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Testing effects of Glatiramer Acetate and Fingolimod in an infectious model of CNS immune surveillance

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Abstract

Immune surveillance of the CNS is critical for preventing infections, however there is no accepted experimental model to assess the risk of infection when utilizing disease-modifying agents. We tested two approved agents for patients with multiple sclerosis (MS), glatiramer acetate and fingolimod, in an experimental model of CNS immune surveillance. C57BL/6 mice were infected with the ME49 strain of the neuroinvasive parasite *Toxoplasma gondii* (*T. gondii*) and then treated with GA and fingolimod. Neither treatment affected host survival, however differences were observed in parasite load and in leukocyte numbers in the brains of infected animals. Here we demonstrate that this model could be a useful tool for analyzing immune surveillance.

1. Introduction

Currently there is no universally accepted experimental model to assess CNS immune surveillance. The lack of such a model is a substantial shortfall in drug development as it allows for the approval of pharmacological agents with unknown effects on CNS infections and neoplastic growth.

We recently developed and tested an experimental model of CNS immune surveillance using the protozoan *Toxoplasma gondii* (*T. gondii*). *T. gondii* is a highly prevalent obligate intracellular pathogen. Like other mouse strains, the C57BL/6 mouse strain is susceptible to the ME49 strain of *T. gondii*. Infection of C57BL/6 mice with *T. gondii* strain ME49 is

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characterized by a rapid expansion of tachyzoites in the host, which differentiate into bradyzoites and form tissue cysts predominantly in the CNS. The development of tissue cysts defines the chronic stage of the infection. Depletion of either CD4⁺ or CD8⁺ T-cell results in reactivation of the parasite, and is associated with rapid mortality of infected animals in this mouse strain ¹. The host survival of C57BL/6 mice following an infection with the ME49 strain of *T. gondii* is well established ¹⁻³.

We infected C57BL/6 mice with the ME49 strain of *T. gondii* and further treated with two pharmacological agents, glatiramer acetate (GA) and fingolimod. GA is approved for the treatment of patients with relapsing-remitting multiple sclerosis (RRMS) and in patients who experience an initial clinical relapse with MRI findings that are compatible with a diagnosis of MS. GA reduces the frequency of disease relapses ⁴, and may decrease disease progression. The mechanism of action of GA is still not fully understood. However, immune responses under GA are shifted from a pro-inflammatory Th1 cytokine profile to an anti-inflammatory Th2 cytokine profile ^{5, 6}. GA also inhibits the activation and proliferation of encephalitogenic T cells, and the modulation of antigen presenting cells ^{7, 8}. GA may also have neuroprotective properties through the induction of brain-derived neurotrophic factor (BDNF) by T cells and other cells within the CNS ^{9, 10}. Currently, GA is the only therapeutic intervention that does not require any laboratory testing while patients are being treated ¹¹. Fingolimod is an agent with a relatively novel mode of action that is currently approved by the FDA to treat patients with RRMS ¹². In clinical studies fingolimod was efficient in reducing the rates of relapse and MRI evidence of inflammatory lesions ¹³. Fingolimod binds to sphingosine-1-phosphate (S1P) receptors on the surface of leukocytes causing the receptors to be internalized thereby inhibiting migration. Consequently, these leukocytes are unable to egress from lymphatic tissues ¹⁴. Neoplastic growths and possible risk of progressive multifocal leukoencephalopathy (PML) has been reported by the FDA ¹⁵.

Here, we assessed the feasibility of a *T. gondii* infectious model of CNS immune surveillance to further test these pharmacological agents and their potential role on leukocyte homeostasis and immune surveillance of the CNS.

2. Methods

2.1. Infection of mice with *T. gondii*

Four groups of five eight-week-old female C57BL/6 mice were infected intraperitoneally with 20 cysts per mouse of the avirulent type II *T. gondii* strain ME49. Recipients were left untreated, treated daily with subcutaneous injections of 150 µg of GA, oral fingolimod (0.3 mg/kg/day), or both for another 50 days. Mice were monitored for survival.

2.2. FACS analysis

After 30 days post-infection, or at time of death, mice were perfused via the left ventricle with cold PBS and brains were harvested. Brains were pressed through a 70-µm nylon mesh cell strainer. Brain cells from all mice in each experimental group were pooled and processed as previously described ^{16, 17}. In brief, brain cells were washed twice in 37% Percoll and CNS mononuclear cells were isolated by centrifugation at 2118 x g for 15 min at 22°C over a 30/70% Percoll gradient. The interphase cells were collected, washed with 0.5%

