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Short Communication

The combination of valacyclovir with an anti-TNF alpha antibody increases survival rate compared to antiviral therapy alone in a murine model of herpes simplex virus encephalitis

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ABSTRACT

The added benefit of combining valacyclovir (VACV), an antiviral agent, with etanercept (ETA), an anti-tumor necrosis factor alpha (TNF-α) antibody, for the treatment of herpes simplex virus type 1 (HSV-1) encephalitis (HSE) was evaluated in a mouse model. BALB/c mice were infected intranasally with 1.85×10^4 plaque forming units of HSV-1. Groups of mice received a single intraperitoneal injection of vehicle or ETA (400 μg/mouse) on day 3 post-infection combined or not with VACV (1 mg/ml of drinking water) from days 3 to 21 post-infection. On day 5 post-infection, groups of mice were sacrificed for determination of viral DNA load, detection of ETA in brain homogenates and for *in situ* hybridization. The survival rate of mice was significantly increased when VACV was administered in combination with ETA (38.5% for VACV *vs* 78.6% for combined treatment; $P = 0.04$) although VACV or ETA alone had no significant effect compared to the vehicle. The benefit of combined therapy was still present when treatment was delayed until day 4 post-infection. The viral DNA load was significantly reduced in mice treated with VACV alone ($P < 0.01$) or combined with ETA ($P < 0.05$) compared to the uninfected group whereas ETA alone had no effect. These results reinforce the notion that both virus-induced and immune-related mechanisms participate in the pathogenesis of HSE and suggest that potent antiviral agent could be combined with immune-based therapy, such as a TNF-α inhibitor, to improve prognosis of HSE.

Herpes simplex virus type 1 (HSV-1) is the most frequent cause of sporadic and potentially fatal viral encephalitis in Western countries (Tyler, 2004). Acyclovir (ACV), an analogue of guanosine, is the antiviral of choice for the treatment of HSV encephalitis (HSE). Despite intravenous administration of ACV, the mortality attributable to HSE is still greater than 28% and almost 60% of surviving patients develop neurological sequelae (Whitley, 2006). The pathogenesis of HSE is not well understood but growing lines of evidence suggest that direct virus-mediated and indirect immune-mediated mechanisms contribute to the damages occurring in the central nervous system (CNS).

Microglial cells are the first line of immune defense in the CNS parenchyma (Rivest, 2009). The progressive activation of resident microglial cells leads to the production of tumor necrosis factor alpha (TNF-α), which acts in autocrine and paracrine manners to activate the immune cells across the brain parenchyma (Nadeau and Rivest, 1999). Using knock-out mice, we demonstrated the protective role of TNF-α and interleukin 1-beta (IL-1β) during the early phase of HSE (Sergerie et al., 2007b). TNF-α also produced a significant benefit when administered up to 8 h after the infection in a lethal mouse model of HSE (Rossol-Voth et al., 1991). However, an over-expression of TNF-α constitutively produced by a recombinant HSV-1 strain inoculated in the eye had deleterious effects due to increased inflammation and viral replication (Fields et al., 2008). High levels of TNF-α were also shown to induce blood–brain barrier (BBB) disruption (Candelario-Jalil et al., 2007).

Etanercept (ETA; Enbrel®), which is mainly indicated for the treatment of different forms of arthritis and psoriasis, is a soluble dimeric recombinant fusion protein that consists of the extracellular ligand-binding domain of the p75 receptor of TNF linked to the Fc portion of human IgG_1. ETA binds to two TNF molecules and prevents the interaction of both TNF-α and TNF-β with cell surface receptors to reduce the biologic effect of excess TNF (Tracey et al., 2008). *In vitro* and *in vivo* studies indicated that ETA also blocks TNF-α of murine origin (Lories et al., 2007). We have previously reported that treatment of mice with dexamethasone on day 3 following intranasal infection with HSV-1 increased survival rate by reducing the expression of several proinflammatory cytokines including TNF-α, CCL2 and CXCL10 in brain homogenates (Sergerie

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et al., 2007a). Therefore, immunomodulatory strategies aimed at reducing the activation of the immune response induced by TNF-α could be beneficial to control late onset detrimental inflammation of the CNS that develops during HSE. In addition, such approach could be combined with antiviral agents to improve treatment against this disease. In this study, the added benefit of ETA combined with valacyclovir (VACV), a prodrug of ACV, was evaluated in a murine model of HSE.

