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Treatment of Acute Experimental Toxoplasmosis with Investigational Poloxamers

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Because of the limited chemotherapeutic approaches available to treat reactivated latent *Toxoplasma gondii* infection manifested as toxoplasmic encephalitis in AIDS patients, investigation of novel chemotherapeutic agents is warranted. Several poloxamers (nonionic block copolymers composed of a central hydrophobic chain of polyoxypropylene flanked by two hydrophilic chains of polyoxyethylene) were tested for their abilities to alter the course of acute infection with a highly virulent *T. gondii* in mice. The effect varied markedly with the length of the constituent chains of the copolymers. The most effective preparations were highly effective when administered after infection and afforded remarkable protection against 10 to 1,000 100% lethal doses of *T. gondii*. Protection was dose dependent, and multiple treatments were more effective than single treatment. These preliminary findings warrant additional studies to determine whether this novel form of antitoxoplasma chemotherapy may prove promising in the treatment or prevention of acute toxoplasmic encephalitis in humans.

The prevalence of *Toxoplasma gondii* infection in humans varies greatly around the world, ranging from low levels to >90% in the adult populations of France and El Salvador (18). In the United States, depending on location, 15 to 68% of the adults are infected with this obligate intracellular protozoan parasite. Once infected, these individuals harbor for life *T. gondii* cysts in their brains and skeletal and cardiac muscles. Although chronic toxoplasmosis is usually uneventful, reactivation of latent infection in the immunocompromised host (e.g., in transplant recipients upon immunosuppressive therapy or in patients with certain types of cancer or receiving immunosuppressive chemotherapeutic regimens) can be life threatening. Patients with AIDS who harbor a chronic *T. gondii* infection are also at risk owing to the presence of *T. gondii* cysts in the central nervous system (10, 13). Evidence that at least 30% of AIDS patients seropositive for *T. gondii* will develop life-threatening recrudescent toxoplasmic encephalitis is accumulating (15).

Current therapy for toxoplasmic encephalitis is a synergistic combination of pyrimethamine and sulfadiazine that inhibits the parasite's folate-metabolizing enzymes (1). Because the diagnosis of toxoplasmic encephalitis requires immediate primary treatment followed by a life-long maintenance regimen of chemotherapy, drug toxicity is a major factor. Pyrimethamine treatment is associated with bone marrow toxicity requiring supplemental administration of folinic acid (3, 17), and the sulfonamide component of the combination can also induce adverse drug reactions such as severe skin rashes, which seem to be exacerbated in AIDS patients (4, 12). Withdrawal of maintenance therapy is associated with a toxoplasmic encephalitis relapse rate of approximately 80% (14, 15). To complicate matters further, azidothymidine, the cornerstone of chemotherapy for AIDS, appears to antagonize the antitoxoplasma effects of pyrimethamine, as shown by in vitro and in vivo studies with mice (11).

Clearly, a more effective and less toxic chemotherapeutic regimen for toxoplasmic encephalitis is required. The present study employed a mouse model of acute *T. gondii* infection and involved preliminary experiments on a novel group of poloxamers, some of which have been shown to be effective adjuvants (7, 8, 22) and to have the potential for use in humans. Certain of these compounds exhibited strong antitoxoplasma effects in vitro in infected mouse macrophages (unpublished data) and, as shown here, afforded remarkable in vivo protection in mice acutely infected with a highly lethal strain of *T. gondii*. The unique physicochemical properties of some of these poloxamers are potentially ideal for use in synergistic combination with other antitoxoplasma drugs as therapeutic drug delivery vehicles.

MATERIALS AND METHODS

Mice. BALB/c mice bred in the animal care unit of the G. W. Long Hansen's Disease Center were employed in all experiments. Males and females were used, and all mice weighed 17 to 20 g.

