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DECREASED INFARCT SIZE AFTER FOCAL CEREBRAL ISCHEMIA IN MICE CHRONICALLY INFECTED WITH *TOXOPLASMA GONDII*

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Abstract—To determine whether *Toxoplasma gondii* infection could modify biological phenomena associated with brain ischemia, we investigated the effect of permanent middle cerebral artery occlusion (MCAO) on neuronal survival, inflammation and redox state in chronically infected mice. Infected animals showed a 40% to 50% decrease of infarct size compared with non-infected littermates 1, 4 and 14 days after MCAO. The resistance of infected mice may be associated with increased basal levels of anti-inflammatory cytokines and/or a marked reduction of the MCAO-related brain induction of two pro-inflammatory cytokines, tumor necrosis factor-alpha and interferon-gamma (IFNγ). In addition, potential anti-inflammatory/neuroprotective factors such as nerve growth factor, suppressor of cytokine signaling-3, superoxide dismutase activity, uncoupling protein-2 and glutathione (GSH) were upregulated in the brain of infected mice. Consistent with a role of GSH in central cytokine regulation, GSH depletion by diethyl maleate inhibited *Toxoplasma gondii* lesion resistance by increasing the proinflammatory cytokine IFNγ brain levels. Overall, these findings indicate that chronic toxoplasmosis decisively influences both the inflammatory molecular events and outcome of cerebral ischemia.

Key words: toxoplasmosis, cerebral ischemia, cytokines, redox factors, rodent.

One of the possible biological consequences of infection is a change of the redox status in both CNS and peripheral tissues (Arsenijevic et al., 2001). Although both animal models and clinical studies showed that infections promote neuronal death following cerebral ischemic injury (Sacco, 2001; Emsley and Tyrrell, 2002), activation of the immune system can also result in neuroprotection (Bordet et al., 2000). In fact, the effect of infection on ischemic damage may largely depend on the regulation of reactive oxygen species by pro-inflammatory and anti-inflammatory cytokines as well as by the main antioxidant state regulators, namely superoxide dismutase (SOD) (Guegan et al., 1998; Murakami et al., 1998), glutathione (GSH) (Nicholls and Budd, 2000; Schulz et al., 2000; Droge, 2002), uncoupling protein-2 (UCP2) (Arsenijevic et al., 2000b; Mattiasson et al., 2003) and nerve growth factor (NGF) (Brodie, 1996; Guegan et al., 1999; Villoslada et al., 2000). In addition, the newly described suppressor of cytokine signaling (SOCS) proteins are induced in peripheral and central models of inflammation (Lebel et al., 2000; Bates et al., 2001; Larsen and Ropke, 2002; Wang and Campbell, 2002; Huang et al., 2003; Park et al., 2003; Jo et al., 2005). SOCS possibly interact with cellular redox determinants (Park et al., 2003) and transgenic SOCS expression has been shown to inhibit inflammation and apoptosis following lipopolysaccharide (LPS) injection (Jo et al., 2005).

Chronic murine toxoplasmosis may be of particular interest in the study of infection-related effects on brain ischemia since it is associated with a tissue-specific regulation of the oxidative state (Arsenijevic et al., 2001) as well as activation of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNFα), interferon-gamma (IFNγ) and interleukin-2 (IL-2), but also anti-inflammatory cytokines such as interleukin-10 (IL-10) (Arsenijevic et al., 1997, 1998). Some of these cytokines are known to enhance neurodegeneration following ischemia (Arsenijevic et al., 2006). In order to determine how chronic infection influences the main biological phenomena associated with acute cerebral ischemia, the present study explores the impact of chronic *Toxoplasma gondii* infection on brain ischemic injury and inflammatory/antioxidant processes induced by permanent middle cerebral artery occlusion (MCAO).

