Journal of Neuroimmunology 295–296 (2016) 68–74

Contents lists available at ScienceDirect

Journal of Neuroimmunology

journal homepage: www.elsevier.com/locate/jneuroim

The contribution of antibiotics, pneumonia and the immune response to stroke outcome

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ARTICLE INFO

Article history:
Received 13 February 2016
Received in revised form 5 April 2016
Accepted 7 April 2016

Keywords:
Pneumonia
Antibiotics
Lymphocytes
TH1
TH17
Stroke
Outcome

ABSTRACT

Background: Infections are common following stroke and associated with worse outcome. Using an animal model of pneumonia, we assessed the effect of infection and its treatment on the immune response and stroke outcome.
Methods: Lewis rats were subjected to transient cerebral ischemia and survived for 4 weeks. One day after stroke animals were exposed to aerosolized *Staphylococcus aureus*, *Pseudomonas aeruginosa* or saline. Antibiotics (ceftiofur or enrofloxacin) were started immediately after exposure or delayed for 3 days. Behavioral tests were performed weekly. ELISPOT assays were done on lymphocytes from spleen and brain to assess autoimmune responses to myelin basic protein (MBP).
Results: Among animals that received immediate antibiotic therapy, infection was associated with worse outcome in ceftiofur but not enrofloxacin treated animals. (The outcome with immediate enrofloxacin therapy was so impaired that further worsening may have been difficult to detect.) A delay in antibiotic therapy was associated with better outcomes in both ceftiofur and enrofloxacin treated animals. Infection was associated with an increased likelihood of developing TH1(+) responses to MBP in non-infarcted brain (OR = 2.94 [1.07, 8.12]; $P = 0.04$), and TH1(+) responses to MBP in spleen and non-infarcted brain were independently associated with a decreased likelihood of stroke recovery (OR = 0.16 [0.05, 0.51; $P = 0.002$ and OR = 0.32 [0.12, 0.84]; $P = 0.02$, respectively).
Conclusions: Infection worsens stroke outcome in ceftiofur treated animals and increases TH1 responses to MBP. These data may help explain how infection worsens stroke outcome and suggest that treatment of infection may contribute to this outcome.

1. Introduction

Patients who become infected in the immediate post-stroke period have increased morbidity and mortality in comparison to patients who remain infection free (Westendorp et al., 2011). We previously showed that exposure to lipopolysaccharide (LPS) during experimental stroke increases TH1 immune responses to myelin basic protein (MBP) and worsens outcome (Becker et al., 2005). In an observational study, individuals with post-stroke pneumonia were at similar risk for developing TH1 responses to MBP and worse clinical outcome (Becker et al., 2011). Whether early antibiotic therapy might prevent TH1 responses and improve outcome is unknown, and trials of prophylactic antibiotics to prevent post-stroke infection have had mixed results (Chamorro et al., 2005; Harms et al., 2008; Kalra et al., 2015; Schwarz et al., 2008; Westendorp et al., 2015). In uninfected animals, treatment with enrofloxacin (a fluoroquinolone antibiotic), but not ceftiofur (a β-lactam antibiotic), leads to worse outcome after stroke (Zierath et al., 2015a). In this study we examined the effects of pneumonia on the immune response to MBP and stroke outcome. *Staphylococcus aureus* and *Pseudomonas aeruginosa* are common respiratory pathogens after stroke and chosen here as prototypes of Gram-positive and Gram-negative infections (Hassan et al., 2006; Hilker et al., 2003; Tanzi et al., 2011; Walter et al., 2007; Yan et al., 2015). Broad spectrum antibiotics used for empiric treatment of infection include β-lactams and fluoroquinolones; we chose ceftiofur and enrofloxacin as representatives of each class. The effects of pathogen, antibiotic and antibiotic timing on the immune response and stroke outcome were explored.

2. Materials and methods

2.1. Animals

Male Lewis rats (275–325 g) were purchased from Taconic Farms. All experiments were approved by the University of Washington Institutional Animal Care and Use Committee.

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http://dx.doi.org/10.1016/j.jneuroim.2016.04.005
0165-5728/

2.2. Middle cerebral artery occlusion (MCAO)

Anesthesia was induced with 5% and maintained with 1.5% isoflurane. After midline neck incision, the right common carotid, internal carotid and pterygopalatine arteries were ligated. A monofilament suture (Doccol©; 4.0) was inserted into the common carotid artery and advanced into the internal carotid artery to block the origin of the middle cerebral artery (MCA). Animals were maintained at normothermia during surgery and reperfused 2 h after MCA occlusion (MCAO). Rectal temperature and body weight were assessed at set times. Animals were sacrificed 4 weeks after surgery.

2.3. Pneumonia induction

Twenty-four hours after MCAO, animals were exposed to aerosolized *S. aureus* (Newman strain), *P. aeruginosa* (PAK strain), or saline in a whole animal exposure chamber with a computer interface to control pressures and flows (Biaera Technologies, Hagerstown, MD). Bacteria were prepared as described (Skerrett et al., 1999) and suspended in PBS at 4×10^{11} CFU/ml (*S. aureus*) or 3×10^{10} CFU/ml (*P. aeruginosa*). Bacterial suspensions were aerosolized using a Mini-Heart Hi-Flo nebulizer driven at 44 psi, with airflow through the chamber maintained at 19.5 L/min during the 10 min exposure. Pilot studies demonstrated that these conditions resulted in bacterial depositions of approximately 5×10^7 CFU/lung (*S. aureus*) and 2×10^6 CFU/lung (*P. aeruginosa*) as determined by culture of homogenized lung tissue harvested immediately after exposure. Infected rats developed transient hypothermia and neutrophilic lung inflammation.