Poloxamers. Investigational poloxamers, manufactured and provided by CytRx Corporation (Norcross, Ga.), are nonionic block copolymers consisting of a single chain of hydrophobic polyoxypropylene sandwiched between two hydrophilic chains of polyoxyethylene (16, 19) (Fig. 1). The study described here employed three native poloxamers that differ in size (average molecular weight [MW]) and relative hydrophilic-hydrophobic balance: CRL-8131 (MW, 4,000; 10% hydrophilic), CRL-8142 (MW, 5,100; 20% hydrophilic), and CRL-85221 (MW, 2,800; 10% hydrophilic). Unformulated poloxamers have negative thermal coefficients of solubility (i.e., they are more soluble at colder temperatures). The compounds were dissolved in cold (4°C) distilled water at 20 mg/ml, sterilized by autoclaving, aliquoted (1 ml), and stored at 4°C. In all experiments, the cold poloxamer aliquots were shaken vigorously, sonicated for 30 s in a cup horn sonicator (highest setting), diluted in cold phosphate-

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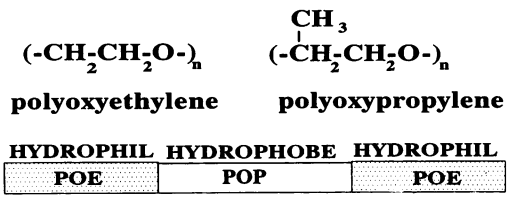

FIG. 1. Schematic representation of the block configuration of poloxamers employed. POE, polyoxyethylene; POP, polyoxypropylene.

buffered saline, and warmed to room temperature before injection.

Because the unformulated poloxamers tend to form emulsions as they warm (16, 19), a formulation of CRL-8131 that would stabilize the emulsion was developed. CRL-8131_{F68} consisted of the native CRL-8131 (20 mg/ml) formulated in 200-mg/ml Pluronic F-68, an 8,400-MW poloxamer consisting of 80% hydrophilic polyoxyethylene. Additional stable formulations of CRL-8131 and CRL-8142, prepared at 30 mg/ml in Tween 80–ethyl alcohol solutions, were prepared as shown in Table 1.

T. gondii. The RH strain of *T. gondii* is maintained by daily passage in BALB/c mice. For an experiment, tachyzoites were harvested in cold heparinized Hanks' balanced salt solution from the peritoneal cavities of passage mice injected 48 h previously with 10^7 RH strain *T. gondii* organisms and filtered through 4-μm Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) as previously described (21). After quantitation in a hemacytometer, tachyzoites were resuspended at the appropriate infecting dose at 4°C in Hanks' balanced salt solution.

Mouse infection. Mice were infected intraperitoneally (i.p.) with the appropriate number of freshly harvested and filtered *T. gondii* tachyzoites suspended in Hanks' balanced salt solution. Treatment and control groups consisted of 10 mice per group. Deaths were recorded daily for 21 days. Only rarely were deaths observed after the 16th day of infection.

Statistics. Statistical analysis was performed by Fisher's exact test.

RESULTS

Time to death after RH *T. gondii* infection. The RH strain of *T. gondii* used in these studies is highly virulent for BALB/c mice. As shown in Fig. 2, i.p. injections with >10 tachyzoites were lethal for all infected mice, and time to death correlated with dose. In our initial studies, an infecting dose of 10^4 organisms, representing at least 1,000 times the 100% lethal dose, was employed. In later studies, the dose was reduced to 10^3 or 10^2 in order to compare treatment doses, routes, and timing.

Aqueous preparations of native poloxamers. In our initial

TABLE 1. Soluble formulations of CRL-8131 and CRL-8142

Poloxamer[a]	Tween 80 (mg/ml)	95% EtOH[b] (%)
$CRL\text{-}8131_{F1}$	10	5
$CRL\text{-}8131_{F2}$	20	1
$CRL\text{-}8142_{F1}$	10	5
$CRL\text{-}8142_{F2}$	20	5

[a] Concentration, 30 mg/ml.
[b] EtOH, ethyl alcohol.

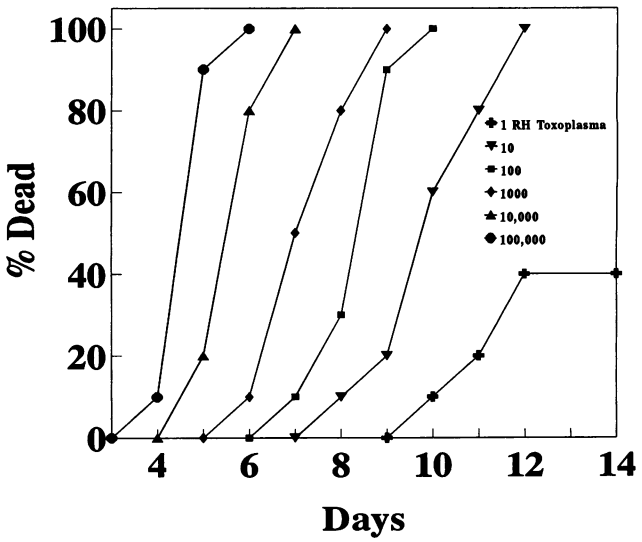

FIG. 2. Effects of various i.p. doses of RH strain *T. gondii* tachyzoites on time to death.

