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Effects of Heterologous Antithymocyte Serum on *Toxoplasma gondii* Infections in Mice. I. Potentiation of Primary, Nonlethal Infection

WALTER STAHL, GREGORY TUREK, AND HASSAN A. GAAFAR
Division of Laboratories and Research, New York State Department of Health, Albany, New York, U. S. A. 12201

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Introduction

The inoculation of mice with nonlethal strains of the intracellular protozoan parasite *Toxoplasma gondii* results in clinically inapparent infections which are characterized by the rapid development of an acquired immunity that terminates the active growth phase of the parasite in the tissues, and protects the mouse against reinfection (Frenkel, 1956; DeRoever-Bonnet, 1963, 1964; Stahl and Akao, 1964). The underlying host factors that control the infection are incompletely understood, but are believed to be a reflection of cell-mediated immunity (CMI) Frenkel, 1967).

In a recent paper (Stahl *et al.*, 1976), we reported on the use of the immunosuppressive drug methotrexate to alter the course and outcome of experimental *Toxoplasma* infections in mice. Depending on the dosage, frequency, and number of injections of the drug, several distinct patterns of disease evolved, encompassing a wide spectrum of symptoms and pathologic changes. The target cell population could not be pinpointed however because of the broad and nonspecific antimetabolic activity of methotrexate.

The present investigation reports on our efforts to better understand the complex interplay between host immune responses and *Toxoplasma*, in terms of parasite proliferation and persistence in the host, cyst formation in the brain, immunity to reinfection, and reactivation of chronic, latent infection. The latter two objectives will be considered separately in a companion paper. Heterologous antithymocyte serum (ATS) was chosen for study because it is generally recognized as one of the most potent and selective of immunosuppressive agents, particularly effective against cell-mediated immunity. Unlike other cytotoxic agents, ATS can be considered a true immunosuppressant, acting directly and specifically on thymus-derived lymphocytes, rather than indirectly through some generalized antimetabolic action (Lance, 1972).

Materials and Methods

Animals

Female, highly inbred Nya: NYLAR albino mice, 8 to 10 weeks of age, were obtained from an animal colony maintained by the New York State Department of Health. The mice were housed up to 10 per cage, and allowed unlimited food and water.

Parasite

The nonlethal Cornell (CS) strain of *Toxoplasma gondii*, originally obtained from Dr. A. Kimball, Cornell University School of Medicine, New York, NY, was used in this study. The CS strain was maintained in the cyst stage in the brains of chronically infected stock mice and was passaged to

normal mice every six months. To obtain CS cysts, brains of stock mice were removed and homogenized in saline in a tissue grinder. Cysts were counted, and the final volume was adjusted to yield approximately 24 cysts per ml of brain suspension. The standard mouse-infecting dose was 12 cysts in a 0.5 ml aliquot, given by intraperitoneal (ip) injection.

Toxoplasma Cyst Counts

The brains of experimental mice were individually emulsified in 4.0 ml of saline in tissue grinders. Four to eight drops of each emulsion (approximately 30 drops/ml) were removed with a pasteur pipette and individually scanned under the low power (100 ×) of a compound microscope. The total number of cysts in each mouse brain was calculated by multiplying the average number of cysts per drop of emulsion by the average number of drops (120) in the emulsion.

Antithymocyte Serum (ATS)

The heterologous ATS was prepared in rabbits according to the method of Levey and Medawar (1966). Rabbits were given 2 intravenous injections of approximately 10^9 mouse thymocytes, 14 days apart. Two weeks after the last injection, the rabbits were bled out by cardiac puncture. Sera were pooled, heat-inactivated at 56 C for 30 minutes, portioned out in 2 and 5 ml aliquots in small glass vials, and stored at −20 C. Normal rabbit serum (NRS), used for control studies, was inactivated and stored in similar fashion. All injections of sera were given by the intraperitoneal route.

Tests for the Immunosuppressive Potency of ATS

The immunosuppressive potency of ATS was assessed by its ability to inhibit the development of contact sensitivity to oxazolone (4-ethoxymethylene-2-phenyl oxazolone, BDH Chemicals, Ltd., Poole, England). Groups of mice were sensitized by applying 0.1 ml of 10% oxazolone in absolute ethanol to an area approximately 3 cm^2 on the shaved dorsum. During the next several days four different regimens of ATS were administered to the mice as described in the results.

