Prevention of behavioral deficits in rats exposed to folate receptor antibodies: implication in autism

A Desai¹, JM Sequeira² and EV Quadros²

INTRODUCTION

Folate is an essential B-complex vitamin required for the transfer of carbon units in the intermediary metabolism of amino acids, purines, pyrimidines and in the production of 5-adenosylmethionine for methylation reactions.¹ Folate is particularly essential during pregnancy as fetal development is a time of continuous DNA synthesis and cell division. Deficiency during pregnancy can produce fetal malformations and miscarriage.²,³ Folate supplementation can prevent neural tube defect pregnancy⁴ and decrease the risk of autism spectrum disorders (ASD).⁵,⁶ Schmidt et al.⁷ reported that the protection afforded by prenatal folic acid supplementation was particularly beneficial in mothers and children with the MTHFR 677 C>T variant genotype.⁷

Dietary folate insufficiency, gene defects or compromised transport can lead to metabolic folate deficiency. The identification of folate receptor alpha (FRA)-specific autoantibodies that can block folate transport provided a potential mechanism for folate deficiency in the fetus.⁸ These FRA autoantibodies have been identified in a majority of women with a history of neural tube defect pregnancy,⁸,⁹ as well as with subfertility and preterm birth.¹⁰,¹¹ Direct proof that FRA-specific antibodies (Abs) are teratogenic to the embryo came from observations in a pregnant rat model of exposure to FRA Ab that caused resorption of embryos at higher doses and neural tube and cranio-facial malformations at lower doses.¹² Folic acid and dexamethasone prevented malformations, suggesting that blocking of folate transport to the embryo and Ab-mediated inflammation may have a role in the pathology.¹²

FRA autoantibodies have also been found in infants with cerebral folate deficiency (CFD), substantiating the important role of folate in brain development, whereby FRA-specific autoantibodies could block folate transport across the choroid plexus.¹³-¹⁵ CFD is a developmental disorder with decreased level of 5-methyltetrahydrofolate (MTHF) in the cerebrospinal fluid.¹⁶ Consequently, these patients suffer from severe neurological symptoms, including marked irritability, cerebellar ataxia, slow head growth, psychomotor retardation and pyramidal tract signs. One-third also suffer from dyskinesia (for example, choreoathetosis and ballismus) and seizures.¹³,¹⁴ These autoantibodies have also been found in other developmental disorders, including low functioning autism,¹⁵ Rett syndrome¹⁷ and in ASD.¹⁸,¹⁹ Owing to the high prevalence of ASD in children with CFD, and favorable response to folic acid, reports have hypothesized that ASD may be a less severe manifestation of CFD.¹⁸,²⁰

Considering the high prevalence of FRA autoantibodies in children with CFD (89%)²¹ and ASD (~70%),²² we sought to establish proof of hypothesis that FRA Abs can produce the pathology of behavioral and cognitive deficits. Preliminary studies indicated that exposure to FRA Abs during gestation and preweaning in a rat model produced severe learning and behavioral deficits.²¹ Therefore, we determined behavioral deficits in pups born to dams exposed to FRA Ab during gestation and evaluated the effect of folic acid, which would provide adequate folate, and dexamethasone, which would suppress inflammation, during gestational Ab exposure.