experiments, a number of different poloxamers, varying in MW and size of hydrophobe composition, were employed to treat mice infected with RH *T. gondii*. Unformulated CRL-85221 was toxic for mice at doses of 5 or 10 mg per mouse (590 and 245 mg/kg of body weight, respectively [data not shown]). However, lower doses of CRL-85221 injected i.p. within a few hours of infection afforded remarkable ($P < 0.01$) protection against this highly lethal dose of RH *T. gondii* (Fig. 3).

CRL-8131 and CRL-8142 have proved to be less toxic than CRL-85221 (data not shown) and were explored further for their antitoxoplasma effects. The efficacies of CRL-8131 and CRL-8142 were compared in the experiment whose results are shown in Fig. 4. In comparison with controls, both

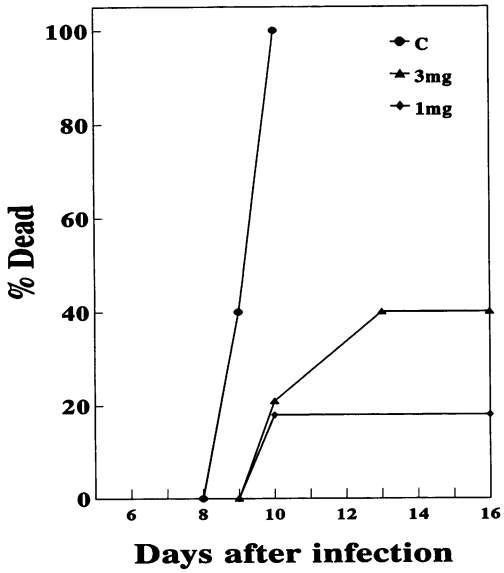

FIG. 3. Effects of treatment with CRL-85221 on infection with 10^4 tachyzoites of *T. gondii*. All treatments were administered at +3 h. C, control.

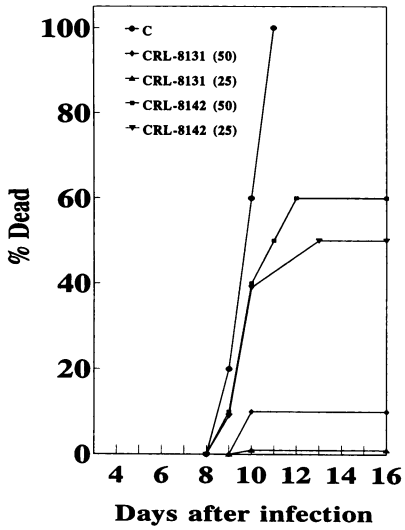

FIG. 4. Effects of treatment with CRL-8131 and CRL-8142 on infection with 10^3 *T. gondii* organisms. All treatments were administered at +1 h. Doses in milligrams per kilogram are given in parentheses. C, control.

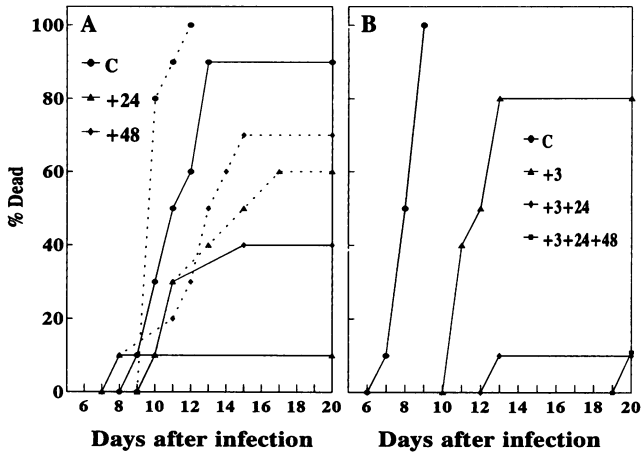

FIG. 6. Effects of timing of treatment with CRL-8131_{F68} on *T. gondii* infection (dose, 25 mg/kg). (A) Infection with 100 (—) or 1,000 (- - - -) *T. gondii* organisms. (B) Infection with 1,000 *T. gondii* organisms. C, control.

compounds afforded significant protection at 25 ($P < 0.0001$ and $P < 0.02$, respectively) and 50 mg/kg ($P < 0.0001$ and $P < 0.05$, respectively), but CRL-8131 was clearly superior to CRL-8142 ($P < 0.05$).