Six days after sensitization, the mice were anesthetized, and the thickness of each ear measured successively 3 times with an engineer's micrometer (Fisher Scientific Co., NY). One drop of 1% oxazolone in olive oil was then applied to both sides of both ears. After 24 hours, the mice were again anesthetized, and the thickness of all ears remeasured and the results expressed as the mean increment in ear thickness in units of 10^{-3} cm.

Test for ATS Toxicity

Prior to administering ATS to *Toxoplasma*-infected animals, several preliminary tests were performed to determine if ATS was toxic to mice. Groups of normal mice were injected daily for 6 days with 0.5 ml aliquots of ATS or NRS then observed for an additional 14 days. The total volume of ATS and NRS administered (3.0 ml) was not demonstrably toxic to the mice. It was felt that this lack of overt *in vivo* toxicity obviated the necessity of determining either hemagglutinin titers or cytotoxic activity *in vitro*, or of absorbing the ATS or NRS with mouse erythrocytes prior to use.

Serology

The indirect hemagglutination test (IHA) for *Toxoplasma* antibodies (Jacobs and Lunde, 1957), was performed according to Center for Disease Control (CDC) procedures, using a commercial *Toxoplasma* antigen purchased from Industrial Biochemical Laboratories, Rockville, Maryland.

Experimental Design and Analysis

The objectives of this study were to note the effects of heterologous ATS on the course and outcome of primary nonlethal *Toxoplasma* infections in mice, with particular emphasis on the persistence of trophozoites in tissues, and numbers of cysts in the brain. The experimental design was as previously described (Stahl *et al.*, 1976); briefly, however, graded doses of ATS were administered to infected mice and the effects assessed by following morbidity, mortality, and pathologic changes.

Results

Verification of cell-mediated immunosuppressive properties of ATS

The inhibitory effect of ATS on cell-mediated immunity was monitored by examining its effects on the development of contact sensitivity to oxazolone. Four regimens of ATS were tested, representing the maximum, intermediate, and minimum doses administered to the *Toxoplasma*-infected mice in the succeeding experiments.

The results are listed in Table 1. It is evident that the higher doses of ATS, given in multiple injections, are potent inhibitors of contact sensitivity. The maximum regimen, 4 alternate-day injections totaling 2.0 ml of ATS, completely inhibited ear swelling. The intermediate regimen reduced the amount of ear swelling approximately two-thirds. The minimum regimens, involving single injections of 0.07 ml of ATS either on day 0 or day 4, had no discernable effects whatever. It is of interest to note that the administration of NRS apparently enhanced the amount of ear swelling recorded, to almost double that seen in the untreated control mice.

Course of primary, nonlethal Toxoplasma infections in untreated mice

Untreated mice infected with the CS strain of *T. gondii* generally ran a mild course during the 30 days they were observed. Signs and symptoms, when present, appeared during the second and third weeks of the infection, and consisted of ruffled fur, huddling, a transient ascites, and a gradual loss of about 10 percent of body weight. No mortalities were recorded in any group of infected, untreated mice (5 groups of 12 mice each).

Toxoplasma cysts were first detected in brain tissue on day 10, and trophozoites persisted in peritoneal exudate up to the 12th day post-infection. Histopathological examination of tissue sections revealed inflammatory lesions in the heart, lungs, liver, kidneys, and brain after 2 weeks of infection. Cysts in the brain were solitary, and generally not associated with the inflammatory lesions. Mice infected for 4 weeks had an average of 900 cysts per mouse brain and an anti-*Toxoplasma* IHA titer mode of 1 : 512. The mode is the titer with the highest frequency of occurrence in the group.

Course and outcome of primary, nonlethal Toxoplasma infections in ATS-treated mice

A series of 5 experiments were performed, employing as variables the following: The number, the timing, and the frequency of serum injections, and the total amount of ATS injected. Table 2 lists the results of the 5 experiments in chronological order, based on the total amount of ATS adminis-

Table 1 Suppressive effects of heterologous antithymocyte serum (ATS) on contact sensitivity to oxazolone in mice

ATS Regimens			Mean ear thicknesses ($\times 10^{-3}$ cm)		
Treatment of sensitized mice	Days serum injected	Total amount (ml)	Before challenge (day 6)[b]	After challenge (day 7)	Increase in thickness
Untreated	—	—	22	34	12
NRS	−1,1,3,5	2.0	24	44	20
ATS	−1,1,3,6	2.0	23	23	0
ATS	0,2,4,6	0.5	22	26	4
ATS	0	0.07	22	36	14
ATS	4	0.07	22	37	15

[a] day 0 : day of sensitization with 10% oxazolone.
[b] day 6 : day of challenge with 1% ozazolone.

