Experimental *Acanthamoeba* Infections in Mice Pretreated With Methylprednisolone or Tetracycline

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Human infections due to free-living amebas of the genus *Acanthamoeba* have been reported sporadically, occasionally in individuals with underlying diseases. To determine if such infections may be considered opportunistic, groups of laboratory mice were pretreated with either methylprednisolone or tetracycline and inoculated intranasally with 1.075×10^4 *Acanthamoeba castellanii* isolated from a natural fresh water well. Results were compared with controls receiving either drug or amebas alone and with controls receiving saline injections with and without amebas. The mortality rate for those animals receiving methylprednisolone and amebas (50%) was found to be greater than the mortality in ameba controls (10%) ($P = 0.074$). Similarly, the mortality rate for animals receiving tetracycline and amebas (60%) was higher than the mortality in the ameba controls (10%) ($P = 0.0286$). Precise mechanisms for the increased mortality were unknown but were suspected to be due to the capacity of either corticosteroids or tetracycline to suppress host defenses, particularly those depending on neutrophils. The findings suggest a potentially pathogenic role for naturally occurring *Acanthamoeba* sp in humans with depressed host immunity. (Am J Pathol 92:733–744, 1978)

HUMAN INFECTIONS due to free-living amebas are receiving increasing attention since the original descriptions of primary amebic meningoencephalitis (PAM) by Fowler and Carter in Australia [1] and Butt in the United States.[2] Although only amebas of the genus *Naegleria* [3] have been cultured in such infections, sporadic reports suggest that *Acanthamoeba* or *Hartmannella* species also may produce central nervous system infections in humans,[4-9] especially in those with underlying debilitating illnesses.[7,9] Experimentally induced infections in mice with *Acanthamoeba* sp [10-13] are associated with a disease picture similar to that described in both natural human [4,6-9] and animal infections.[14-16] The capacity of *Acanthamoeba* sp to produce human infections is without question, for such organisms have been culturally proved to produce keratoconjunctivitis and uveitis, occasionally leading to destructive lesions of the cornea and subsequent blindness.[17,18]

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Host factors favoring the establishment or influencing the course of acanthamoebic infections are unknown, for organisms have been isolated from the nasopharynx of presumably healthy individuals.[19] The fact that humans may harbor free-living amebas as part of their "normal" upper respiratory flora suggests that these organisms may have the potential to cause disease, especially under circumstances of depressed host immunity. Corticosteroids are drugs frequently used to alter host immune responsiveness, and although certain diseases find their most dramatic expression in the "compromised host," information regarding the occurrence of protozoan diseases in "compromised" patients is scarce. For example, a recent review of the effect of corticosteroids on infections mentions only that protozoan diseases are "made worse by steroid therapy and the drugs are contraindicated except in rare circumstances."[20]

To determine if treatment with corticosteroids would alter host response to challenge with *Acanthamoeba* sp, laboratory mice were inoculated intranasally with *A castellanii* after pretreatment with methylprednisolone and were then studied histologically after death or on sacrifice to determine the frequency and extent of infection. Additional groups of mice were studied in a similar manner after pretreatment with tetracycline, a broad-spectrum antimicrobial agent reported to have adverse effects in several areas of host defense, including alteration in normal host flora.[21-24] Results of this study suggest that in experimental *Acanthamoeba* infections in mice treated with either corticosteroids or tetracycline, host immune responsiveness is altered in favor of the amebas, producing a disease similar to certain reported clinical experiences with infections presumably due to these organisms.

Materials and Methods

Seventy male, white Swiss mice (Dublin ICR strain, Flow Laboratories, Dublin, Va.) 2 to 3 months old and weighing an average of 22 g were divided randomly into seven groups of 10 each and designated to receive the following: Group I, tetracycline; Group II, tetracycline and amebas; Group III, methylprednisolone; Group IV, methylprednisolone and amebas; Group V, amebas; Group VI, amebas and saline; Group VII, saline. Drug therapy for Groups I through IV was begun on the first day of the experiment (designated Day 0) and continued throughout for a maximum of 35 days or until death. Each animal in Groups I and II received a daily intraperitoneal injection of 1.1 mg (50 mg/kg) tetracycline hydrochloride (Tetracyn, Reorig, New York, N.Y.) in 0.2 ml of 0.9% saline, in hopes of altering normal nasal and oropharyngeal flora. Mice in Groups III and IV received daily intraperitoneal injections of 0.22 mg (10 mg/kg) methylprednisolone (Solumedrol, Upjohn, Kalamazoo, Mich.) in 0.1 ml of 0.9% saline. This dose of methylprednisolone was equivalent in potency to the dose of cortisone acetate previously used in rodent experiments to produce hypercortisolism and experimental opportunistic *Pneumocystis carinii* infections.[25] Animals in Groups VI and VII served as saline controls, receiving daily intraperitoneal injections of 0.2 ml of 0.9% saline.

