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Evidence for an immune signature of prenatal alcohol exposure in female rats

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ABSTRACT

Evidence for immune/neuroimmune disturbances as a possible root cause of a range of disorders, including neurodevelopmental disorders, is growing. Although prenatal alcohol exposure (PAE) impacts immune function, few studies to date have examined immune function in relation to long-term negative health outcomes following PAE, and most have focused on males. To fill this gap, we utilized a rat model to examine the effects of PAE on immune/neuroimmune function during early-life [postnatal day 1 (P1), P8, and P22] in PAE and control females. Due to the extensive interplay between the immune and endocrine systems, we also measured levels of corticosterone and corticosterone binding globulin (CBG). While corticosterone levels were not different among groups, CBG levels were lower in PAE offspring from P1 to P8, suggesting a lower corticosterone reservoir that may underlie susceptibility to inflammation. Spleen weights were increased in PAE rats on P22, a marker of altered immune function. Moreover, we detected a unique cytokine profile in PAE compared to control offspring on P8 – higher levels in the PFC and hippocampus, and lower levels in the hypothalamus and spleen. The finding of a specific immune signature in PAE offspring during a sensitive developmental period has important implications for understanding the basis of long-term immune alterations and health outcomes in children with Fetal Alcohol Spectrum Disorder (FASD). Our findings also highlight the future possibility that immune-based intervention strategies could be considered as an adjunctive novel therapeutic approach for individuals with FASD.

1. Introduction

There is increasing evidence for immune and neuroimmune abnormalities in the etiology and pathophysiology of numerous neurodevelopmental disorders including schizophrenia (Noto et al., 2015) and autism spectrum disorder (ASD) (Krakowiak et al., 2015). Evidence for underlying immune/neuroimmune abnormalities in Fetal Alcohol Spectrum Disorder (FASD), which includes the broad range of deficits/disorders that arise following *in utero* alcohol exposure, is also emerging [reviewed in (Drew and Kane, 2014)]. Children with FASD have a higher incidence of both minor (e.g., recurrent otitis media, respiratory infections), and major, (e.g., sepsis) infections compared to non-exposed children (Gauthier et al., 2004; Johnson et al., 1981) as well as an increased incidence of malignancies, including cancers of embryonic origin (neuroblastoma, ganglioneuroblastoma, medulloblastoma) [reviewed in (Gottesfeld and Abel, 1991)], and leukemias (Latino-Martel et al., 2010). Animal models of prenatal alcohol exposure (PAE) support and extend the clinical findings. Increased susceptibility to infections (Grossmann et al., 1993; McGill et al., 2009) and malignancies (Gottesfeld and Abel, 1991), deficits in immune organ development (Bray et al., 1993; Ewald and Frost, 1987; Ewald and Walden, 1988; Redei et al., 1989), decreased splenic lymphocyte, T lymphoblast, and B cell proliferative responses to stimulation (Gottesfeld et al., 1990; Jerrells and Weinberg, 1998; Weinberg and Jerrells, 1991; Wolcott et al., 1995), blunted LPS-induced febrile responses (Taylor et al., 1999), dampened cytokine responses to immune challenge (Chiappelli et al., 1997; Kim et al., 1999; Lee and Rivier, 1993), and a more severe and prolonged course of inflammation in an adjuvant-induced arthritis model (Zhang et al., 2012) have been reported in models of *in utero* alcohol exposure [reviewed in Bodnar and Weinberg, 2013].

It is well established that chronic alcohol consumption increases proinflammatory cytokine levels (Crews et al., 2006; He and Crews, 2008). In this context, if alcohol is consumed during pregnancy, the developing fetus is likely exposed not only to

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alcohol but also to heightened cytokine signals. In addition to their function in the immune response, cytokines are essential to brain development. Cytokine receptors are expressed on neuronal cells in the fetal brain (Gilmore et al., 2004) and play important roles in key neuronal processes, such as neurogenesis (Smith et al., 2007), myelination (Jakovcevski et al., 2009), synaptogenesis, synaptic pruning, and modulation of synapse strength (Stephan et al., 2012) [reviewed in (Deverman and Patterson, 2009)]. As such, deviations in the normal cytokine balance have been shown to alter the course of normal brain development (Cai et al., 2000). Further support of this notion comes from the "prenatal cytokine hypothesis", put forth in the field of schizophrenia, which links increased cytokine exposure during the prenatal period and alterations in the trajectory of brain development (Howard, 2013).

Only a few studies to date have focused on neuroimmune function/inflammation following prenatal alcohol exposure, and of these, most have focused primarily on males or have pooled the data from males and females (Drew et al., 2015; Topper et al., 2015). Of note, in relation to neuroimmune function in general, females often show an increased incidence of autoimmune/inflammatory diseases or disorders compared to males (Whitacre, 2001), and in many cases, increased rates of inflammatory mediated depressive-like behaviors (Tonelli et al., 2008).