Special formulations of poloxamers. Because of the instability of emulsions of unformulated poloxamers at room and body temperatures, two compounds that afforded high and intermediate levels of protection against RH *T. gondii* infection (CRL-8131 and CRL-8142, respectively) were further explored with formulations that either stabilized the emulsion or rendered the poloxamers soluble at body temperature. As shown in Fig. 5A, treatment with different doses of CRL-8131_{F68} within 3 h of infection afforded protection against 10^4 *T. gondii* organisms in a dose-dependent manner (75 mg/kg, $P < 0.002$; 25 mg/kg, $P < 0.02$; 8 mg/kg, $P < 0.3$). Time elapsed between infection and treatment was critical,

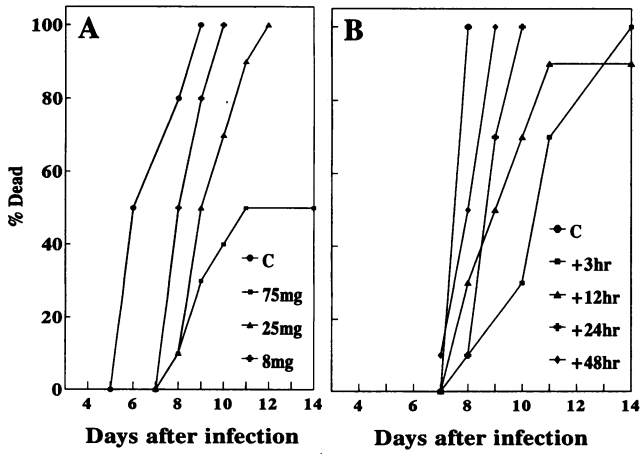

FIG. 5. Effects of dose (per kilogram) and timing of treatment with CRL-8131_{F68} on *T. gondii* infection. The infecting dose of *T. gondii* was 10^4 organisms. (A) All treatments were administered at +1 h. (B) Dose, 25 mg/kg. C, control.

as shown in Fig. 5B, with early treatment with 25 mg/kg (+3 and +12 h) resulting in a marked delay in time to death compared with controls and mice treated at +24 and +48 h ($P < 0.05$). In the experiment whose results are shown in Fig. 6, the infecting dose of *T. gondii* was reduced, and the effects of timing of treatment with CRL-8131_{F68} became more discernible. With an infecting dose of only 100 organisms, it was clear that treatment administered at 24 h provided markedly more protection than that at 48 h (Fig. 6A) ($P < 0.01$). With a higher challenge dose (1,000 organisms), protection was marked ($P < 0.05$), but the differences between the two treatment groups were indistinguishable. Similarly, multiple treatments were far superior to a single administration (Fig. 6B) of CRL-8131_{F68} ($P < 0.001$). The control carrier for CRL-8131_{F68}, Pluronic F-68, was not protective (data not shown).

In the experiments whose results are shown in Fig. 7 and 8, soluble formulations of both CRL-8131 and CRL-8142 were evaluated. Mice infected with 1,000 *T. gondii* organisms were treated i.p. at +1 h with one of several doses (25, 50, or 100 mg/kg) of CRL-8131_{F1} (Fig. 7A) or CRL-8142_{F1} (Fig. 7B). Untreated controls and groups of mice treated with the appropriate placebo formulation were included and afforded no protection. As was observed with the unformulated forms of CRL-8131 and CRL-8142, it was clear that CRL-8131_{F1} was also superior to CRL-8142_{F2}, although the latter did afford a significant intermediate level of protection. In Fig. 8, four soluble formulations are compared. CRL-8131_{F1} and CRL-8131_{F2} at 50 mg/ml both provided protection (Fig. 8) ($P < 0.0001$), but both CRL-8142_{F1} and CRL-8142_{F2} were poorly protective, as shown by only a modest delay in time to death.