On Day 7, animals in Groups II, IV, V, and VI were inoculated intranasally with a 72-

hour axenic culture of *A castellanii*. The *A castellanii* was supplied by Dr. Shih L. Chang (Water Supply Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio), who isolated the organism from a private well near Cincinnati. Initial isolations were done on plain agar using *Enterobacter aerogenes* as the sole source of nutrients. Subsequently, the culture was cloned by isolating a single ameba and transferring it to Nelson's liquid axenic media containing kanamycin and penicillin. An inoculum of less than the LD_{50} was utilized, ie, 1.075×10^4 organisms were suspended in 0.1 ml of liquid axenic growth media and instilled intranasally after intraperitoneal administration of pentobarbital (0.06 mg/g body weight) for anesthesia. The inoculums employed contained the maximum concentration of amebas obtainable at an incubation of 30 C for 48 hours. Nelson's liquid axenic medium consisted of a proteose–peptone broth containing yeast and liver extract and fetal calf serum, supplemented with the antibiotics penicillin and kanamycin.[26] The virulent character of these amebas has been a stable trait despite repeated passages in artificial medium approximately every 2 weeks for the past 6 years.[27]

All animals were examined daily for clinical signs of pulmonary or nervous system disease. Those dying during the experiment were autopsied within 24 hours, while those surviving the full 35 days were killed. On death, the brain, lungs, heart, liver, and spleen were removed, placed in 10% buffered formalin solution (pH 7.4), and processed for histologic examination in a manner previously described.[13] Axenic and monaxenic (*E aerogenes*) cultures of heart, blood, and brain tissue from animals in Groups II and IV were obtained on sacrifice at 35 days, incubated on 2.5% agar for 7 days at 37 C, subcultured for an additional 7 days at 37 C, and examined on an inverted microscope for the presence of amebic trophozoites and cysts. All animals in Group VII (saline controls) were well at 35 days and were not studied histologically.

To study changes in oropharyngeal flora, throat swabs were done on mice in Group I immediately before therapy with tetracycline was begun and on the last day of therapy. The swabs were cultured on blood and eosin–methylene-blue (EMB) agar plates. Gram staining and morphology were recorded but no attempt was made to precisely speciate isolates.

Results

Tetracycline-Treated Animals (Groups I and II)

All animals tolerated tetracycline alone without obvious adverse effects (Table 1). However, mortality rates for Groups II (tetracycline and amebas) and V (amebas alone) were 60% (6 of 10 animals) and 10% (1 of 10

Table 1—Comparison of Mortality Rates in Groups of Mice Treated Intraperitoneally With Either Tetracycline or Methylprednisolone and Inoculated Intranasally With 1.075×10^4 *Acanthamoeba castellanii*

Regimen	No. of mice in group	No. of mice dying in each group (%)
Tetracycline (Group I)	10	0 (0)
Tetracycline and amebas (Group II)	10	6 (60)
Methylprednisolone (Group III)	10	1 (10)
Methylprednisolone and amebas (Group IV)	10	5 (50)
Amebas (Group V)	10	1 (10)
Saline and amebas (Group VI)	10	1 (10)
Saline (Group VII)	10	0 (0)

animals), respectively, a difference significant at $P = 0.0286$ by Fisher's Exact Test. One animal in Group VI died (Table 1), giving a mortality rate similar to those animals receiving amebas alone (Group V). Thus, the intraperitoneal injection per se did not account for the difference in mortality rates between Groups II and V. Of those animals in Group II that died spontaneously before completion of the experiment, the mean duration of survival was 14.2 days after inoculation of amebas or 21.2 days after starting drug therapy.