Four-week-old BALB/c mice (Charles River, St-Constant, Quebec, Canada) were infected intranasally with 1.85×10^4 plaque forming units (PFU) of a neurovirulent HSV-1 clinical strain (H25; (Sergerie et al., 2007b)). Four groups of mice received a single intraperitoneal injection of the vehicle (0.9% benzyl alcohol) or ETA (Amgen Canada, Mississauga, Ontario, Canada; 400 μg/mouse) on day 3 post-infection combined or not with VACV (1 mg/ml *ad libitum* in drinking water) from days 3 to 21 post-infection. Mice were monitored during 21 days for occurrence of HSE-related signs, ruffled fur and ocular swelling as well as for mortality. Animals were sacrificed when a ⩾20% weight loss or two obvious neurological signs (shaking movement, hind limb paralysis, prostration and convulsion) were observed. Fig. 1A shows that the survival rate of mice treated with VACV alone (38.5%) was greater than that of the vehicle group (8.3%) but the difference was not statistically significant. ETA alone slightly delayed the onset of HSE-related signs and mortality compared to the vehicle group. Interestingly, treatment of mice with VACV combined with ETA delayed the onset of HSE-related signs and significantly increased survival rate compared to the three other groups (78.6% *vs* 8.3% ($P < 0.0001$), 14.3% ($P < 0.01$) and 38.5% ($P = 0.04$) for vehicle, ETA and VACV groups, respectively). The weight loss or gain for the different groups of mice over the study period is also shown in Fig. 1B.

Animals were sacrificed on day 5 post-infection by intracardiac perfusion with 0.9% cold saline to determine cerebral viral load. Real-time polymerase chain reaction was performed using DNA extracted from brain homogenates as previously described (Boivin et al., 2006). Fig. 1C shows that the brain viral DNA load was significantly reduced by about 0.7 log in mice treated with VACV alone ($P < 0.01$) or with VACV combined with ETA ($P < 0.05$) compared to the vehicle group whereas ETA alone had no significant effect on this parameter.

TNF-α signaling stimulates microglia to produce CCL2 (chemokine receptor ligand 2) which mediates monocytes/macrophages recruitment in the CNS (D'Mello et al., 2009). Moreover, TLR2 (Toll-like receptor 2) is a reliable marker of activation of resident microglia and infiltrating monocytes/macrophages (Nguyen et al., 2002). To further characterize the effect of the combination ETA/VACV on viral replication and inflammatory response, the expression of HSV thymidine kinase (TK), CCL2 and TLR2 was evaluated in brain slices of the different groups of mice by *in situ* hybridization. Animals were sacrificed on day 5 post-infection by intracardiac perfusion with 0.9% saline followed by 4% paraformaldehyde in 0.1 M borax buffer (pH 9.5) at 4 °C. Brains were post-fixed in a 4% paraformaldehyde solution for 20–24 h and then placed in 10% sucrose in 4% paraformaldehyde buffer at 4 °C for several days. Brains were cut on dry ice and rostrocaudal coronal sections (25-μm thick) were collected in a cold cryoprotectant solution (0.05 M phosphate buffer at pH 7.3 containing 30% ethylene glycol and 20% glycerol). The three transcripts were detected on brain slices using specific [^{35}S]-labeled cRNA probes as previously described (Boivin G. et al., 2002; Boivin N. et al., 2008). The micrographs which correspond to the pons region and part of the cerebellum (Dorr et al., 2007) are shown in Fig. 2. In both vehicle and ETA groups, transcripts for viral TK were found in the cerebral cortex, the pons and medulla regions and the olfactory bulb whereas they were barely detectable and mainly restricted to the brainstem in the groups treated with VACV alone or combined with ETA. Transcript levels for CCL2 were found in the same regions as viral TK transcripts for the vehicle and ETA groups. Compared to the vehicle group, hybridization level was slightly reduced in the ETA group, more potently suppressed in the VACV group and almost absent in mice treated with VACV combined with ETA. Transcript levels for TLR2 were detected in all brain regions in the vehicle group. Hybridization signal was slightly reduced and was confined to the pons and medulla in the ETA group whereas it was barely detectable in mice treated with VACV alone or combined with ETA.