Table 2 Effects of normal rabbit serum (NRS) and heterologous antithymocyte serum (ATS) on the course and outcome of nonlethal CS-strain *Toxoplasma gondii* infections in mice

Treatment of mice	Day(s)[a] serum given	Total amount (ml)	Course of infection	*Percent mortality* (No. dead/no. tested) Day 14	Day 28
Untreated[b]	—	—	Subclinical	0(0/60)	0(0/60)
NRS	−1,1,3,5	2.0	Subclinical	0(0/10)	0(0/10)
ATS			Fulminating	85(12/14)	100(14/14)
NRS	−1 thru 6	1.0	Subclinical	0(0/ 9)	11(1/ 9)
ATS			Fulminating	100(16/16)	—
NRS	0,2,4,6	0.5	Subclinical	0(0/ 8)	0(0/ 8)
ATS			Acute	70(10/14)	100(14/14)
NRS	0,4,8	0.2	Subclinical	0(0/12)	0(0/12)
ATS			Acute	0(0/10)	60(6/10)
NRS	15,18,21	0.2	Subclinical	0(0/10)	0(0/10)
ATS			Subclinical	0(0/14)	7(1/14)
ATS	0	0.07	Subclinical	0(0/10)	0(0/10)
ATS	4		Acute	0(0/10)	40(4/10)
ATS	8		Subclinical	0(0/10)	10(1/10)
ATS	15		Subclinical	0(0/10)	0(0/10)

[a] Day of infection is day 0.
[b] Pooled data from 5 groups of untreated control mice.

tered to the experimental animals, from a maximum of 2.0 ml (4 injections, 0.5 ml each, on days −1, 1, 3, 5) down to the minimum of 0.07 ml of ATS, given in a single injection either on day 0, 4, 8, or 15 of the infection.

The first 3 experiments, involving the higher levels of ATS (0.5 to 2.0 ml), given via multiple injections early in the infection, yielded comparable results. The treated mice began to exhibit morbid changes by the 4th and 5th days post-infection. Huddling, ruffled fur, diarrhea, and loss of body weight were common findings. These were soon followed by symptoms indicating severe and progressive neurological involvement: Head tilting, circling, aimless writhing and rolling, arched spine, weakness of limbs, and posterior paraplegia. This was followed rapidly by lethargy, coma, and death. In the first 3 experiments, the mortality rate reached 100 percent in the ATS-treated mice, with most of the deaths occurring within the second week of the infection. A comparison of the data in Table 2 underlines the importance of both the number and timing of the ATS injections. Although the mice in experiment 2 received only one half the total amount of ATS administered in the first experiment (1.0 ml vs 2.0 ml), their disease syndrome evolved more rapidly and was more fulminating in nature. The schedule of 8 smaller injections at 24 hr intervals, as opposed to 4 large injections at 48 hr intervals, apparently was more efficient in suppressing host defenses to the parasite. Due to the high mortality rate noted in the first three experiments, it was not possible to obtain serum anti-*Toxoplasma* titers for these animals.

In Experiment 4, the total amount of ATS administered was reduced to 0.2 ml, given in 3 injections of 0.07 ml each. There were 2 parts to this experiment, relating to the timing of the injections. When the ATS was given on days 0, 4 and 8 (Exp. 4a) a

Table 3 Modification of several parameters of nonlethal CS-strain *Toxoplasma gondii* infections in mice treated with normal rabbit serum (NRS) and heterologous antithymocyte serum (ATS) (Data from experiment 4a)

Treatment [a] of CS-infected mice	Trophozoites in peritoneal exudate (days)	*Cysts in brain* day first detected	cyst clusters	*Cyst counts* average range	*Serology* IHA[b] titer (mode)[c]
Untreated	12	10	rare	900(650–1,500)	1 : 512
NRS	14	10	rare	1,050(825–1,300)	1 : 512
ATS	23	6	abundant	4,000(3,400–5,200)	1 : 512

