10^8
10^2	9×10^8	8×10^8
10^3	1×10^9	$5 \times 10^{7\dagger}$
10^4	$2 \times 10^{8\dagger}$	$1 \times 10^{8\dagger}$
5×10^4	8×10^8	$5 \times 10^{7\dagger}$

* Mice were infected with *T. cruzi* for 7 days at the time of intravenous challenge with *A. hydrophila*. C3H(He) and C57BL/6 mice were inoculated with 2.5×10^8 and 2.5×10^8 *A. hydrophila*, respectively.

† Statistically different from uninfected control. The Dunnett's multiple comparison standard errors were 0.3720 and 0.2950 for C3H(He) and C57BL/6 mice, respectively.

were then challenged with *A. hydrophila* simultaneously or 1 or 2 days later. As shown in Table III, the passively induced immunosuppression did not alter susceptibility to *A. hydrophila* in C3H(He) mice, but C57BL/6 mice which received SSS and *A. hydrophila* at the same time were rendered more resistant.

The anti-*A. hydrophila* agglutinating antibody titers peaked 6 days following inoculation in normal C3H(He) and C57BL/6 mice (data not shown). The kinetics of the anti-*A. hydrophila* antibody response in C3H(He) and C57BL/6 mice infected with *T. cruzi* for 14 days did not differ from the kinetics of antibody formation in uninfected controls (data not shown). However, in mice infected with *T. cruzi*, the agglutinating antibody titers to *A. hydrophila* increased in step with the progression of the *T. cruzi* infection (Fig. 3). Even though passive immunosuppression of mice did not increase susceptibility to *A. hydrophila* in recipient mice, the agglutinating antibody titers to *A. hydrophila* were significantly lower than in normal controls (Table IV).

DISCUSSION

Suppression of host immune responses by parasites is a nearly ubiquitous finding (Ogilvie and Wilson, 1976). Mice experimentally infected with *T. cruzi* are exemplary of the phenomenon of parasite-induced immunosuppression, and are characterized by irreversibly impaired cellular and humoral responsiveness. It is paradoxical, then, that mice infected with *T. cruzi* successfully resist