MATERIALS AND METHODS

Rat model of FRA Ab exposure

Timed-pregnant postnatal day (PND) 50 Long Evans hooded rats (Charles River Laboratories, Wilmington, MA, USA) were anesthetized on gestational day (GD) 8 and a laparotomy was performed to record the number of implanted embryos. FRA Ab at a dose of 4 or 12 µg per embryo in 1 ml normal rat serum was administered by intraperitoneal (IP) injection, 1 h after the laparotomy. These doses were chosen because they allowed the implanted embryos to be carried to term and produce live pups. Treatment groups received 1 mg of folic acid (GD7–GD12) IP and/or 0.5 mg...
Folate uptake and FRα Ab localization studies
To determine FRα Ab localization and its effect on folate uptake, GD14 rats were administered IP with 12 μg of FRα Ab or NRIgG per embryo and 5 μCi of [3H]HPGA in 1 ml saline on GD15. The rats were killed on GD16 (48 h after the Ab dose). In another set of rats, a similar protocol was followed except the SuG of [3H]HPGA was administered on GD16 and killed on GD17 (72 h after Ab dose). Tissue was homogenized in 0.1 M sodium phosphate buffer pH 7.4. Half of this homogenate was added to scintillation fluid to determine folinic acid ([3H]HPGA) uptake. The other half of the tissue was treated with glycine/HCl pH 2.5 to detach Ab from receptor, supernatant fraction neutralized with 1 M dibasic sodium phosphate, followed by measuring immunoprecipitation of FRαs labeled with [3H]HPGA.

In order to examine the effect of dexamethasone treatment, GD14 rats were given 0.5 mg dexamethasone intramuscularly. Three hours later, they received FRα Ab or NRIgG (12 μg per embryo). All animals received a second dose of Ab 16 h later. Twenty hours after the first Ab or NRIgG injection, all rats were injected with 5 μCi of [3H]HPGA. All animals were killed 4 h later and tissues were collected (placenta, embryo, yolk sac, uterus). Radioactivity in the tissues was then determined as above.

Statistical analysis
Statistical analysis of behavioral studies, which involved ≥3 groups was carried out using one-way analysis of variance (ANOVA). If the one-way ANOVA showed statistical significance (P < 0.05), post-hoc analysis was carried out using Tukey's Honest Significant Difference. For analysis between two groups, Student's t-test was used to determine statistical significance (GraphPad Software, La Jolla, CA, USA). Values plotted are the mean and error bars represent s.e.m. Sample sizes (n) are indicated in the corresponding figure or figure legend. Based on our published data (2% of controls and 75% of Ab-exposed affected pups),12,23 we calculated the necessary sample sizes. We needed pups from two dams in each group to yield a statistically significant result with 95% statistical power, accepting a 95% confidence interval in a two-tailed post-hoc test following a significant main effect ANOVA. This calculation was carried out using the Statistical Power Calculator from DSS Research, Fort Worth, TX, USA.

RESULTS
In the various experimental and treatment groups, no decrease in the number of live pups born was observed in any group. All pups were examined for any gross abnormalities, and none were noted. There was also no difference in the ratio of males to females in litters of each group. Pups were weighed on PND10 and PND25 and showed no significant weight differences in the various groups (18.6 ± 0.5 and 70.8 ± 2.0 g, respectively).

Early communication deficits
Following administration of the rat FRα Ab at 4 or 12 μg per embryo to pregnant dams on GD8, a dose-dependent decrease in isolation-induced vocalizations was observed on PND4 compared with sham controls (P < 0.001; Figure 1a). A significant improvement in the number of vocalizations was seen when folinic acid, dexamethasone or folic acid and dexamethasone (P < 0.001) were administered along with the Ab. Additionally, these pups also presented with a significant delay to first call; corrected in all treatment groups (Figure 1b, P < 0.001) by folinic acid and dexamethasone.

Preventing folate receptor antibody-induced deficits
A Desai et al
Adult communication deficits
Communication deficits on PND4 among in utero Ab exposed rats persisted into adulthood as Ab exposed male rats significantly decreased vocalizations around a female, compared with controls. Folinic acid and dexamethasone normalized this behavior (Figure 1c, P < 0.01).

Sociability deficits
When adult male rats exposed in utero to Ab were tested in a sociability paradigm, they demonstrated significantly decreased social interaction as compared with controls (P < 0.001). This impaired behavior was prevented by folinic acid (P < 0.01), dexamethasone (P < 0.02) or folinic acid plus dexamethasone (P < 0.01; Figure 1d).