[a] Aliquots of 0.07 ml of serum administered ip, on days 0, 4, 8 of infection.
[b] Indirect Hemagglutination Test. Titers obtained after 4 weeks of infection.
[c] Mode : The titer with the highest frequency of occurrence.

protracted disease evolved, with 60 percent mortality within 4 weeks. Press preparations of brain revealed numerous cysts, developing singly and in clusters. Pathologically, an extensive meningoencephalitis was observed, which is believed to be the cause of death. *Toxoplasma* trophozoites were detected in peritoneal exudate into the fourth week of the infection. These data are summarized in Table 3.

On the other hand, if the 3 injections of ATS were delayed until days 15, 18, and 21 (Exp. 4b), the *Toxoplasma* infection remained subclinical and the mortality fell to only 7 percent.

The objective of experiment 5 was to correlate the day of ATS administration (timing) with the degree of infection-modification. To focus on the importance of timing, it was deemed necessary to minimize the strong infection-enhancement effects obtained with the higher doses of ATS, administered via multiple injections. Accordingly, the several groups of infected mice received a single injection of only 0.07 ml of ATS, either on day 0, day 4, day 8, or day 15 of the infection (Table 2).

Only the administration of ATS on day 4 modified the primary subclinical CS infection into clinical disease and death. The effect of ATS was less marked if the injection was delayed until day 8 and there was no enhancement if the injection was given either on day 0, or delayed until day 15.

Serological tests on mice surviving into the fourth week revealed that antibody titers were unaffected by a prior single injection of ATS, whether given on day 0, 4, or 8 of the *Toxoplasma* infection. The anti-*Toxoplasma* IHA titer mode was 1 : 512, comparable to that of the untreated controls.

Discussion

Our results indicate clearly that the administration of ATS to mice during the first several days of *Toxoplasma* infection can greatly alter the course of the infection. Depending on the amount and timing of the ATS injections, several distinct clinical forms of acute toxoplasmosis emerged, ranging from a protracted, slowly evolving neurological syndrome to a rapid, progressive, fatal disease. In all the ATS-treated mice, *Toxoplasma* trophozoites persisted for extended periods of time, demonstrating that the tissue-clearing mechanisms of the host were impaired. Previously, Strannegard and Lycke (1972) had shown that ATS prolonged parasitemia in mice infected with an avirulent strain of *Toxoplasma*, and reduced the survival time of mice infected with a virulent strain.

Mice receiving the higher doses of ATS in multiple injections suffered the more rapid and severe forms of disease, marked by a profusion of trophozoites in peritoneal exudate, lungs. heart, liver, and spleen. Histopathologic examination disclosed pneumonitis and myocarditis. The smaller the amount

of ATS given, and the fewer the injections, the less severe the resultant disease. It is noteworthy that the smaller doses of ATS, although unable to suppress either serum antibody titers or contact sensitivity to oxazolone, still possessed the ability to modify the *Toxoplasma* infection.

These observations are very similar to those recorded by Stahl *et al.* (1976), who examined the effects of treating *Toxoplasma*-infected mice with methotrexate (MTX), a folate antagonist with immunosuppressive properties. They found that MTX administered during the first week converted the subclinical infection into several distinct patterns of clinical disease. However, it was not possible to fully interpret their findings due to the broad antimetabolic and immunosuppressive properties of MTX. In contrast, the use of ATS as a highly specific suppressant of CMI permits a possible explanation. With ATS, it was possible to determine the time of greatest vulnerability of the hosts' immune system to manipulation and to pinpoint the type of cells involved. For example, one injection of ATS on day 4 markedly potentiated the *Toxoplasma* infection whereas if given either on day 0 or day 8, the ATS was largely ineffective. Since ATS operates selectively against T cells in the circulation (Blanden, 1974), these data indicate that effector T cells left their ATS-inaccessible sites in lymphoid tissues and entered the blood by day 3 or 4, wherein they became inactivated by the recently administered ATS. The destruction of effector T cells would, in turn, block the activation of sufficient numbers of macrophages to control the parasite, and subclinical infection thereby progressed into clinically evident disease. By day 8, immunogenesis apparently was too far advanced for ATS reversal.