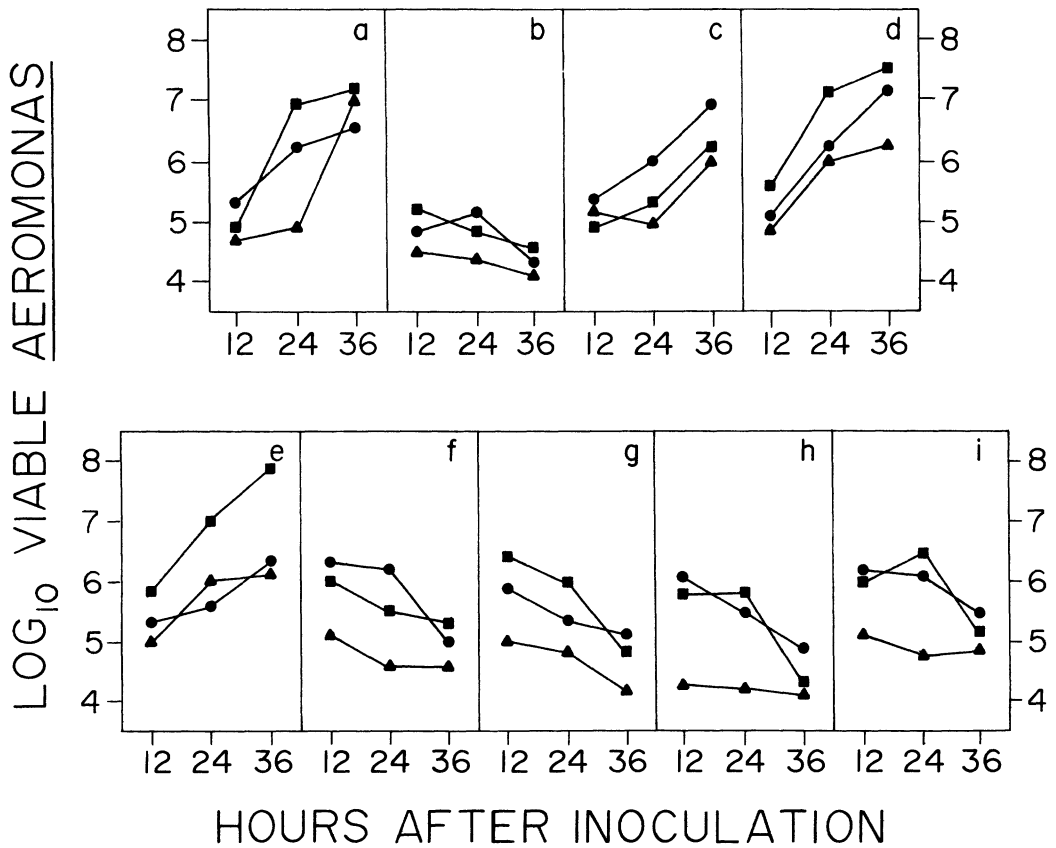


FIGURE 2. Growth curves of *A. hydrophila* in the blood (circles), spleen (squares), and liver (triangles) of mice following intravenous challenges. Bacteria were counted in normal C3H(He) (a) and C57BL/6 mice (e) and mice infected with *T. cruzi* for 7 (b, C3H(He); f, C57BL/6), 14 (c, C3H(He); g, C57BL/6), 21 (d, C3H(He); h, C57BL/6) or 28 days (i, C57BL/6). C3H(He) and C57BL/6 mice were challenged with 2.5×10^5 and 2.5×10^6 bacteria, respectively. The Dunnett's multiple comparison standard errors were 0.2080, 0.2160, and 0.2930 (C3H(He) blood, spleen, and liver, respectively) and 0.3110, 0.2670, and 0.2990 (C57BL/6 blood, spleen, and liver, respectively).

infections with secondary pathogens, and, as shown in the present investigation, acquire a greater than normal capacity to do so. Humans afflicted with chronic Chagas' disease also have been reported not to be more susceptible to infectious diseases than the indigenous population (Lozzio, in Albright and Albright, 1976). Our studies in Colombia, South America, revealed that patients with chronic Chagas' disease typically had multiple intercurrent infections, although no information on the relative susceptibility of these patients to secondary pathogens was available (Cunningham et al., 1980). The phenomenon of increased antimicrobial activity concurrent with an unrelated companion infection is not unprecedented (Collins et al., 1972; Bomford and Wedderburn, 1973; Cypess et al., 1974);

however, it is by no means the rule (Greenwood et al., 1972). The basis for the phenomenon of increased resistance to secondary infection, therefore, needs further elucidation,

TABLE III. Effect of *T. cruzi*-induced suppressor substance (when administered to normal mice) on resistance to *A. hydrophila*.*

Mouse strain	LD ₅₀			
	Control (no serum)	Day 0	Day 1	Day 2
C3H(He)	5×10^6	6×10^6	5×10^6	5×10^6
C57BL/6	5×10^6	4×10^6 †	5×10^6	5×10^6

* Mice received *A. hydrophila* i.v. at the same time, 1 day, or 2 days after administration of SSS. C3H(He) and C57BL/6 mice were injected with 2.5×10^5 and 2.5×10^6 *A. hydrophila*, respectively.

† Statistically different from uninfected control. The Dunnett's multiple comparison standard errors were 0.1230 and 0.3450 for C3H(He) and C57BL/6 mice, respectively.

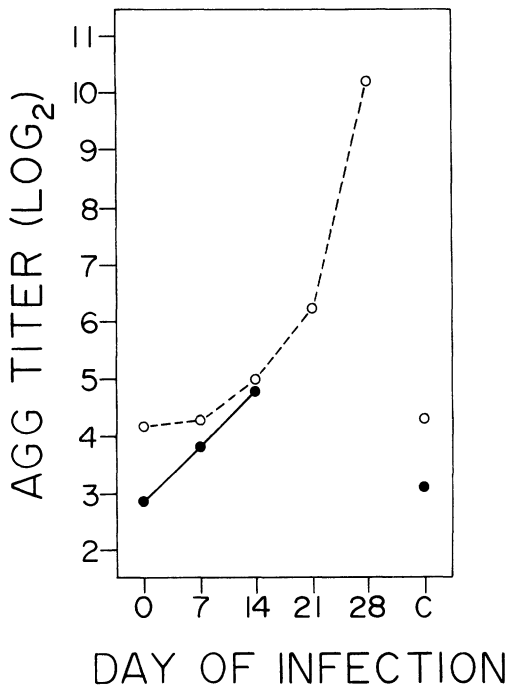


FIGURE 3. Agglutinating antibody titers in sera of C3H(He) (closed circles) and C57BL/6 mice (open circles) to *A. hydrophila* during the course of infection with *T. cruzi*. C3H(He) and C57BL/6 mice received an intravenous challenge of 2.5×10^6 and 2.5×10^6 *A. hydrophila*, respectively, and were bled for sera 6 days later. The Dunnett's multiple comparison standard errors were 0.0660 and 0.1440 for C3H(He) and C57BL/6 mice, respectively. C = uninfected control mice; Agg = agglutination.

