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Fetal Exposure to Ethanol Has Long-Term Effects on the Severity of Influenza Virus Infections

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Alcohol use by pregnant women is a significant public health issue despite well-described risks to the fetus including physical and intellectual growth retardation and malformations. Although clinical studies are limited, they suggest that in utero alcohol exposure also results in significant immune deficiencies in naive neonates. However, little is known about fetal alcohol exposure (FAE) effects on adult infections. Therefore, to determine the long-term effects of FAE on disease susceptibility and the adult immune system, we infected FAE adult mice with influenza virus. In this study, we demonstrate that mice exposed to ethanol during gestation and nursing exhibit enhanced disease severity as well as increased and sustained pulmonary viral titers following influenza virus infection. Secondary exposure to alcohol as an adult further exacerbates these effects. Moreover, we demonstrate that FAE mice have impaired adaptive immune responses, including decreased numbers of virus-specific pulmonary CD8 T cells, a decreased size and frequency of pulmonary B cell foci, and reduced production of influenza-specific Ab following influenza infection. Together, our results suggest that FAE induces significant and long-term defects in immunity and susceptibility to influenza virus infection and that FAE individuals could be at increased risk for severe and fatal respiratory infections. The Journal of Immunology, 2009, 182: 7803–7808.

Despite the well-described risks of alcohol use during pregnancy (1–5), alcohol abuse and binge drinking by pregnant women continues to be a significant public health problem, with an estimated 35% of pregnant women consuming alcohol each month and one in nine women binge drinking during the first trimester of pregnancy (6–8). Prenatal ethanol (EtOH) exposure is known to cause a variety of physical and mental abnormalities in the exposed fetus including growth retardation, muscular and skeletal abnormalities, and intellectual and behavioral impairments (1–5). In addition to these developmental lesions, in utero and prenatal EtOH exposure has been shown to cause a variety of immune deficits both in humans and animal models. Although limited, clinical research has demonstrated that in utero alcohol exposure results in an increased incidence of both minor and life-threatening bacterial infections as well as decreased white cell counts in the cord blood (9). Furthermore, even limited alcohol use during gestation has been shown to increase a newborn’s risk for neonatal infections by 2.5-fold, whereas excessive alcohol abuse rendered newborns 3–4 times more likely to acquire severe neonatal infections (10). Animal models have demonstrated that in utero EtOH exposure can result in delayed T and B cell development, reduced lymphocyte numbers in the blood and spleen, and decreased cellular responses to mitogen stimulation up to early postweaning age (4, 9, 11–18).

The results of several studies have suggested that lymphocyte numbers and proliferation in responses to mitogen stimulation are also reduced in adult rats exposed to EtOH in utero (4, 12, 13, 19). Furthermore, young adult mice exposed to EtOH in utero also have decreased contact hypersensitivity and graft-vs-host responses (14), suggesting the effects of EtOH exposure on immunity can last into adulthood. Similarly, fetal alcohol exposure (FAE) in humans appears to have long-term effects because in utero EtOH-exposed adolescents exhibit impaired immune responses and increased susceptibility to infection (20, 21). Together these results suggest that offspring exposed to EtOH in utero may be at increased risk for infections even into adulthood.

Whereas the effects of in utero EtOH exposure are well documented in the setting of an immunologically naive animal, little has been done to examine the effects of fetal alcohol exposure on immunity following pathogen infection and, more specifically, viral infection. It is known that adult alcoholics are at increased risk for acquiring community-acquired pneumonia and are more prone to severe disease outcomes (22–26). We have recently demonstrated that chronic consumption of EtOH as an adult likewise results in an increased severity of influenza virus infection and impaired antiviral immune responses (27). Given the current threat of potential pandemic influenza (i.e., H5N1 avian influenza, etc.) and the constant threat of seasonal and epidemic influenza (28–30), we have determined the effects of fetal alcohol exposure on the severity and outcome of influenza virus infection. In this study, we demonstrate that adult mice exposed to alcohol only during gestation and nursing have increased influenza-associated morbidity and mortality, increased pulmonary viral titers, and decreased numbers of both B cells and influenza-specific CD8 T cells in the lungs following influenza infection. Secondary exposure to alcohol as an adult significantly exacerbated these effects. Finally, we
demonstrate that mice from a different inbred background that were exposed to EtOH as a fetus and during nursing 6 mo previously exhibit similarly increased susceptibility to a second strain of influenza virus. These results demonstrate that the severe immune deficiencies resulting from alcohol exposure in utero and during nursing are long lived even in the absence of further EtOH consumption and are independent of the host and viral background.