EXPERIMENTAL PROCEDURES

All procedures were approved by the Veterinary Office of the Canton of Zurich Health Directorate and the Veterinary Office of Geneva in accordance with the Swiss Animal Care Guidelines. All efforts were made to minimize the number of animals used in this study and every effort was taken to reduce any suffering.

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Abbreviations: cDNA, complementary DNA; DEM, diethyl maleate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; IFNγ, interferon-gamma; IL-2, interleukin-2; IL-10, interleukin-10; LPS, lipopolysaccharide; MCAO, middle cerebral artery occlusion; NGF, nerve growth factor; SOCS, suppressor of cytokine signaling; SOCS-3, suppressor of cytokine signaling-3; SOD, superoxide dismutase; TNFα, tumor necrosis factor-alpha; TUNEL, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling; UCP2, uncoupling protein-2.

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Mice and diets

Male Swiss Webster mice of 4 months of age from Charles River Laboratories (Wilmington, MA, USA) were used. Mice were chronically infected by i.p. injection of 10 cysts of *Toxoplasma gondii* (Me49 strain obtained from Dr A. Hemphill, University of Bern, Switzerland) (Arsenijevic et al., 1997). Less than 5% of mice died due to infection during weeks 2 and 3. After this time point all infected mice survived. Acute infection with *Toxoplasma gondii* results in anorexia and body weight loss (Arsenijevic et al., 1997). We followed infected mice body weight and food intake 7 days before infection and up to 28 days (chronic phase) after infection ($n=10$). In the chronic phase of infection, some of these mice may show a partial weight regain (50%) or no weight regain (50%) (Arsenijevic et al., 1997). For all experiments, we used only the latter type of mice since these animals had higher basal brain cytokine levels and were expected to maximally respond to a new inflammation (Arsenijevic et al., 1998). A group of non-infected mice ($n=18$) was chronically underfed to mimic the food intake level of infected mice from days 1–28. This group was used to determine: 1) if the reduced food intake of the infected mice may influence basal brain GSH levels ($n=6$); 2) the effect of underfeeding on brain GSH levels after MCAO ($n=6$) and 3) the effect of underfeeding on ischemic lesion size ($n=6$). All mice were individually weighed and food intake was measured daily. For the MCAO study, infected and non-infected mice with and without MCAO ($n=18$ mice per group) were monitored daily from 7 days prior to and up until 3 days after operation. Daily food intake (g/mouse/day) and body weight changes after MCAO were measured, and food intake changes after MCAO were calculated as a percentage of pre-ischemia average food intake for each mouse group.

Histology of infected brains compared with non-infected controls

Histological analysis was performed in infected (28 days following infection) ($n=4$) and non-infected control brains ($n=4$) prior to MCAO. Brain slices (20 μm) were stained with hematoxylin and eosin for histological identification of *Toxoplasma gondii* cysts and infiltrating immune cells (Frenkel and Escajadillo, 1987; Arsenijevic et al., 2007a). Detection of apoptosis in the brain of chronically infected mice was made using terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) labeling as previously described (de Bilbao et al., 2000). The suppressor of cytokine signaling-3 (SOCS-3) mRNA expression was determined by *in situ* hybridization in the brains of these mice ($n=6$ per group). Riboprobe preparation and *in situ* hybridization histochemistry were kindly performed by Dr S. Rivest (Laval University, Canada). The rat SOCS-3 complementary DNA (cDNA) fragment that was initially inserted in a pEF-FLAG-1 vector (provided by Dr. Doug Hilton, The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia) was extracted with *Xba*I and reinserted into a pCRII (Invitrogen, Carlsbad, CA, USA). The new construct was then linearized with *Xho*I. 35S-UTP was used to label the probe (for the complete *in situ* hybridization protocol conditions see reference by Lebel et al., 2000).