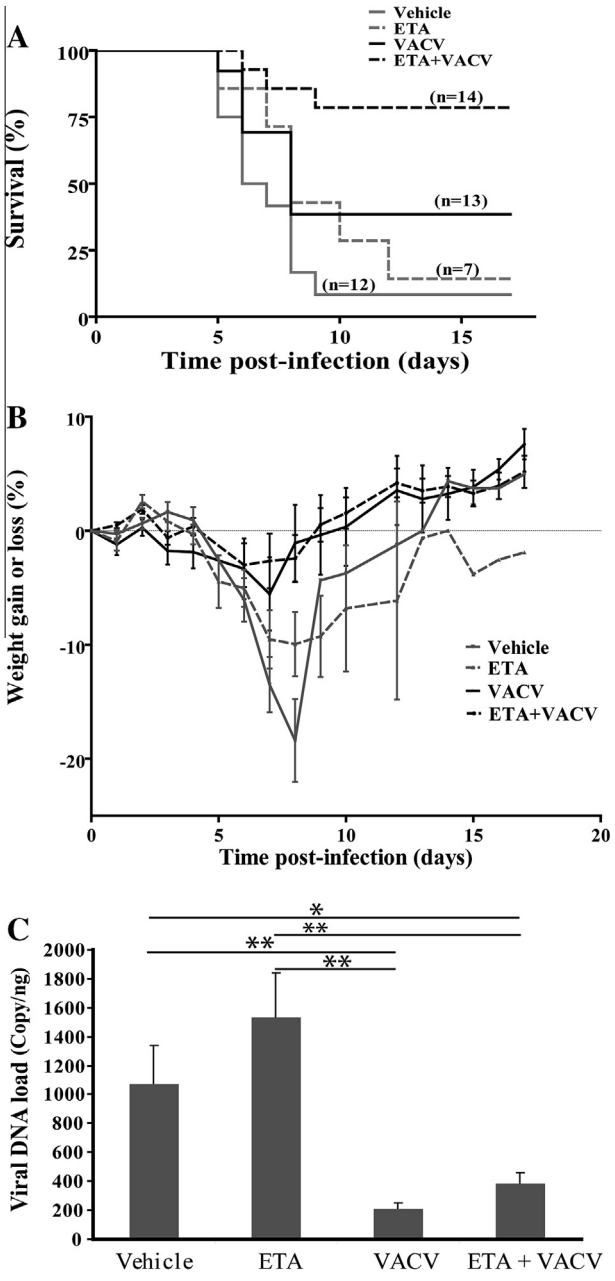

Fig. 1. Effect of valacyclovir (VACV) combined or not with etanercept (ETA) on survival rates (Panel A), percentage of body weight loss or gain (Panel B) and viral DNA load in brain homogenates (Panel C) of HSV-1-infected mice. Four groups of infected mice received a single intraperitoneal injection of vehicle (0.9% benzyl alcohol) or ETA (400 μg/mouse) on day 3 post-infection combined or not with VACV (1 mg/ml *ad libitum* in drinking water) from days 3 to 21 post-infection. Mice were monitored once daily for 21 days. Differences in group survival rates were compared using a log-rank (Mantel–Cox) test. Viral DNA loads were analyzed by a one-way analysis of variance (ANOVA) with Newman–Keuls post-test. All statistical analyses were carried out using GraphPad Prism version 5.00 (GraphPad Software, San Diego, CA). $^{*}P < 0.05$ and $^{**}P < 0.01$.