Materials and Methods

**Mice**

Six- to seven-week-old C57BL/6 and BALB/c mice were obtained from The National Cancer Institute (Frederick, MD). All mice were housed and maintained in the specific pathogen-free animal care facility at the University of Iowa (Iowa City, IA). All experiments were performed in accordance with regulatory standards and guidelines and were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

**Fetal alcohol exposure**

After 1 wk of acclimation, female mice were placed on 5% (v/v) EtOH (pharmacological grade) in drinking water, as the only source of drinking water, for 2 wk (see Fig. 1 for model). Mice were provided with solid mouse chow ad libitum at all times during the studies (31, 32). After 2 wk, EtOH was increased to 10% and male mice were added for 5–7 days. Following breeding, males were removed and the females remained on 10% EtOH until parturition. At birth, litters were standardized to six pups per dam (water:water) and EtOH was increased to 12%. After weaning, mice were maintained on water (without EtOH) until their body weight reached 17 g (5–7 wk). Half of the FAE mice were then conditioned onto 20% EtOH over the course of a week (10% for 2 days, 15% for 5 days, 20% for the duration of the study) (EtOH:EtOH), whereas the other half remained on water (EtOH:water). After 6 wk of chronic adult EtOH exposure (~14–16 wk of age), mice were used for experiments. Pups from water-conditioned mothers (water:water) as well as adult-only, EtOH-exposed (6 wk of EtOH: water:EtOH) mice, similar to our previous studies (27), were used as controls.

**Influenza virus infection**

Stocks from the mouse-adapted influenza A viruses A/PR/8/34 (H1N1) and A/JAPAN/305/57 (H2N2) were prepared as described (27). Mice were anesthetized with isoflurane and C57BL/6 mice and BALB/c mice infected intranasally with a 3.2 × 10^6 PFU dose of A/PR/8/34 or a 2.0 × 10^5 PFU dose of A/JAPAN/305/57, respectively. Mortality and morbidity were monitored daily.

**Pulmonary virus titer**

Lungs from infected mice were rapidly homogenized and viral titers were determined as previously described (33) using an end point dilution assay and expressed as 50% tissue culture infectious dose (TCID50). Briefly, 10-fold dilutions of homogenized and clarified lungs from influenza virus-infected mice were mixed with 10^5 Madin-Darby canine kidney (MDCK) cells in DMEM. After 24 h of incubation at 37°C, the inoculum was removed and DMEM containing 0.0002% L-1-(tosylamido-2-phenyl)ethyl chloromethyl ketone-treated trypsin (Worthington Diagnostics) and penicillin (100 U/ml)/streptomycin (100 mg/ml) was added to each well. After 3 days of incubation at 37°C in a humidified atmosphere of 5% CO2, supernatants were mixed with an equal volume of 0.5% chicken RBC, the agglutination pattern was read, and the TCID50 values were calculated.

**T cell analysis**

Single-cell suspensions of lungs were stained with MHC tetramers or assayed for intracellular IFN-γ as previously described (27). Responses to the peptides PA224 (where PA is influenza acid polymerase protein) and NP366 (where NP is influenza nucleocapsid protein) for C57BL/6 mice were measured. Tetramers were obtained from the National Institutes of Allergy and Infectious Diseases Tetramer Core Facility (Atlanta, GA).

**Immunohistochemistry**

Lungs were inflated and fixed in PBS-formalin on days 0, 4, 8, 10, and 14 postinfection (p.i.) and then sectioned. Sections were deparaffinized in xylene and rehydrated in graded alcohols. Ag unmasking was performed for B220 (in citrate buffer (pH 6.0) by microwave for 3 min at 1000 watts) and influenza nucleocapsid protein (NP) Ag (for 5 min; proteinase K from DakoCytomation). Endogenous peroxidase activity and background staining were blocked with hydrogen peroxide and Fab blocker (Jackson ImmunoResearch), respectively, with background buster reagent (Innovex Biosciences). Sections were immersed with anti-mouse B220 monoclonal (Serotec, Co) or anti-NP H116 mAb. A Vector Laboratories biotinylated anti-rat, a mouse absorbed reagent (B220 staining), or a mouse DakoCytomation EnVision HRP system reagent (NP staining) was applied to slides and allowed to incubate. A Vector Laboratories Vectastain Elite ABC (avidin:biotinylated enzyme complex) reagent was then applied to the slides followed by a DakoCytomation DAB Plus and then the DAB Enhancer (where DAB is diaminobenzidine). Slides were counterstained with hematoxylin, routinely dehydrated, and coverslipped.