Induction of permanent focal cerebral ischemia and volume of the infarct

Mice were operated 28 days after infection, when their body weight and food intake had stabilized (Arsenijevic et al., 1998). We performed permanent MCAO in infected and non-infected control mice ($n=6$ for each group and post-MCAO time) as described in details elsewhere (de Bilbao et al., 2000). All mice survived and showed infarction after MCAO. One day, 4 days and 14 days later, the animals were perfused through the ascending aorta with a solution of paraformaldehyde 4% in phosphate-buffered saline (PBS, pH 7.35). Brains were removed and processed for paraffin embedding. Sections (7 μm) of the whole infarct area were cut on slides pretreated with 3-aminopropyltriethoxy-silane (Sigma, MO, USA), counterstained with Cresyl Violet for the histological identification of the nuclear boundaries and peri-infarct areas and mounted in Eukitt. For each animal, quantification of the infarcted area was performed on the Cresyl Violet–stained sections at five representative levels throughout the rostro-caudal extent of the lesion (A 0.26, −0.22, −0.40, −0.70 and −1.2–4 mm relative to Bregma) (Franklin and Paxinos, 1997). The rostro-caudal extent of the infarct was the same in both groups of mice. The infarcted area of each section was calculated by the subtraction of healthy tissue areas of the contralateral to the ipsilateral side of the section in order to compensate for the effect of brain edema (Guegan et al., 1998) using a computer-assisted image analyzing system (Software Morphometry, Samba 2005 TITN, Alcatel). Volumes of infarct (mm^3) were calculated for each animal after integration of areas with the distance between each level (de Bilbao et al., 2000).

To evaluate whether local alterations in cerebral vascular anatomy contribute to different susceptibility to injury in infected mice, an additional series of five non-infected and five infected mice were killed on day 1 after ischemia. Cerebral vasculature was studied in non-infected and infected mice (non-operated and on day 1 after ischemia) after intracardial perfusion of a mixture of an equal proportion of gelatinous water (5%) and China ink (Sennelier, France) warmed at 40 °C (1 ml). Brains were removed and immersed for 24 h in 4% paraformaldehyde at 4 °C (Chen et al., 2005). Cerebral vasculature was observed with a Zeiss stereo zoom microscope. The absence of cerebral blood flow in the infarct area was assessed visually and by transcranial measurements of cerebral blood flow that were made using laser Doppler flowmetry (Oxford Optronix Ltd., UK) just before and after MCAO. Animals were placed under a stereotactic head frame and then a fine needle probe (MNP110XP, 0.48 mm diameter) was lowered onto the temporal bone surface 0.5–1 mm dorsal to the opening giving access to the MCA and wetted with a small amount of physiological saline.

Physiological parameters

Physiological parameters including arterial blood pressure (Kent mouse tail blood pressure system RTBP2000, Kent Scientific Corporation, Torrington, USA), plasma glucose (using Roche Glucotrend Active, Rotkreuz, Switzerland) and hematocrit were measured daily ($n=5$ for each type of mice) before MCAO and on day 1 and day 4 after injury. During surgery, mice were placed on a warm mat and rectal temperature was measured. During the operation, all mice had a body temperature of 38 °C.

Northern blot for UCP2 mRNA

Infected and non-infected mice subjected or not to ischemia (1 day post-MCAO) ($n=6$ mice per group) were anesthetized i.p. with xylazine (20 mg/kg)/ketamine (100 mg/kg) in 0.9% NaCl (100 μl/10 g body weight). They were intracardially perfused without delay with ice-cold isotonic saline. At the end of the perfusion the whole brains were quickly dissected out and frozen. Total RNA was prepared as described before (Arsenijevic et al., 1997). Northern blot analyses were performed using the mouse UCP2 or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA labeled with ^{32}P under standard conditions. A similar amount of total RNA (20 μg) was used in every lane.

Cytokines and NGF levels in brain

We measured TNFα, IFNγ, IL-10, IL-2 and NGF in the brain of infected and non-infected mice subjected or not to ischemia (1 day post-MCAO; $n=6$ mice per group). Brains were analyzed 1 day