Figure 1. Vocalizations and social behavior in rats exposed to FRα antibodies in utero. (a) Isolation induced ultrasonic vocalization by pups when separated from the mother on postnatal day 4 and correction of this deficit by dexamethasone (Dex), folinic acid (FA) and combination of the two, (b) latency to first call, (c) male vocalizations in the proximity of a female, (d) male sociability (e) rearings and (f) movement in the open field test. FR, folate receptor. *P < 0.05; **P < 0.01; ***P < 0.001.
Open field testing for anxiety
Rats exposed to Ab in utero showed increased anxiety in the open field test. These animals had significantly decreased numbers of rearings compared with controls (Figure 1e, \( P < 0.02 \)). They did not present with any significant decrease in total distance traveled, indicating no motor abnormalities (Figure 1f).

Learning, memory and set-shifting deficits
The rats were further tested for learning and memory in a series of tests of increasing complexity that evaluated their ability to learn and remember a task over an extended period and then be able to switch to a new task. When animals exposed to FRα Ab in utero were tested in a hierarchy of place avoidance tasks between PND60 and PND70, all animals could learn the PPA task by successfully avoiding a stationary shock sector using olfactory and visual cues. However, when they continued on to the APA task, 50% of the 4 \( \mu \)g, and 75% of 12 \( \mu \)g Ab-exposed animals failed to learn the task (Figure 2a). Protection from these deficits was provided by administration of folinic acid, dexamethasone and folinic acid plus dexamethasone along with the FRα Ab (Figure 2a). A majority of the animals in all treatment groups could successfully acquire the APA task, with the learning curve for animals treated with folinic acid plus dexamethasone being almost identical to control animals (Figure 2b).

Figure 2. Learning deficits in rats exposed to FRα antibody in utero. (a) Active place avoidance test results and (b) learning curves for various treatment groups as indicated by decreased entrances into the shock zone in subsequent trials. *** \( P < 0.001 \) compared with all the groups. APA, place avoidance; Dex, dexamethasone, FA, folinic acid.

Figure 3. Set-shifting deficits in rats exposed to FRα antibody in utero. (a) Conflict place avoidance test results for various treatment groups. Of the animals exposed to antibody during gestation, only 25% could pass the active place avoidance task and 40% of these could not complete the conflict avoidance task. This is indicative of impairment in completing set-shifting tasks as 93% of sham animals that passed active place avoidance could pass conflict avoidance. This impairment was corrected by dexamethasone (Dex), folinic acid (FA) and folinic acid plus dexamethasone treatment. (b) Tracing the movement of the rat in the fourth trial of the active and conflict place avoidance tasks.

Not all of the 12 \( \mu \)g Ab/embryo-exposed rats that passed the active avoidance task were able to pass the CPA task, (Figure 3a). Further, administration of folinic acid and dexamethasone rescued these rats from the CPA learning deficits (Figure 3a). Representative tracings illustrating the movement of rats in the APA and CPA tasks are shown in Figure 3b, demonstrating the inability of the Ab-exposed animals to learn the tasks as indicated by repeated entry into the shock sector and the ability of the animals in the treatment groups to learn the tasks. No significant difference between the treatment groups of folinic acid, dexamethasone or folinic acid plus dexamethasone following Ab exposure was noted in any of the behavioral and cognitive studies, suggesting no detrimental effect of the steroid use.
Folate uptake, localization of Ab and inflammation

During fetal development, maternal folate is the only source of folate and this has to traverse the placental barrier in order to enter fetal circulation. One potential mechanism by which the Abs can affect brain development is by blocking folate transport to the fetus. Ab localization was seen mostly in the placenta and in the uterus and much less in the embryo (Figure 4b-1). Within the embryo, Ab was most localized to epithelial cells in the head.
region (Figure 4b-2) and to the epithelial cells of thechoroidplexus (Figure 4b-3). During this period, folate transport to theembryo was significantly compromised as indicated by a 56% and41% decrease in $^{3}$HPGA accumulation in the placenta at 48 and72 h, respectively, and ~76% decrease in the embryo (Figure 4c).Ab localization within the placenta and yolk sac appeared to affectfolate transfer from the mother to the fetus and folate uptakeinfetuses. Localization of IgG was not seen in the NRIgG-injecteddame.