**Hemagglutination inhibition assay**

The hemagglutination inhibition Ab titer in serum was determined by first heat inactivating 50 μl of serum at 56°C for 30 min. The sera were then absorbed by adding 200 μl 1% chicken RBC suspension (1/5 dilution of original serum sample) for an additional 30 min at room temperature. After incubation, the RBC were pelleted for 5 s at 14,000 rpm. Sera were then transferred to 96-well plates and diluted serially in duplicate in PBS with 0.05% BSA. The influenza virus was then added to wells containing serially diluted sera, mixed, and incubated at room temperature. After 30 min, 1% chicken RBC were added to each well and plates were incubated at room temperature for 30–40 min allowing hemagglutination to occur. The hemagglutinin inhibition titer are defined as the highest serum dilution capable of preventing hemagglutination.

**Results**

Establishing a model of FAE

In humans, the majority of alcohol-exposed newborns do not exhibit the physical and behavioral changes that are often associated with fetal alcohol syndrome (FAE), resulting in a underestimation of the adverse consequences of FAE (10). In this study we examined the effects of EtOH exposure in a model that mimics FAE but does not cause external physical defects. To this end, we have established a model of FAE by using an adaptation of the EtOH-in-drinking-water mouse model of chronic EtOH exposure (27, 32). Using this EtOH feeding model, adult mice exhibit a range of immune and tissue lesions normally observed in chronic alcoholics without the overt stress response often associated with other models of EtOH exposure (32). For the FAE model (Fig. 1), female C57BL/6 mice were exposed to 5% (w/v) alcohol in drinking water and mouse chow ab libitum as previously described (27, 32) for 2 wk before breeding.
For breeding, female and male mice were placed together and alcohol was increased to 10% EtOH for 5–7 days. After 1 wk, the male was removed and the pregnant female remained on 10% EtOH for the length of gestation. At birth, EtOH litter sizes were culled to six pups to avoid competition for milk and the dam was given 12% EtOH until weaning. At these concentrations of EtOH we did not observe any evidence of the physical malformations often associated with fetal alcohol syndrome, suggesting that this is an appropriate model for human FAE. Control pups were obtained from females provided only water (without EtOH) during breeding, pregnancy, and nursing. Litters from control dams were likewise culled to six pups. At weaning, all pups were placed on water and chow for 5–7 wk until they were a minimum of 17 g. Upon reaching sufficient weight the pups were subdivided and half remained on water (EtOH:water, also referred to as FAE mice) whereas the other half were conditioned as adults to EtOH over the course of a week, reaching a final concentration of 20% EtOH (27, 32) to model FAE children who go on to chronically consume EtOH as adults (EtOH:EtOH). These mice were maintained on 20% EtOH for 6 wk before the initiation of the influenza experiments. Pups from water exposed mothers were age and weight matched to serve as non-EtOH-exposed controls (water:water).

**FAE results in increased influenza-associated morbidity, mortality, and pulmonary viral titers**

We infected adult EtOH:water, EtOH:EtOH, and water control mice with a sublethal dose of influenza A (mouse-adapted A/PR/8/34) and monitored the mice daily for morbidity (as measured by weight loss) and mortality. Alcohol exposure in utero and during nursing (EtOH:water/FAE) resulted in significantly increased morbidity and mortality following influenza virus infection as compared with adult water:water controls (Fig. 2). This increase in mortality was similar to that which we have previously described (27) and is observed in mice consuming EtOH for 6 wk only as adults (water:EtOH; supplemental Fig. 1). It is important to note however, that unlike the chronic adult drinking water:EtOH mice, the EtOH:water FAE mice had not been exposed to EtOH in the previous 11–13 wk before influenza virus infection. This suggests that exposure to EtOH in utero and during nursing may cause long-term changes in pulmonary virus susceptibility and underlying immune responses. Interestingly, the increase in virus-induced mortality seen in FAE mice is exacerbated if the FAE mice are further exposed to chronic EtOH again as an adult (EtOH:EtOH; Fig. 2).