The pathologic findings in Groups I, II, and V are summarized in Table 2. Amebic pneumonitis and encephalitis occurred more frequently and were more extensive in Group II animals than in those in Group V. Although pathologic changes occurred in the lungs of all 9 animals in Group II, the changes associated with the presence of amebas appeared more severe than in the absence of organisms. The lungs of animals infected with amebas contained areas of interstitial and alveolar inflammation, usually with polymorphonuclear leukocytes (PMNs) and mono-

Table 2—Comparison of Pathologic Findings in the Lungs and Brains of Mice in Groups I, II, and V

Regimen	Mouse	Day of sacrifice or death (after drug therapy)	Lung	Brain
Tetracycline (Group I)	1-10	35	Normal	Normal*
Tetracycline and amebas (Group II)	1	7†	—	—
	2	13	Amebic pneumonitis‡	Normal
	3	17	Amebic pneumonitis	Amebic encephalitis§
	4	21	Amebic pneumonitis	Amebic encephalitis§
	5	22	Amebic pneumonitis	Amebic encephalitis§
	6	33	Amebic pneumonitis	Normal
	7-10	35	Focal interstitial pneumonitis; no amebas seen	Normal
Amebas (Group V)	1	18	Amebic pneumonitis	Amebic encephalitis§
	2-10	35	Focal interstitial pneumonitis; no amebas seen	Normal*

* Brains were not studied microscopically because all animals in these groups appeared clinically well; brains were grossly normal.

† Died on the first day after inoculation

‡ Unless otherwise specified, amebic pneumonitis is defined by the presence of an interstitial and/or alveolar infiltrate composed of polymorphonuclear leukocytes and mononuclear cells in the presence of typical trophozoites and/or cysts of *Acanthamoeba* sp.

§ Amebic trophozoites and/or cysts present

cytes (Figure 1). Of the 5 animals with amebic pneumonitis, 3 demonstrated ulcerated, necrotic bronchiolar epithelia. All 3 animals in Group II with amebic encephalitis had an associated amebic pneumonitis. In all 3, the encephalitic process was confined to the olfactory lobes of the brain and was characterized by marked microglial, histiocytic, and lymphocytic infiltration, particularly in the presence of trophozoites (Figure 2). Meningeal involvement was minimal or absent.

No pathologic changes were noted in the lungs of animals in Group I (tetracycline alone). However, pathologic changes were evident in the lungs of all 10 animals in Group V; the process in 9 of these animals was minimal, usually revealing scattered foci of either peribronchial monocytic cell infiltrations or simply collections of mononuclear cells. One animal in Group V died of amebic pneumonitis and encephalitis. The infection was similar histologically to those in Group II but was not as severe. The brains of the remaining 9 mice in Group V were normal.

Regardless to which group (ie, I through V) the animals belonged, whenever amebic pneumonitis and/or encephalitis occurred, both cysts and trophozoites of *A castellanii* could be identified easily. Histologic changes in livers, spleens, and hearts were minimal and in all instances were considered insignificant. No amebas were identified in these organs nor were any other pathogens. All amebic cultures of lung and brain tissues of surviving animals in Groups II and IV were negative.

Oropharyngeal cultures from animals in Group I showed a marked shift in flora after administration of tetracycline. Initially, cultures consisted principally of gram-negative bacilli, but following 7 days of tetracycline, 8 of 10 animals demonstrated a shift of flora to gram-positive cocci.

Methylprednisolone-Treated Animals (Groups III and IV)

All animals tolerated methylprednisolone without obvious adverse effects. The mortality rate (50%) for Group IV animals (methylprednisolone and amebas) was significantly higher than the 10% mortality rates for Group V (amebas alone) or for Group III (methylprednisolone alone) (P = 0.0704, Table 1). Of those mice in Group IV dying spontaneously, the mean duration of survival was 15.8 days after inoculation of amebas or 22.8 days after starting drug therapy.

Pathologically, the lungs of all animals in Group III were normal. One animal died of unknown causes on Day 21, was cannibalized, and, hence, was not available for study. However, the lungs of all animals in Group IV were abnormal (Table 3). Those dying with amebic pneumonitis had extensive pathologic changes similar to those in Group II (Figure 1). Only 1 animal in Group IV developed amebic encephalitis, the histopathology

Table 3—Comparison of the Pathologic Findings in the Lungs and Brains of Mice in Groups III and IV

Regimen	Mouse	Day of sacrifice or death (after drug therapy)	Lung	Brain
Methylprednisolone (Group III)	1*	21	—	—
	2–10	35	Normal	Normal†
Methylprednisolone and amebas (Group IV)	1	15	Amebic pneumonitis‡	Normal
	2	19	Amebic pneumonitis	Normal
	3	24	Amebic pneumonitis	Normal
	4	27	Amebic pneumonitis	Amebic encephalitis§
	5	28	Amebic pneumonitis	Normal
	6–10	35	Focal interstitial pneumonitis; no amebas seen	Normal

* Died of unknown causes (see text).