BSA/PBS, resuspended in complete RPMI 1640 and counted. The following monoclonal antibodies were used: Anti-CD3-AF700 (17A2), anti-CD45-PE-Cy7 (30-F11), anti-CD4-APC (RM4-5), anti-CD8-Pacific Orange (MCD0830), anti-Gr-1-APC-Cy-7 (RB6-8C5), and anti-CD11c-Pacific Blue (N418). Cells were resuspended in staining buffer (4% FCS and 0.1% sodium azide in PBS) and Fc receptors blocked with anti-CD16/32 (BD Biosciences) for 15 minutes at 4°C before staining with mAbs for 30 min at 4°C. Cells were then washed, resuspended in staining buffer, and fixed in 1% paraformaldehyde. Up to 500,000 events were acquired on a FACS Aria (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

2.3. Quantification of parasite cysts

To quantify *T. gondii* cysts in the brain, we removed brains after 30 days of infection or at time of death and parasite load was determined by the number of cysts in the brain.¹⁸

2.4 Statistical analysis

Correlations between continuous and categorical variables were assessed using the Mann-Whitney U-test. The means of two normally distributed samples were compared by Student t-test. All other statistical comparisons between groups were examined using one-way multiple range analysis of variance (ANOVA) for multiple comparisons or Student-Newman-Keuls multiple comparison test. A P-value <0.05 was considered significant.

3. Results

Neither treatment group had a significant adverse effect on host survival (Figure 1a). Compared to untreated mice, the number of parasites in the brains of GA-treated animals was not increased (Figure 1b). In contrast, the number of parasite cysts more than doubled in fingolimod-treated animals. Interestingly, addition of GA to fingolimod reduced the number of brain cysts back to baseline (Figure 1b). The addition of GA to fingolimod reverses a minor decrease of CNS leukocyte numbers seen with fingolimod monotherapy (Figure 2). The combination also appears to result in an expansion of some myeloid subsets (Figure 2).

4. Discussion

Infection of C57BL/6 mice with the ME49 strain of *T. gondii* is a valid experimental model to test CNS immune surveillance for MS pharmacotherapies. C57BL/6 mice are susceptible to *T. gondii* infection and develop encephalitis. Innate and adaptive immune responses against the pathogen are well characterized in this mouse strain, and the survival rate is established. Parasite cysts accumulate within the brain, and there is an association between parasite numbers and clinical outcomes. Thus, the effect on any pharmacological intervention on survival, parasite numbers, and the composition on immune cells can be assessed.

In preliminary experiments, we confirmed that GA is a safe agent that does not alter survival of experimental animals, and that is not associated with an expansion of parasite cysts in the brain. Fingolimod, which was designed to sequester T cells into lymph nodes and away from

the CNS, also did not adversely affect host survival. However, fingolimod led to an increase in CNS parasite load, which was reversed through the combination of GA and fingolimod.

These results are perhaps not surprising, given that GA has an outstanding track record with regard to safety, and as stated above, is currently the only agent that does not require any safety monitoring. Also, GA was shown to expand the CD8⁺ T cell compartment^{19, 20}, which is critical in host defense against *T. gondii* infection^{1, 2}, and which may explain a reversal of high parasite counts in the brain under fingolimod when GA was added.

In the future, more extensive experiments should be included to further assess this model. Given our knowledge on the role of IFN γ and IL-12 in host defense against *T. gondii*, one should evaluate the expression of these cytokines in the periphery and CNS. Also, given the effects of agents that are currently approved or in clinical development on different leukocyte subsets, a more thorough phenotyping of these cells in different compartments would provide useful information.

Any experimental model that tests CNS immune surveillance will have to take several aspects into consideration, including pharmacodynamics and pharmacokinetic differences between species. Thus, different dosages and different treatment paradigms should be employed.

In summary, we believe that a CNS immune surveillance experimental model that involves infection of C57BL/6 mice with the ME49 strain of *T. gondii* provides meaningful data on the composition and homeostasis of immune-competent cells. Results derived from this model would provide insight into potential adverse events of monotherapies and combination therapies in MS and related neurological disorders.

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Reference List

1. Gazzinelli R, Xu Y, Hieny S, Cheever A, Sher A. Simultaneous depletion of CD4⁺ and CD8⁺ T lymphocytes is required to reactivate chronic infection with *Toxoplasma gondii*. *J Immunol*. 1992; 149:175–180. [PubMed: 1351500]
2. Gazzinelli RT, Hakim FT, Hieny S, Shearer GM, Sher A. Synergistic role of CD4⁺ and CD8⁺ T lymphocytes in IFN- γ production and protective immunity induced by an attenuated *Toxoplasma gondii* vaccine. *J Immunol*. 1991; 146:286–292. [PubMed: 1670604]
3. Yarovsky F, Kanzler H, Hieny S, Coffman RL, Sher A. Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4⁺ T cell response. *Immunity*. 2006; 25:655–664. [PubMed: 17000122]
4. La Mantia L, Munari LM, Lovati R. Glatiramer acetate for multiple sclerosis. *Cochrane Database Syst Rev*. 2010:CD004678. [PubMed: 20464733]
5. Duda PW, Schmied MC, Cook SL, Krieger JJ, Hafler DA. Glatiramer acetate (Copaxone) induces degenerate, Th2-polarized immune responses in patients with multiple sclerosis. *J Clin Invest*. 2000; 105:967–976.