Increased morbidity and mortality following influenza virus infection is often associated with increased viral loads. In our previous studies examining the effects of adult chronic EtOH consumption on influenza immunity, we observed significantly increased pulmonary viral titers in adult EtOH mice compared with water controls (27). To determine whether EtOH exposure in utero results in a similar increase in pulmonary viral titers, we again infected C57BL/6 EtOH:water, EtOH:EtOH, and water:water control mice with a sublethal dose of influenza virus. As shown in Fig. 3, we observed significantly increased pulmonary viral titers in the lungs of both EtOH:water and EtOH:EtOH mice as measured by endpoint dilution assay in MDCK cells on day 8 p.i. (Fig. 3A) or by immunohistochemistry for NP Ag (Fig. 3B) as compared with controls on days 4–14 p.i. Although water:water control mice cleared the majority of the influenza NP protein from the pulmonary epithelium between days 8–10 p.i. (Fig. 3B), EtOH:water mice continued to have significant levels of NP protein through day 10, suggesting a delay in the ability of the EtOH:water mice to clear the virus. A similar increase in NP protein load on day 10 p.i. was observed in control adult-only, chronic EtOH-consuming mice (water:EtOH; supplemental Fig. 2). Correlated with the increased mortality demonstrated in Fig. 2, EtOH:EtOH mice had significant levels of NP protein even through day 14 p.i., a time when both controls and surviving EtOH:water mice have cleared the infection. Together, these results show that FAE EtOH
exposure has a profoundly negative effect on influenza virus clearance and that additional EtOH exposure as an adult further exacerbates the length of time before viral clearance in FAE mice. Additionally, the results suggest that the enhanced mortality of EtOH:water and EtOH:EtOH mice may relate to higher and sustained levels of virus within the lungs.

**FAE results in impaired adaptive immunity following influenza virus infection**

Clearance of a primary influenza virus infection requires the killing of infected epithelial cells by influenza-specific CTL (33, 34). A range of studies have shown that in utero exposure to EtOH can reduce T cell responses (4, 12), so we determined what effects in utero EtOH exposure had on FAE mice and controls were infected with influenza virus at the times shown in Fig. 1, and their lungs were inflated and fixed on days 8 and 14 p.i.; *p < 0.05 compared with water:water controls. The hemagglutinin inhibition dilution represents the highest serum dilution capable of preventing influenza virus hemagglutination of RBC.

**FIGURE 5.** Reduced B cell immunity in FAE mice. A, Lungs from influenza-infected water:water, EtOH:water, and EtOH:EtOH mice were inflated, fixed, and stained for B220<sup>+</sup> cells on day 10 and day 14 p.i. Data are representative of one to two separate experiments, n = 4–7/group. B, Water:water, EtOH:water, and EtOH:EtOH mice were infected with influenza and the titer of influenza-specific Ab in the serum was determined by hemagglutination inhibition (HAI) assay as described in Materials and Methods on days 8 and 14 p.i.; *p < 0.05 compared with water:water controls.
influenza virus. We addressed both concepts by infecting 6-mo-old mice with multiple strains of influenza virus strains (27). Therefore, we determined whether older FAE mice exhibit increased disease severity after influenza infection compared with their age-matched control counterparts, and whether FAE-induced immune defect is sustained. Furthermore, no studies have been performed in older adults to examine whether FAE adult humans have the same increased susceptibility to viral infections observed in our murine model, but these results underscore the need for longitudinal clinical studies to assess the effects of in utero EtOH exposure on long-term immunity.

Discussion

Our above results suggest that there are long-term changes in the immune response in FAE mice. It is important to note that in the latter experiments shown in Fig. 6, the mice have not been exposed to EtOH in the previous 6 mo. Therefore, it is unlikely that the effects observed are due to direct effects of EtOH on T cells, B cells, or other immune cells such as dendritic cells (DC) and macrophages, as the natural turnover of these cells or the seeding of substantial numbers of new naive cells would have occurred after the removal of EtOH. Therefore our results suggest that fetal and neonatal EtOH exposure alters the development of the peripheral immune system in a manner independent of lymphocyte and accessory cell turnover. Two potential explanations are epigenetic changes in immune cell genes or loss of immune organ integrity. Consistent with these theories, current studies suggest that in utero exposure to EtOH results in long-term changes in the hypothalamic-pituitary-adrenal axis through epigenetic modifications (4). Like the hypothalamic-pituitary-adrenal axis, the presence and development of lymph nodes and spleens with normal architecture is dependent upon programming early during development, and in the absence of key signals these organs fail to develop correctly (35–37). Consistent with this idea, studies have demonstrated that in utero exposure to EtOH inhibits the development of the thymic epithelium (4). In addition, our preliminary histologic assessment of spleens from 12-wk FAE mice indicates the altered development of white pulp units, with smaller follicles and T cells zones (data not shown).