† Brains were not studied microscopically because all animals in this group appeared clinically well; brains were grossly normal.

‡ Unless otherwise specified amebic pneumonitis is defined by the presence of an interstitial and/or alveolar infiltrate composed of polymorphonuclear leukocytes and mononuclear cells in the presence of typical trophozoites and/or cysts of *Acanthamoeba* sp.

§ Amebic trophozoites and/or cysts present

of which was similar to that seen in animals in Group II (Figure 2). The brains of the other 9 animals in Group IV were normal.

Discussion

These experiments were designed to ascertain if free-living amebas of the genus *Acanthamoeba*, similar to those isolated from the nasopharyngeal passages of humans,[19] could behave as opportunistic pathogens. Mice pretreated with and maintained on a representative corticosteroid (methylprednisolone) or a representative broad-spectrum antibiotic (tetracycline) and then inoculated 7 days later intranasally with a specific inoculum of *A castellanii* appeared to suffer a significantly higher mortality rate than either untreated mice inoculated with the same numbers of organisms or control mice receiving either drug along.

Pathologic findings corroborated what was apparent clinically, ie, that amebic infection, either pneumonitis, encephalitis, or both, was the cause of every death occurring in Groups II (tetracycline and ameba), IV (methylprednisolone and amebas), and V (amebas). Histologically, the disease process was more severe in Groups II and IV, suggesting enhancement of virulence and/or pathogenicity by either tetracycline or methylprednisolone.

The precise mechanisms responsible for the increased mortality and severity of infections in Groups II and IV were not defined, but speculation in several cases seems justified by our results. Corticosteroids are known to enhance the progression and spread of virtually every infection, including those due to protozoans.[28-31] Therefore, mice receiving corticosteroid treatment might be expected to have a lower survival rate when challenged with *Acanthamoeba* (Group IV) than might untreated mice similarly challenged (Group V). Clinical and experimental data indicate that one defense system affected by steroids is neutrophilic responsiveness, especially neutrophilic migration into the site of infection.[32,33] In addition, phagocytosis and killing may be impaired,[34] as might delayed hypersensitivity.[35] Although the facet of an animal's host defense system most responsible for containing acanthamoebic infections is not known, invasion and infection by these organisms appears to be more frequent and severe in the presence of exogenously administered corticosteroids than in their absence.

Tetracycline, although ineffective against *Acanthamoeba*,[11,12] alters the normal oropharyngeal flora of animals, including humans. Such alterations in flora may upset the natural balance of microorganisms in this ecologic niche.[36] A striking shift in oropharyngeal flora was observed in most animals in Group I treated with tetracycline, as gram-positive cocci appeared to replace a natural flora consisting mainly of gram-negative bacilli. Free-living amebas readily use bacteria, particularly those of the family Enterobacteriaceae, as a source of nutrient.[3] In the absence of such gram-negative organisms, amebas may possibly turn to host tissues for sources of nutrients. Such species of amebas are known to accidentally infect tissue cultures and produce cytopathic effects.[3] In addition to an effect on bacterial flora, tetracycline has been shown to interfere with leukocyte migration,[23] with the capacity of human neutrophils to phagocytose yeast and bacteria [22] and with the alternate complement pathway (via C3 activation).[29] Any alteration in these various parameters of host defense also might impair the animal's ability to prevent the establishment and progression of acanthamoebic infections.

In humans, infections presumably due to *Acanthamoeba* often have appeared to occur in the setting of impaired host immune responsiveness, specifically in patients with alcoholism,[8,9] diabetes mellitus,[37] and Hodgkin's disease.[6] Because of the increasing number of patients who are "immunosuppressed" or "debilitated," it is surprising that human infections due to *Acanthamoeba* or related species have not been more frequent, especially since surveys of large, presumably "normal" population groups have revealed these organisms to be present at times in the upper respiratory passages.

