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Early and late acute lung injury and their association with distal organ damage in murine malaria

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ABSTRACT

Severe malaria is characterised by cerebral oedema, acute lung injury (ALI) and multiple organ dysfunctions, however, the mechanisms of lung and distal organ damage need to be better clarified. Ninety-six C57BL/6 mice were injected intraperitoneally with 5×10^6 *Plasmodium berghei* ANKA-infected erythrocytes or saline. At day 1, *Plasmodium berghei* infected mice presented greater number of areas with alveolar collapse, neutrophil infiltration and interstitial oedema associated with lung mechanics impairment, which was more severe at day 1 than day 5. Lung tumour necrosis factor-α and chemokine (C-X-C motif) ligand 1 levels were higher at day 5 compared to day 1. Lung damage occurred in parallel with distal organ injury at day 1; nevertheless, lung inflammation and the presence of malarial pigment in distal organs were more evident at day 5. In conclusion, ALI develops prior to the onset of cerebral malaria symptoms. Later during the course of infection, the established systemic inflammatory response increases distal organ damage.

1. Introduction

Malaria remains a major global health problem, causing approximately 2 million deaths every year, particularly in tropical areas (Mohan et al., 2008). Several pathological events, such as parasitised erythrocytes, leucocyte adhesion to organ microvasculature, systemic production of cytokines, and cytotoxic lymphocyte activation, induce a condition of systemic activation, which leads to severe malaria. Severe malaria is characterised by increased intracranial pressure, acute lung injury (ALI)/acute respiratory distress syndrome (ARDS), and multiple organ dysfunction (Abdul Manan et al., 2006; Mohan et al., 2008; de Souza et al., 2010). Notably, ALI/ARDS is observed in 5% of patients with uncomplicated malaria and 20–30% of patients with severe malaria (Mohan et al., 2008). Post-mortem examination of fatal malaria patients revealed lung oedema, congested pulmonary capillaries, thickened alveolar septa, intraalveolar haemorrhages, and hyaline membrane formation, which are characteristic of diffuse alveolar damage in ALI/ARDS (James, 1985).

The pathogenic mechanisms that lead to ALI/ARDS during severe malaria are poorly understood, as most studies of lung injury have been performed in patients who were concurrently under treatment (Maguire et al., 2005). The importance of ARDS during severe malaria highlights the need for studies describing the pathophysiology of this syndrome during malarial infection.

Several features of lung injury during experimental severe malaria have previously been described, such as increased expression of circulating vascular endothelial growth factor (VEGF) (Epiphanio et al., 2010), leucocyte accumulation (Van den Steen et al., 2010), and diminished expression of epithelial sodium channels (Hee et al., 2011) in lung tissue.

However, the mechanisms of lung inflammation and its association with distal organ damage during experimental severe malaria require further clarification. This study sought to analyse the impact of severe malaria on lung and distal organ damage in the early and late phases of the disease.

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2. Methods

This study was approved by the Research Ethics Committee of the Federal University of Rio de Janeiro Health Sciences Centre (CEUA-CCS-019) and the Committee on Ethical Use of Laboratory Animals of the Oswaldo Cruz Foundation (L-0004/08). All animals received humane care in compliance with the – Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the *Guide for the Care and Use of Laboratory Animals* prepared by the U.S. National Academy of Sciences.

2.1. Animal preparation and experimental protocols

Ninety-six C57BL/6 mice (weighing 18–20 g) were provided by the Oswaldo Cruz Foundation breeding unit (Rio de Janeiro, Brazil) and kept in cages in a room at the Farmanguinhos experimental facility, with free access to food and fresh water, temperature ranging from 22 to 24 °C, and a standard 12 h light/dark cycle, until experimental use. All animals were randomly assigned to two groups:control (SAL) or *Plasmodium berghei* ANKA infection (*P. berghei*). Both groups were analysed at days 1 and 5 post-inoculation. Mice were infected by intraperitoneal (i.p.) injection of *P. berghei*-infected erythrocytes withdrawn from a previously infected mouse (5×10^6 infected erythrocytes diluted in 200 μl of sterile saline solution). Control mice received saline alone (200 μl, i.p.). After infection, a thick blood smear was performed for determination of parasitemia by Panotico Rápido (Laborclin, Paraná, Brazil) staining. Symptoms of cerebral malaria were evaluated by SHIRPA protocol modified by Martins et al. (2010).