Although adaptive immunity is essential for the clearance of a primary influenza challenge, innate immunity also plays an important role in controlling the viral load before the establishment of an adaptive immune response. Recently, in utero EtOH exposure has been shown to have adverse effects on the function of newborn alveolar macrophages (28, 39). Oxidative stress is increased in the fetal alcohol-exposed lung, resulting in decreased aMφ phagocytosis and increased apoptosis. In a model of Staphylococcus aureus infection, the decreased function of fetal alcohol-exposed aMφ resulted in increased susceptibility to neonatal infection (39). Although the role of aMφ in influenza virus infection is not as well understood, these cells are part of the first line of defense in the lungs and are known to have an important role in cytokine production and in the recruitment of innate and adaptive immune cells following respiratory challenge. To date, although we have not observed a decrease in aMφ or pulmonary DC numbers in EtOH:water FAE mice, we have observed alterations in splenic DC function (data not shown). Therefore it currently remains unknown whether EtOH exposure in utero and during nursing has similar adverse effects on adult aMφ and pulmonary DC function (38–40) and whether such potential functional changes contribute to the altered adaptive immune response observed in FAE mice.

In conclusion, we demonstrate that FAE results in serious long-term impairment of both B and T cell adaptive immune responses and that this impairment results in increased risk for severe disease and death. Given the distinct requirement for adaptive immunity following primary influenza virus infections, our studies underscore the need to develop potent strategies to boost immunity and viral strain specific. It is unclear from the available literature whether FAE adult humans have the same increased susceptibility to viral infections observed in our murine model, but these results underscore the need for longitudinal clinical studies to assess the effects of in utero EtOH exposure on long-term immunity.

Long-term defects in antiviral immunity following FAE

The above experiments demonstrate that FAE results in significant and long-term impairment of primary adaptive immunity (i.e., T cell and B cell responses). Protection from secondary homologous influenza virus infection is primarily mediated by Ab responses, whereas recovery from heterologous challenge relies on the presence of cross-reactive CTLs. Because EtOH: water and EtOH:EtOH mice are impaired in both primary T and B cell immunity, it will be important to determine in future studies what effect this adaptive immune cell deficiency has on the development of immune memory and the ability of the FAE mice to repel secondary homologous and heterologous influenza virus challenges in the few FAE animals that survive primary influenza virus challenge.

FIGURE 6. Effects of FAE on influenza infection in older mice. Six-month-old BALB/c EtOH:water and water:water mice were infected with mouse-adapted influenza (A/JAPAN/305/57) and monitored daily for morbidity (A) and mortality (B), n = 11–13/group; *, p < 0.05 compared with water:water controls.

showed a decrease in size and number of B cell foci as well as influenza-specific Ab production (supplemental Fig. 4).

Together, these results suggest that in utero EtOH exposure results in significant and long-term impairment of primary adaptive immunity (i.e., T and B cell responses). Protection from secondary homologous influenza virus infection is primarily mediated by Ab responses, whereas recovery from heterologous challenge relies on the presence of cross-reactive CTLs. Because EtOH: water and EtOH:EtOH mice are impaired in both primary T and B cell immunity, it will be important to determine in future studies what effect this adaptive immune cell deficiency has on the development of immune memory and the ability of the FAE mice to repel secondary homologous and heterologous influenza virus challenges in the few FAE animals that survive primary influenza virus challenge.

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In conclusion, we demonstrate that FAE results in serious long-term impairment of both B and T cell adaptive immune responses and that this impairment results in increased risk for severe disease and death. Given the distinct requirement for adaptive immunity following primary influenza virus infections, our studies underscore the need to develop potent strategies to boost immunity and
immune-mediated protection to this and potentially other important respiratory pathogens in these at risk individuals (9, 20).

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Disclosures
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