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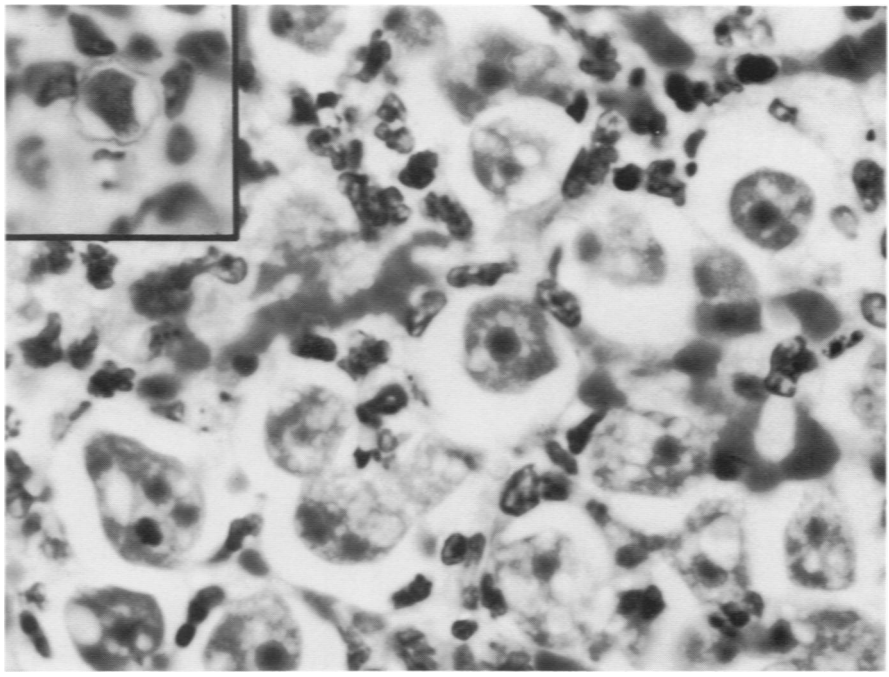
[*Illustrations follow*]



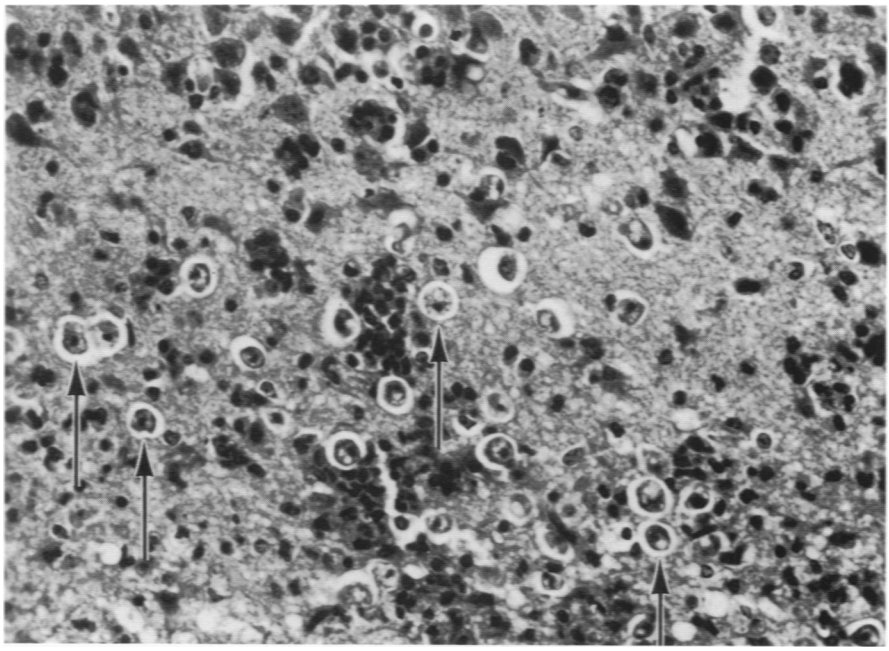


Figure 1—Photomicrograph of the lung of an animal from Group II, showing numerous amebic trophozoites and a moderate interstitial and alveolar inflammatory reaction composed mainly of polymorphonuclear leukocytes and monocytes. Note the characteristic spherical, dense karyosome surrounded by a nuclear halo in the amebic trophozoites. **Inset**—Cyst of *A castellanii* with characteristic double-walled envelope surrounding the polyhedral or stellate endocyst. (Hematoxylin & eosin, ×1000) **Figure 2**—Amebic encephalitis involving mainly the olfactory and frontal lobes in an animal from Group II. A marked microglial, histiocytic, and lymphocytic infiltrate is present with numerous amebic trophozoites (*arrows*), some of them without an accompanying inflammatory response. (Hematoxylin & eosin, ×375)

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