especially when one considers the profound immunosuppressed condition engendered by the primary pathogen.

In the present study, we have shown both *T. cruzi*-susceptible (C3H(He)) and -resistant (C57BL/6) mice to be more resistant to challenge with *A. hydrophila* following infection with *T. cruzi*. The increased resistance to *A. hydrophila* depended on the duration of the primary infection and the dose of *T. cruzi* used to establish infection, but was generally independent of the immunosuppressed condition. The lack of association between resistance and immunosuppression may indicate the involvement of accessory defense mechanisms, such as those mediated by the reticuloendothelial system, as a primary means for combating *A. hydrophila*. Indeed, Ketover et al. (1973) showed the opsonic activity of serum from patients with *A. hydrophila* infec-

TABLE IV. Effect of *T. cruzi*-induced suppressor substance (when administered to normal mice) on the agglutinating antibody response to *A. hydrophila*.*

	Agg titer (log ₂)†
C3H(He) normal	3.2
C3H(He) suppressed	1.4‡
C57BL/6 normal	4.2
C57BL/6 suppressed	0.8‡

* Mice received *A. hydrophila* i.v. at the same time SSS was administered, and were bled for sera 6 days later. C3H(He) and C57BL/6 mice were injected with 2.5×10^6 and 2.5×10^6 *A. hydrophila*, respectively.

† Agg = agglutination.

‡ Statistically different from uninfected control by a two-tailed paired-sample *t*-test.

tion to be correlated with the severity of the disease, with the highest titers of opsonins demonstrable in convalescent sera. With respect to *T. cruzi*-infected mice, it is known that the infection has little effect, if any, on the functioning of the reticuloendothelial system (Ramos et al., 1978) and, as reported herein, humoral activity directed against the parasite is not blunted (at least the agglutinating antibody response). The unimpaired reticuloendothelial system and elevated humoral activity may collectively account for the observed increased resistance.

It is not evident why the agglutinating antibody titers to *A. hydrophila* increased as the infection progressed. Perhaps this might be attributed to polyclonal activation, which is known to occur during experimental Chagas' disease (Ortiz-Ortiz et al., 1980; Cunningham et al., 1981b), except that an anti-*A. hydrophila* antibody response was not demonstrable in *T. cruzi*-infected mice prior to challenge with *A. hydrophila* (data not shown). The fact that passively suppressed mice were not more susceptible to *A. hydrophila*, yet had reduced agglutinating antibody titers to the organism, suggests that antibody probably plays a minor role in controlling this bacterial infection. The effect, if any, of immunosuppression on the production of opsonic activity remains to be determined. If opsonic activity is not affected during active (parasite-induced) or passive (SSS-induced) suppression, "suppressed" mice would not be expected to be more susceptible to *A. hydrophila*.

The suppression of immune responses in

mice infected with *T. cruzi* affects primarily the induction of responses to new antigens and has only a minimal effect on activation of anamnestic responses (Cunningham et al., 1980b). That is, pristine clones of lymphocytes are suppressed readily, while low-level exposure to antigens prior to infection leads to enhanced immune responsiveness. With the cosmopolitan distribution of *A. hydrophila* (Hazen et al., 1978) a preinfection experience with homologous or cross-reacting antigens is a likely event, and may have led to the development of resistance during *T. cruzi* infection. Alternatively, and as suggested by the disparate level of *A. hydrophila* resistance in C3H(He) and C57BL/6 mice infected with *T. cruzi*, there may exist a genetic basis to the immunoregulatory alterations occurring in hosts infected with *T. cruzi*. This enigma might be resolved by conducting similar experiments on resistance to secondary pathogens in gnotobiotic mice and/or genetically similar mice of differing susceptibility to *T. cruzi*.

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