Reinfection of survivors of poloxamer treatment. After the experiments reported here and confirmatory studies, 27 mice that survived because of poloxamer treatment were rechallenged with 10^4 RH tachyzoites in groups of 3 to 7 mice. These reinfection studies were usually carried out within 21 to 28 days of the original infection and in parallel with controls from new experiments. Without exception, all rechallenged mice died at the same rate as controls (data not shown).

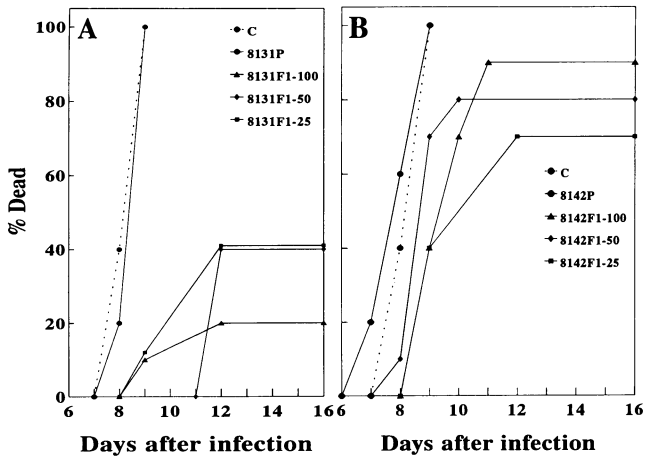

FIG. 7. Effects of doses (in milligrams per kilogram) of CRL-8131_{F1} and CRL-8142_{F1} on *T. gondii* infection. Experiments A and B were run in parallel. The infecting dose was 1,000 *T. gondii* organisms. (A) CRL-8131_{F1} placebo (8131P) was equivalent to 100 mg/kg. (B) CRL-8142_{F1} placebo (8142P) was equivalent to 100 mg/kg. C, control.

DISCUSSION

The present studies provide evidence for a novel form of antitoxoplasma chemotherapy that may prove promising in the treatment or prevention of acute toxoplasmic encephalitis in AIDS patients and other immunocompromised hosts. Protection was seen with mice previously infected with 10 to 1,000 times the 100% lethal dose of RH strain *T. gondii*. Although the RH strain of *T. gondii* is extremely virulent, the rapid course of infection in mice is limited to growth of the tachyzoite stage of the parasite. Cysts are not formed in this model. In our follow-up experiments, we showed that survivors of poloxamer therapy rechallenged with RH *T. gondii* were completely susceptible, suggesting that the initial treatment was sterilizing and did not induce a chronic infection that would protect mice from a secondary challenge (20). However, additional murine studies are required to evaluate whether poloxamers can also kill bradyzoites within cysts, especially those located across the blood-brain barrier in the central nervous system. Strains of *T. gondii* with pathogenesis in mice that resembles toxoplasmic encephalitis have been described and could be explored (23).

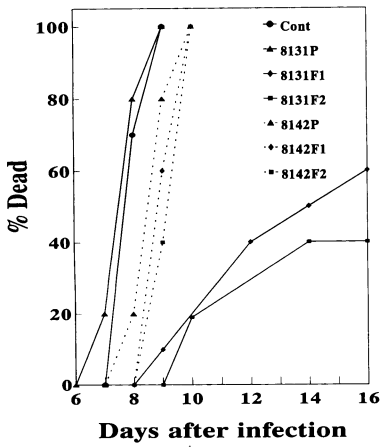

FIG. 8. Comparison of effects of treatment with CRL-8131_{F1}, CRL-8131_{F2}, CRL-8142_{F1}, and CRL-8142_{F2} on infection with 10^3 *T. gondii* organisms. A dose of 50 mg/kg (or its equivalent in the case of placebo [P]) was given 1 h after infection. Cont, control.