In the present study, we utilized our well-established animal model of prenatal alcohol exposure to investigate whether PAE affects development of neuroimmune function during early life. In the context of the discussion above, and as a first step in examining neuroimmune/ inflammatory changes in our model, we conducted the present study in females. Specifically, we examined levels of key pro- and anti-inflammatory cytokines in peripheral and central compartments, at a number of key developmental ages [postnatal day 1 (P1), P8, and P22] in order to probe for a unique, immune-based signature of alcohol exposure. Furthermore, due to the extensive interplay between the immune and endocrine systems, with shared ligands, receptors, and regulatory feedback (Haddad et al., 2002), we also examined hypothalamic-pituitary-adrenal (HPA) parameters (plasma corticosterone and corticosterone binding globulin [CBG]) to determine whether they might have a modulatory role on immune function.

We hypothesized that *in utero* alcohol exposure would alter the developmental immune profile, as indexed by alterations in pro- and anti-inflammatory cytokine levels. Based on recent findings of increased cytokine levels in the hippocampus, cortex, and cerebellum following alcohol administration during the early postnatal period (third trimester equivalent model) (Drew et al., 2015; Topper et al., 2015), we predicted that PAE would result in overall increased cytokine levels within the brain and blood.

2. Materials and methods

2.1. Breeding

Male and female Sprague-Dawley (SD) rats (Charles River Laboratories, St. Constant, Quebec, Canada) were pair-housed by sex in clear polycarbonate cages with corn-cob bedding, and handled daily for a one week habituation period. Colony rooms were maintained on a 12:12 h light/dark cycle (lights on at 0700 h), at 20–23 °C, and rats were given *ad libitum* access to water and standard laboratory chow (18% Protein Extruded Rodent Diet, #2018, Teklad Global). Nulliparous females (250–325 g; n = 43) were pair-housed with a male and vaginal lavage samples were collected daily for estrous cycle staging and to check for the presence of sperm, indicating gestation day 1 (GD1). All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and approved by the University of British Columbia Animal Care Committee.

2.2. Prenatal diets and feeding

On GD1, females were single housed and assigned to one of three treatment groups: (1) Prenatal alcohol exposure (PAE) – *ad libitum* access to an alcohol-containing liquid diet with 36% of total calories derived from ethanol, 6.37% v/v, n = 13; (2) Pair-fed (PF) – liquid control diet with maltose dextrin isocalorically substituted for ethanol, in the amount consumed by a PAE partner (g/kg/body wt/day of gestation, n = 15); (3) Control (C) – pelleted version of the liquid control diet, *ad libitum,* n = 15. All diets were formulated to provide optimal nutrition (Weinberg/Keiver High Protein Experimental Diet #710324, Control (PF) Diet #710109, and Pelleted Control Diet #102698, Dyets Inc. Bethlehem, PA, USA) and were presented daily, one hour prior to lights off (1800–1900 h) in order to maintain the normal corticosterone circadian rhythm in PF dams, which are fed a restricted ration (Gallo and Weinberg, 1981; Krieger, 1974). Rats in all groups had *ad libitum* access to water and were weighed weekly throughout gestation. On GD17 a blood sample was collected from the tail vein from a subset of PAE, PF, and C dams (n = 3–6) at lights on (0700 h). Blood alcohol levels were measured as previously reported (Hellemans et al., 2010; Uban et al., 2010) and ranged from ~80 to 150 mg/dl in PAE rats. On GD21, diets were replaced with standard laboratory chow (19% Protein Extruded Rodent Diet, #2019, Teklad Global), *ad libitum*, and rats were continued on this diet throughout lactation.

2.3. Tissue collection

On the day of birth (postnatal day 1; P1), litters were culled to 6 males and 6 females, when possible. Female pups from each prenatal group were randomly assigned to one of three termination ages, with tissue collected on P1, P8, or P22 (n = 7–11/group). Litters were maintained at a minimum of 8 pups (4 males, 4 females), with a balanced sex ratio, through the end of the experiment (P22), in order to control for changes in maternal behaviour, which can occur with small litters or litters with an unbalanced sex ratio (Alleva et al., 1989; Moore and Morelli, 1979). The dam and remaining pups were weighed weekly during the lactation period and any pup deaths or abnormalities were noted. On the day of tissue collection (1400–1700 h), female rats were quickly removed from the home cage and decapitated. Trunk blood was collected, serum allowed to separate for 2 h, and samples centrifuged at 2190g for 10 min at 4 °C. Serum samples were stored at −80 °C until assayed for hormone and cytokine levels. Due to the small brain size on P1, the whole brain was removed from the skull, the cerebellum and olfactory bulbs removed, and the brain weighed and quickly frozen on dry ice. On P8 and 22, the whole brain was again removed and weighed following removal of the cerebellum and olfactory bulbs, and the hypothalamus, PFC, and hippocampus were dissected on ice and frozen on dry ice. At all ages, the spleen was removed, weighed, and frozen on dry ice. To control for litter effects, only one rat from each litter was used per age.

2.4. Corticosterone radioimmunoassay

Total serum corticosterone levels were measured using the ImmuChem Double Antibody Corticosterone ^{125}I radioimmunoassay kit (MP Biomedicals, LLC, Orangeburg, NY, USA), according the manufacturer's instructions with one modification – the lowest standard was further diluted to detect lower corticosterone concentrations (Taves et al., 2015). The minimum detectable corticosterone concentration was approximately 7.7 ng/mL, and