Strongly supporting this line of reasoning are the findings of Hapel and Gardner (quoted by Blanden, 1974) who reported that a single injection of 0.2 ml of ATS on days 3–5 following ectromelia infection in mice reduced the total number and cytotoxic potency of harvested mononuclear cells. The same dosage given on days 1, 2, or 6 was ineffective. Similarly, resistance to *Listeria monocytogenes* was inhibited by ATS given 2–5 days after infection, but not if the ATS was administered on days 6 or 7 (Mackaness and Hill, 1969 ; Pearson and Osebold, 1973).

Once again, as in previous reports (Stahl *et al.*, 1966a, b ; 1976), *Toxoplasma* cysts were detected in the brains of the immunosuppressed mice earlier and in much greater numbers, with many cysts developing in large, grape-like clusters. In spite of much speculation, the origin of these cyst clusters remains elusive. One possibility, suggested by Frenkel (1961), involves the rupturing of cysts in the brain and the release of many merozoites (intracystic trophozoites) which then invade neighboring cells and eventually form new cysts. Supporting Frenkel's view are recent studies by Nakayama (1974), who subjected chronically infected mice to long-term cortisone treatment. A four-fold increase in the numbers of cerebral cysts was achieved, as well as the appearance of bud-like protrusions of the cyst. Nakayama suggests this "budding" process may be a prelude to cyst rupture. However, the microscopic examination of numerous gross and histopathologic preparations of brain tissue has not yielded corroborative evidence, in that neither ruptured cysts nor cyst wall detritus has ever been found (Waaij, 1959 ; Stahl *et al.*, 1966b).

Another possibility is that the number of cysts in the brain may be directly related to the number of trophozoites reaching the CNS via hematogenous transport. If so, any manipulation of the host that would interfere with the efficient elimination of trophozoites from the tissues and circulation should then lead to increased numbers of cysts in the brain. This hypothesis is substantiated by the dramatic results obtained after only a single injection of ATS on day 4 of the infection. In the immunosuppressed mice, the observed persistence of trophozoites in tissue and blood likely resulted in a constant stream of parasites to the brain. There,

glial invasion was followed by cyst formation. Cyst clusters may represent fortuitous gatherings of parasites entering the brain through common portals, or the encystment en masse of scattered packets of trophozoites proliferating in the CNS of the immunosuppressed mice.

Summary

Groups of mice were infected with a nonlethal strain of *Toxoplasma gondii* and then treated with heterologous antithymocyte serum (ATS), a potent suppressant of cell-mediated immunity. The administration of ATS during the first week of infection (during immunogenesis) quickly converted subclinical infection into clinical disease with pronounced morbidity and high mortality.

Depending on the dose of ATS administered, and the number and frequency of injections, several distinct clinical entities of increasing severity emerged, ranging from a protracted neurological syndrome of several weeks duration to a fulminating visceral disease killing within several days. In the ATS-treated mice, *Toxoplasma* trophozoites persisted in peritoneal exudate and tissues for extended periods of time ; cyst development in the brain occurred earlier and in much greater numbers, and many cysts were found developing in large, grape-like clusters.

Delaying the administration of ATS until two weeks postinfection did not modify the ongoing *Toxoplasma* infection.

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マウスにおける *Toxoplasma gondii* 感染に対する異種 Antithymocyte Serum の効果

I. 弱毒株初感染の増強

WALTER STAHL, GREGORY TUREK, AND HASSAN A, GAAFAR
ニューヨーク州保健局

Toxoplasma gondii の弱毒株感染マウスに異種 Antithymocyte Serum を投与し，その効果を観察した．初感染後，一週の間は ATS を投与すると不顕性感染から，顕著な症状をもち高い死亡率を示す顕性感染へと速やかに移行した．ATS の投与量，投与回数，及びその頻度によつて明らかに多様な臨床所見が出現した．すなわち，一番軽症のもので数週間つづく神経症状を呈し，最も重症のものでは数日以内に死亡する激しい内臓症状を呈した．ATS 投与マウスでは Toxoplasma 栄養型虫体は腹水中，組織中に生存し続け，ATS 非投与マウスに較べて，脳の cyst はより早く形成され，数も増加し，集合し，塊状に発育した．ATS の投与を感染2週以後に行つても Toxoplasma 感染の経過に何の効果も認められなかつた．