Poloxamers are thought to be among the least toxic of known nonionic surface-active agents. These poloxamers have been synthesized to achieve a high degree of homogeneity relative to MW and structural characteristics. Variation in the absolute and relative sizes of the constituent hydrophile and hydrophobe blocks of the poloxamers produces a diversity of surface physicochemical properties which have been correlated with a remarkable array of biologic activities including use as immunologic adjuvants and hemoreologic agents (16, 19). They are among the least toxic of known nonionic surface-active agents, and several have been used as inactive ingredients in pharmaceutical preparations (19). Certain of the large hydrophobic poloxamers are potent immunologic adjuvants (7, 8, 22), and it has been reported (6) that CRL-85221 is a potent upregulator of macrophage afferent effector function. These novel compounds suggest effectiveness with acceptable toxicity and have a propensity for accumulation in cells of the mononuclear phagocyte system, key cells in the host cell-mediated immune response to *T. gondii* (unpublished data).

It was clear from the present studies that CRL-8131 was superior to CRL-8142 as an antitoxoplasma agent, in both the unformulated form and the Tween 80–ethanol-soluble formulations. The present studies have not addressed the possible mechanism of action of these compounds, but results of in vitro studies suggest that the compounds are not directly lethal for *T. gondii* but act only on parasites that are intracellular in macrophages (unpublished data). Interestingly, in vitro in infected macrophages, CRL-8131 and CRL-8142 seem to be comparable in their antitoxoplasma effects at high doses, but the superior efficacy of CRL-8131 over CRL-8142 in vivo is mirrored in vitro when low doses or short treatment intervals are employed.

Obviously, additional work is required to better understand the mechanisms of action and take advantage of the special physicochemical and pharmacokinetic properties of these novel compounds that might allow them to be formulated and used for even greater antitoxoplasma efficacy (16, 19). The hydrophobic-hydrophilic block configurations of poloxamers may allow these compounds not only to be antitoxoplasma agents but to be used in a potent synergistic combination with other hydrophilic or hydrophobic drugs. The solubility characteristics of poloxamers are related to properties of the hydrophobic polyoxypropylene block which consists of ether-linked oxygen molecules sterically hidden by methyl groups. In the cold, the kinetic energy of water molecules is reduced, and they form weak hydrogen bonds with the oxygen in polyoxypropylene. This hydration of the hydrophobe promotes solubility at low temperatures. As the temperature is raised and the "cloud point" of the poloxamers is reached (+5°C for the unformulated poloxamers employed here), the increased kinetic energy of water breaks the hydrogen bonds and the poloxamer becomes insoluble and forms micelles (16). These micelles may be capable of capturing existing hydrophilic or hydrophobic antitoxoplasma drugs, and poloxamers could perhaps be used as a therapeutic drug delivery vehicle to present drugs at the site of *T. gondii* infection (i.e., infected macrophages). Poloxamers with potential for use as antitoxoplasma therapeutic agents have an acceptable toxicity profile, and because they are chemically defined polymers, they may be

designed to specifically capture a specified antimicrobial agent.

Finally, although we report here that certain poloxamers may be potent anti-*T. gondii* drugs against multiplying tachyzoites, in order for poloxamer chemotherapy to be effective in toxoplasmic encephalitis alone or in combination with other antitoxoplasma drugs the compound must be able to cross the blood-brain barrier and target the encysted bradyzoites as well as tachyzoites (2). Recent reports from Remington's group (5, 9) establish in vitro and in vivo experimental models for evaluating new therapeutic agents against *T. gondii* in brain cysts. Poloxamer chemotherapy must be assessed in these models, and additional pharmacokinetic data are required to custom-design poloxamers to be used alone or in combination with an existing anti-*T. gondii* drug.