2.2. Mechanical parameters

One or five days following saline or *P. berghei* administration, mice were sedated (diazepam, 1 mg i.p.), anaesthetised (sodium thiopental, 20 mg/kg i.p.), tracheotomised, paralysed (vecuronium bromide, 0.005 mg kg^{-1} i.v.), and mechanically ventilated with a constant flow ventilator (Samay VR15; Universidad de la Republica, Montevideo, Uruguay) using the following settings: respiratory rate = 100 breaths/min, tidal volume (V_T) = 0.2 ml, and fraction of inspired oxygen (FiO_2) = 0.21. The anterior chest wall was surgically removed, and a positive end-expiratory pressure (PEEP) of 2 cmH_2O was applied. After a 10-min ventilation period, lung mechanics were computed. Airflow and tracheal pressure (Ptr) were measured (Burburan et al., 2007). In an open chest preparation, Ptr reflects transpulmonary pressure (PL). Lung resistive (ΔP1) and viscoelastic/inhomogeneous (ΔP2) pressures, as well as static elastance (Est), were computed by the end-inflation occlusion method (Bates et al., 1988). Lung mechanics measurements were performed 10 times in each animal. All data were analysed using the ANADAT data analysis software (RHT-InfoData, Inc., Montreal, Quebec, Canada).

2.3. Lung histology

Laparotomy was performed immediately after determination of lung mechanics, and heparin (1000 IU) was injected into the vena cava. The trachea was clamped at end-expiration (PEEP = 2 cmH_2O), and the abdominal aorta and vena cava were sectioned, producing massive haemorrhage and rapid death. The right lung was then removed, fixed in 4% buffered formaldehyde and embedded in paraffin. Slices (thickness = 4 μm) were cut and stained with haematoxylin and eosin. Lung morphometric analysis was performed using an integrating eyepiece with a coherent system consisting of a grid with 100 points and 50 lines (known length) coupled to a conventional light microscope (Olympus BX51, Olympus Latin America, Inc., Brazil). The volume fractions of the lung occupied by collapsed alveoli and normal pulmonary areas were determined by the point-counting technique (Weibel, 1990) at a magnification of 200× across 10 random, non-coincident microscopic fields. Neutrophils and mononuclear (MN) cells and lung tissue were evaluated at 1000× magnification. Points falling on neutrophils and MN cells were counted and divided by the total number of points falling on lung tissue in each field of view. For quantification of interstitial oedema, 10 arteries were transversely sectioned. The number of points falling on areas of perivascular oedema and the number of intercepts between the lines of the integrating eyepiece and the basement membrane of the vessels were counted at a magnification of 400×. The interstitial perivascular oedema index was calculated as follows: number of points/number of intercepts (Hizume et al., 2007).

2.4. Lung wet/dry (W/D) weight ratio

At days 1 and 5, the W/D ratio was determined in a separate group of mice (n = 6/group), which was subjected to an identical protocol to the one described above. These mice were euthanized in a CO_2 chamber, after which the lungs were removed, weighed (wet weight) and kept at 80 °C overnight for dry weight determination. The wet/dry weight ratio was then calculated.

2.5. Distal organ histology

Brain, heart, liver and kidney were removed, fixed in 4% buffered formaldehyde, and paraffin-embedded. Slices were cut and stained with haematoxylin and eosin. Sections from the regions exhibiting pathologic findings were examined under 400× magnification. A five-point, semiquantitative, severity-based scoring system was used to assess the degree of injury as follows: 0 = normal tissue; 1 = 1–25%; 2 = 26–50%; 3 = 51–75%; and 4 = 76–100% damage out of total tissue examined (Chao et al., 2010).

2.6. Determination of cytokine production by ELISA

Interferon (IFN)-γ, tumour necrosis factor (TNF)-α and chemokine (C-X-C motif) ligand 1 (CXCL1) levels were quantified. Briefly, the lungs, kidney, liver, brain and heart of control and *P. berghei*-infected mice were excised and homogenised in cell lysis buffer (20 mM TRIS, 150 mM NaCl, 5 mM KCl, 1% Triton X-100, protease inhibitor cocktail (1:1000, Sigma–Aldrich, USA), and immediately frozen at −80 °C. The total protein content of each tissue homogenate was evaluated by the Bradford method, followed by determination of cytokine production by a standard sandwich ELISA, performed according to manufacturer's instructions (BD Pharmingen, USA). Plates were read at 490 nm in an M5 Spectrophotometer (Molecular Devices, USA).

2.7. Evaluation of blood–brain barrier disruption

Blood–brain barrier (BBB) disruption was evaluated as previously described (Pamplona et al., 2007). Briefly, mice received an intravenous (i.v.) injection of 1% Evans blue (Sigma–Aldrich, São Paulo, Brazil). One hour later, mice were euthanized, and their brains were weighed and placed in formamide (2 ml, 37 °C, 48 h) to extract the Evans blue dye from the brain tissue. Absorbance was measured at 620 nm (Spectramax 190, Molecular Devices, CA, USA). The concentration of Evans blue was calculated using a standard curve. The data are expressed as mg of Evans blue per g of brain tissue.