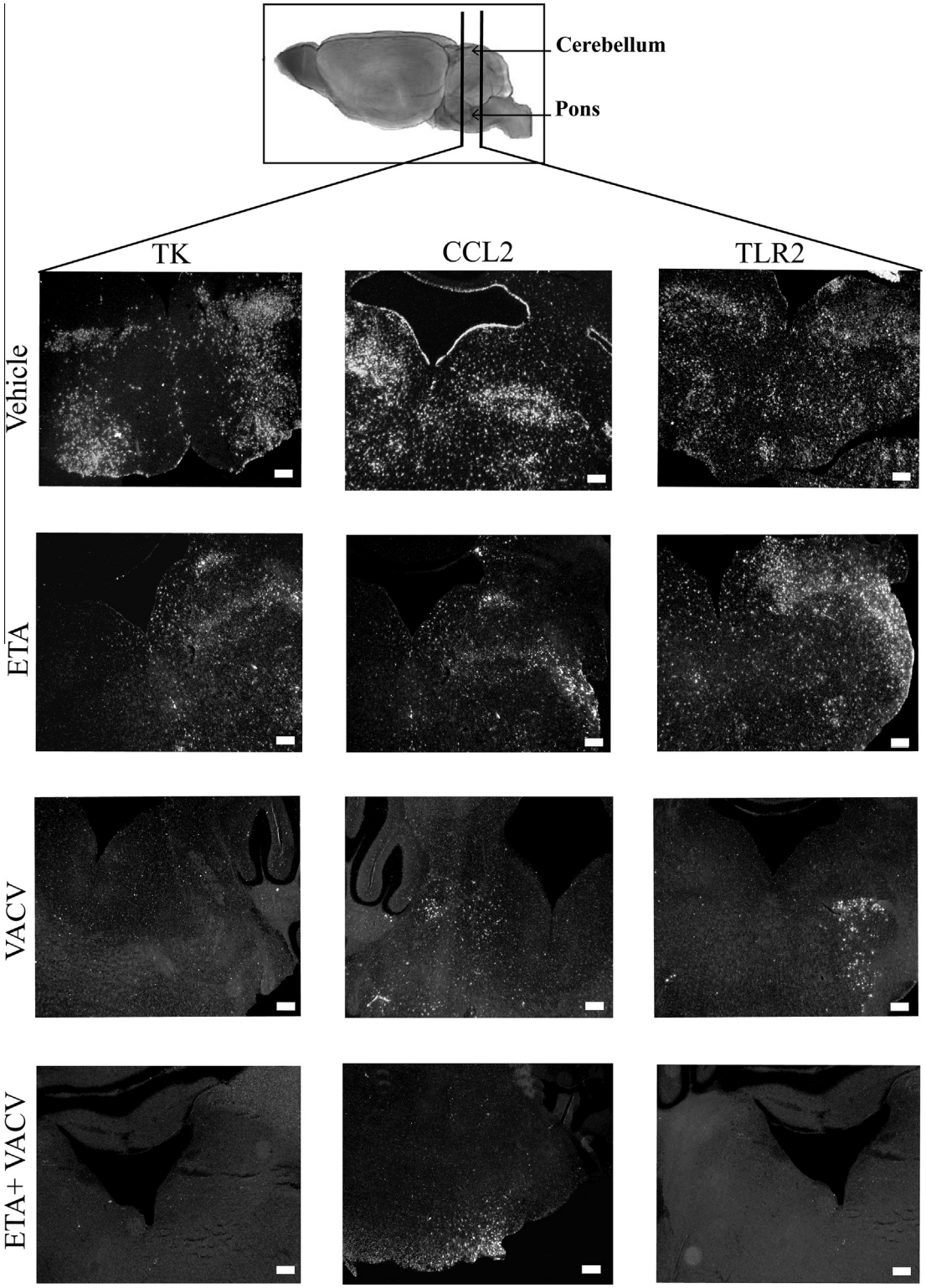

Fig. 2. Representative micrographs illustrating the detection of the viral thymidine kinase (TK), chemokine receptor ligand 2 (CCL2) and Toll-like receptor 2 (TLR2) transcripts by *in situ* hybridization in brain sections of mice infected with HSV-1 and treated with valacyclovir (VACV) combined or not with etanercept (ETA). Four groups of mice received a single intraperitoneal injection of vehicle (0.9% benzyl alcohol) or ETA (400 μg/mouse) on day 3 post-infection combined or not with VACV (1 mg/ml *ad libitum* in drinking water) from days 3 to 21 post-infection. Mice (4–5 per group) were sacrificed on day 5 post-infection and rostrocaudal coronal brain sections (20–25 per mouse) were processed for *in situ* hybridization and examined. The micrographs correspond to the pons region and part of the cerebellum (Dorr et al., 2007). Original magnification, 40×. Bars 250 μm.

The presence of ETA in brain homogenates was evaluated by Western blotting with a primary chicken anti-human ETA antibody (diluted 1:5000; Agrisera, Vannas, Sweden) and a secondary horseradish peroxidase goat anti-chicken antibody (diluted 1:20,000; Jackson ImmunoResearch Laboratories, West Grove, PA). Horseradish peroxidase activity was then revealed using an

ECL detection kit (Amersham, Buckinghamshire, UK). ETA was detected, approximately at its theoretical molecular weight (i.e., 150 kDa), in brain homogenates from all infected mice that received the antibody alone or combined with VACV (data not shown) suggesting that it was able to cross the BBB.