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REFERENCES

1. **Eyles, D. E., and N. Coleman.** 1955. An evaluation of the curative effects of pyrimethamine and sulfadiazine alone and in combination, on experimental mouse toxoplasmosis. Antibiot. Chemother. (Washington, D.C.) **5**:529–539.
2. **Frenkel, J. K., and A. Escajadillo.** 1987. Cyst rupture as a pathogenic mechanism of toxoplasmic encephalitis. Am. J. Trop. Med. Hyg. **36**:517–522.
3. **Frenkel, J. K., and G. H. Hitchings.** 1957. Relative reversal by vitamins (*p*-aminobenzoic, folic and folinic acids) of the effects of sulfadiazine and pyrimethamine on Toxoplasma, mouse and man. Antibiot. Chemother. (Washington, D.C.) **7**:630–638.
4. **Haverkos, H. W.** 1987. Assessment of therapy for *Toxoplasma* encephalitis. Am. J. Med. **82**:907.
5. **Hofflin, J. M., and J. S. Remington.** 1986. Clindamycin in a murine model of toxoplasmic encephalitis. Antimicrob. Agents Chemother. **31**:492–496.
6. **Howerton, D. A., R. L. Hunter, H. K. Ziegler, and I. J. Check.** 1990. Induction of macrophage Ia expression in vivo by a synthetic block copolymer, L81. J. Immunol. **144**:178–184.
7. **Hunter, R. L., and B. Bennett.** 1984. The adjuvant activity of non-ionic block polymer surfactants. II. Antibody and inflammation related to the structure of triblock and octblock copolymers. J. Immunol. **133**:3167.
8. **Hunter, R. L., F. Strickland, and F. Kezdy.** 1981. The adjuvant activity of non-ionic block surfactants. I. The role of hydrophile lipophile balance. J. Immunol. **127**:1244–1251.
9. **Huskinson-Mark, J., F. G. Araujo, and J. S. Remington.** 1991. Evaluation of the effect of drugs on the cyst form of *Toxoplasma gondii*. J. Infect. Dis. **164**:170–177.
10. **Israelski, D. M., and J. S. Remington.** 1988. Toxoplasmic encephalitis in patients with AIDS. Infect. Dis. Clin. N. Am. **2**:429–445.
11. **Israelski, D. M., C. Tom, and J. S. Remington.** 1989. Zidovudine antagonizes the action of pyrimethamine in experimental infection with *Toxoplasma gondii*. Antimicrob. Agents Chemother. **33**:30–34.
12. **Leport, C., F. Raffi, S. Matheron, et al.** Treatment of central nervous system toxoplasmosis with pyrimethamine-sulfadiazine combination in 35 AIDS patients: efficacy of long-term continuous therapy. Am. J. Med., in press.
13. **Luft, B. J., R. G. Brooks, F. K. Conley, et al.** 1984. Toxoplasmic encephalitis in patients with AIDS. JAMA **252**:913.
14. **Luft, B. J., and J. S. Remington.** 1985. Toxoplasmosis of the central nervous system. Curr. Top. Infect. Dis. **6**:315–356.
15. **Luft, B. J., and J. S. Remington.** 1988. Toxoplasmic encephalitis. J. Infect. Dis. **157**:1–6.
16. **Lundsted, L. G., and I. R. Schmolka.** 1976. The synthesis and properties of block copolymer polyol surfactants, p. 1–103. *In* R. J. Ceresa (ed.), Block and graft copolymerization, vol. 2. John Wiley & Sons, London.
17. **Myatt, A. V., G. R. Coatney, T. Hernandez, et al.** 1958. A further study of the toxicity of pyrimethamine (Darapram) in man. Am. J. Trop. Med. Hyg. **2**:1000–1001.
18. **Remington, J. S., and J. L. Krahenbuhl.** 1981. Immunology of Toxoplasma infections, p. 327–371. *In* A. J. Nahmias and R. J. O'Reilly (ed.), Immunology of human infections. Plenum Press, New York.
19. **Schmolka, I. R.** 1991. Poloxamers in the pharmaceutical industry, p. 190–208. *In* P. J. Tarcha (ed.), Polymers for controlled drug delivery. CRC Press, Inc., Boca Raton, Fla.
20. **Schwartzberg, J. E., J. L. Krahenbuhl, and J. S. Remington.** 1975. Dichotomy between macrophage activation and degree of protection against *Listeria monocytogenes* and *Toxoplasma gondii* in mice stimulated with *Corynebacterium parvum*. Infect. Immun. **12**:1037–1043.
21. **Sibley, L. D., and J. L. Krahenbuhl.** 1988. Defective activation of granuloma macrophages from *Mycobacterium leprae*-infected nude mice. J. Leukocyte Biol. **43**:60–66.
22. **Snippe, H., M. J. De Reuver, F. Strickland, J. M. Willers, and R. L. Hunter.** 1981. Adjuvant effect of non-ionic block surfactants in humoral and cellular immunity. Int. Arch. Allergy Appl. Immunol. **65**:390–396.
23. **Suzuki, Y., F. K. Conley, and J. S. Remington.** 1989. Differences in virulence and development of encephalitis during chronic infection vary with the strain of *Toxoplasma gondii*. J. Infect. Dis. **159**:790–794.