2.4. Antibiotic administration

Antibiotics were started immediately after exposure to bacteria (or saline) or delayed for 3 days and dosed according to protocol. Ceftiofur was given subcutaneously daily (10 mg/kg) for 7 days and enrofloxacin was given subcutaneously in 2 doses 3 days apart (20 mg/kg per dose).

2.5. Behavioral outcomes

Animals were trained on the rotarod prior to MCAO and performance assessed weekly thereafter (Kunze et al., 2014). Only animals with a neurological score ≥3 at 24 h after MCAO were randomized to infection/antibiotic therapy (Bederson et al., 1986). The experimental protocol is detailed in Fig. 1. Behavioral testing was done by an investigator masked to treatment status.

2.6. ELISPOT assays

At the time of sacrifice, lymphocytes were isolated from the brain and spleen (Becker et al., 2005; Zierath et al., 2015b). ELISPOT assays were used to detect MBP and ovalbumin (OVA) specific secretion of interferon (IFN)-γ, interleukin (IL)-17 and transforming growth factor (TGF)-β1. Rat MBP was manufactured by NeoBioSci™. OVA was purchased from Sigma. Antigens were used at a concentration of 50 μg/ml; responses were assessed in triplicate.

Lymphocytes (1×10^5 cells/well) were cultured in media alone or in media supplemented with antigen for 48 h in 96 well plates (Multiscreen®-IP, Millipore). Plates were developed using standard protocols (R & D Systems). Spots were counted with the aid of a semi-automated system (AID iSPOT®) and expressed as the ratio of the relative increase in antigen-specific IFN-γ secreting cells to that of TGF-β1 secreting cells (TH1 response) or as the ratio of the relative increase in antigen-specific IL-17 secreting cells to that of TGF-β1 secreting cells (TH17 response). For purposes of this study, animals were considered to be TH1(+) or TH17(+) if the TH1 or TH17 response to the antigen (MBP or OVA) was greater than the 75th percentile of uninfected animals treated with the same antibiotic. Analyses of ELISPOT plates were done by an investigator masked to treatment status.

2.7. Statistics

Parametric data are displayed as mean ± standard deviation (sd) and compared using the *t*-test. Non-parametric data are displayed as median (interquartile range [IQR]) and compared using the Mann–Whitney U test or Kruskal–Wallis H test. Categorical data are compared using the likelihood ratio. Multivariate logistic regression was used to determine the effect of infection (pathogen), antibiotic therapy and the immune response on return to baseline rotarod performance at 1 month. Significance was set at $P < 0.05$.

3. Results

3.1. Effect of infection and antibiotics on outcome

Mortality was 4/150 (3%) and did not differ by treatment group. Infection was associated with relative hypothermia from days 2–6 after pathogen exposure, but change in body weight did not differ between infected and uninfected animals (Fig. 2). Since we previously showed that antibiotics affected outcome in uninfected animals (Zierath et al., 2015a), these data are stratified by antibiotic. In ceftiofur treated animals, infection was associated with worse performance on the rotarod when antibiotics were given concomitant with infection but not when

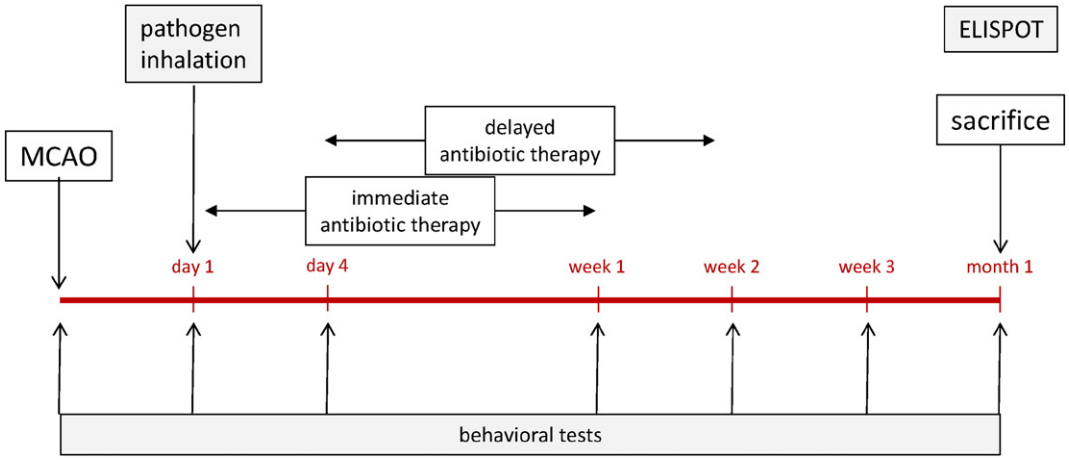

Fig. 1. Experimental protocol.