To validate and extend the beneficial effect of ETA combined with VACV on HSE, new groups of mice were infected intranasally with 1.00×10^4 PFU of HSV-1. Infected mice received a single intraperitoneal injection of the vehicle alone or ETA (200 μg/mouse) on day 4 post-infection combined with VACV (1 mg/ml of drinking water) from days 4 to 21 post-infection or VACV alone. The survival rate of mice treated with ETA combined with VACV (66.7%) was still significantly higher than those of mice treated with the vehicle (18.2%; $P = 0.005$) and VACV alone (30.0%, $P = 0.04$) (Fig. 3).

In the present study, we demonstrated, by using two different experimental conditions, that a combination of VACV and ETA initiated on day 3 or 4 post-infection improved the outcome of HSE compared to antiviral therapy alone in a mouse model. We showed that ETA was able to cross the BBB but the antibody by itself did not reduce viral replication suggesting that it might control the exaggerated inflammatory response that develop following infection. However, only a modest effect of ETA was found on CCL2 and TLR2 brain transcripts. The reduced expression of CCL2 and TLR2 in mice treated with VACV combined with ETA was thus mostly due to the reduced viral replication caused by the antiviral. This also suggests that the antibody could decrease other mediators of the immune response such as IL-1β and IL-6 as observed in rats with traumatic brain injury treated with ETA (Chio et al., 2010).

It could thus be beneficial to combine a potent antiviral agent with immune-based therapy, such as a TNF-α inhibitor, to improve treatment against this disease. Of note, TNF-α is still critical to control early HSV replication in the brain as shown by HSE development in patients under chronic anti-TNF treatment (Bradford et al., 2009). The cellular functions of TNF are mediated by TNF receptor 1 (TNFR1) and 2 (TNFR2), which differ in expression profiles and downstream signaling pathways. Binding of soluble and transmembrane TNF to TNFR1 mediates apoptosis and chronic inflammation whereas binding of transmembrane TNF to TNFR2 regulates gene programs important for cell survival, resolution of inflammation, maintenance of immunity to pathogens and myelination (Van Hauwermeiren et al., 2011). ETA is a non-selective inhibitor which blocks both soluble and transmembrane TNF binding (McCoy and Tansey, 2008). Therefore, it would be interesting to evaluate inhibitors that target specifically TNFR1-mediated signaling while sparing TNFR2 activation (Faustman and Davis, 2010; Van Hauwermeiren et al., 2011) such as XPro®1595 (Brambilla et al., 2011) and to assess their effects on viral DNA load, mediators of inflammatory response and infiltration of immune cells in the brain over time. Moreover, the identification of biomarkers for timely administration of such immunomodulatory therapy is also needed.

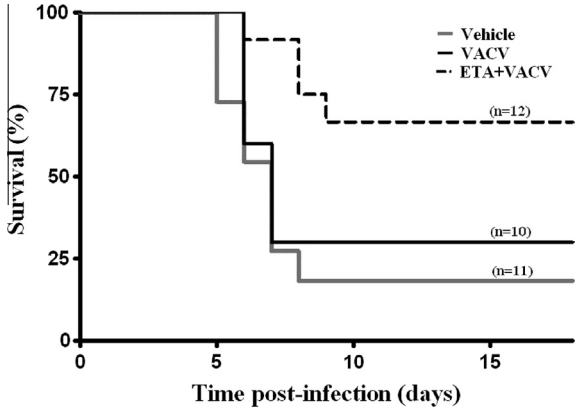

Fig. 3. Effect of valacyclovir (VACV) combined or not with etanercept (ETA) on survival rates of HSV-1-infected mice. Three groups of infected mice received a single intraperitoneal injection of vehicle (0.9% benzyl alcohol), ETA (200 μg/mouse) on day 4 post-infection combined with VACV (1 mg/ml *ad libitum* in drinking water) from days 4 to 21 post-infection or VACV only. Mice were examined once daily for 21 days. Differences in group survival rates were compared using a log-rank (Mantel–Cox) test.

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