6. Neuhaus O, Farina C, Yassouridis A, et al. Multiple sclerosis: comparison of copolymer-1- reactive T cell lines from treated and untreated subjects reveals cytokine shift from T helper 1 to T helper 2 cells. *ProcNatlAcadSciUSA*. 2000; 97:7452–7457.
7. Weber MS, Starck M, Wagenpfeil S, Meinl E, Hohlfeld R, Farina C. Multiple sclerosis: glatiramer acetate inhibits monocyte reactivity in vitro and in vivo. *Brain*. 2004; 127:1370–1378. [PubMed: 15090474]
8. Weber MS, Prod'homme T, Youssef S, et al. Type II monocytes modulate T cell-mediated central nervous system autoimmune disease. *NatMed*. 2007; 13:935–943.
9. Ziemssen T, Kumpfel T, Klinkert WE, Neuhaus O, Hohlfeld R. Glatiramer acetate-specific T-helper 1- and 2-type cell lines produce BDNF: implications for multiple sclerosis therapy. Brain-derived neurotrophic factor. *Brain*. 2002; 125:2381–2391. [PubMed: 12390966]
10. Aharoni R, Eilam R, Domev H, Labunskay G, Sela M, Arnon R. The immunomodulator glatiramer acetate augments the expression of neurotrophic factors in brains of experimental autoimmune encephalomyelitis mice. *Proceedings of the National Academy of Sciences of the United States of America*. 2005; 102:19045–19050. [PubMed: 16365293]
11. Rommer PS, Zettl UK, Kieseier B, et al. Requirement for safety monitoring for approved MS therapies - An overview. *Clinical and experimental immunology*. 2013
12. http://www.accessdata.fda.gov/drugsatfda_docs/label/2010/022527s000lbl.pdf. Available.
13. Brinkmann V, Billich A, Baumrucker T, et al. Fingolimod (FTY720): discovery and development of an oral drug to treat multiple sclerosis. *Nature reviews Drug discovery*. 2010; 9:883–897.
14. Brinkmann V, Davis MD, Heise CE, et al. The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *JBiolChem*. 2002; 277:21453–21457.
15. <http://www.fda.gov/Drugs/DrugSafety/ucm366529.htm>. Available.
16. Cravens PD, Hussain RZ, Zacharias TE, et al. Lymph node-derived donor encephalitogenic CD4+ T cells in C57BL/6 mice adoptive transfer experimental autoimmune encephalomyelitis highly express GM-CSF and T-bet. *JNeuroinflammation*. 2011; 8:73. [PubMed: 21702922]
17. Cravens PD, Kieseier BC, Hussain R, et al. The neonatal CNS is not conducive for encephalitogenic Th1 T cells and B cells during experimental autoimmune encephalomyelitis. *Journal of neuroinflammation*. 2013; 10:67. [PubMed: 23705890]
18. Yarovsky F, Zhang D, Andersen JF, et al. TLR11 activation of dendritic cells by a protozoan profilin-like protein. *Science*. 2005; 308:1626–1629. [PubMed: 15860593]
19. Karandikar NJ, Crawford MP, Yan X, et al. Glatiramer acetate (Copaxone) therapy induces CD8(+) T cell responses in patients with multiple sclerosis. *JClinInvest*. 2002; 109:641–649.
20. Racke MK, Lovett-Racke AE, Karandikar NJ. The mechanism of action of glatiramer acetate treatment in multiple sclerosis. *Neurology*. 2010; 74(Suppl 1):S25–S30. [PubMed: 20038760]

Figure 1.

A. Treatment with GA or fingolimod, or both had no adverse effect on host survival. GA = Glatiramer acetate. Mice were infected with 20 cysts per mouse of ME49 and treated with 150 μ g of GA s.c., oral fingolimod (0.3 mg/kg/day), or both and monitored. B. Compared to untreated mice, the number of parasites in the brains of Glatiramer acetate (GA)-treated animals was not increased after 30 days. In contrast, the number of parasite cysts more than doubled in fingolimod-treated animals. Interestingly, addition of GA to fingolimod reduced the number of brain cysts back to baseline.

Figure 2.

Treatment with a combination of GA and fingolimod reverses a decrease of CNS leukocyte numbers seen with fingolimod monotherapy and might also result in expansion of myeloid subsets. Flow cytometry was utilized to examine the percentage of CD45⁺ cells, T cells, dendritic cells, and monocytes from infected brains.