Rodent studies of maternal inflammation during pregnancy thatincrease risk of neurodevelopmental deficits in the offspring haveshown increased CD68$^{+}$ cells and interleukin (IL)-1$^{\beta}$ in theplacenta.$^{32,33}$ This prompted us to examine whether the localizationof this Ab in the placenta induced a similar inflammatoryresponse. Consistent with the presence of Ab on the placenta,increased expression of CD68$^{+}$ cells and IL-1$^{\beta}$ was seen insimilar areas (Figure 4d). Dexamethasone treatment significantlydecreased the expression of both these markers for inflammation(Figures 4d-1 and d-2). Dexamethasone treatment also significa ntlyincreased folate transport to the placenta, yolk sac and amnion and theembryo (Figure 4e). Rats exposed to Ab in utero were killed after PND 60 and examined for the presence of anystructural changes in the brain using coronal sections stained withhematoxylin-eosin and for markers of inflammation. No discernablehistological changes were observed compared with sham or simples controls.

**DISCUSSION**

In this study, we provide evidence that exposure to FRα-specificAb during gestation in a rat results in the birth of pups with severebehavioral and learning deficits. Many of these deficits mirror coredeficits of ASD, including deficits in communication, sociabilityand difficulty with set-shifting tasks. Lack of bonding, socialinteraction and verbal communication are some of the earlyindicators of autism development although these may not fullymanifest until later in life. In the rodent model, ultrasonicvocalizations are one of the earliest forms of communicationbetween the pups and their mother.$^{22}$ Thus evaluating therat pups on PND4 is ideal. As observed in this study, lack ofcommunication has been reported in many rodent models ofautism-like behavior.$^{34-36}$ Additionally, we have shown thepresence of variable symptoms of ASD, including increasedanxiety in the open-field test and learning deficits in the placeavoidance tasks. We have shown that both of these core deficitsand variable symptoms produced by Ab exposure can beprevented, or at the least attenuated, using gestational treatmentwith folinic acid and dexamethasone.

Further, intellectual disability has been found to be highlycomorbid with ASD.$^{37}$ We have previously shown that exposure toAb during gestation and the preweaning period in rats leadstowards learning deficits.$^{21}$ We have confirmed this effect of gestationalexposure in the current study and, more importantly, shown thatthese learning deficits can be prevented by treatment with folinicacid and dexamethasone.

Overall, it appears that FRα Abs exert their effect on the fetus bydecreasing transplacental transport of folate, which results in fetalfolate deficiency. This explains why protection is afforded byfolinic acid treatment where folinic acid, a reduced form of folate,is taken up by the reduced folate carrier, rather than by FRα, whichis hindered by the Abs from transporting folate.

Epidemiological studies suggest a clear association betweenmaternal exposure to infection and inflammation and increasedrisk of ASD in the offspring.$^{38}$ Maternal cytokine expression,including expression in the placenta, can affect the developingembryo or fetus, resulting in neurological and behavioralabnormalities.$^{39}$ These studies suggest that FRα Ab exposure inan animal model leads to a type II autoimmune reaction,$^{40}$ wherethere are increased Fc-receptor-bearing phagocytes, as demonstratedby the increased presence of CD68$^{+}$ macrophages. These macrophagesappear to secrete increased amounts of IL-1$^{\beta}$ cytokine in the presence of FRα Ab, leading to increasedinflammatory response. When the inflammatory response issuppressed with dexamethasone, there is more folate uptakealong with rescue of behavioral deficits. Hence, the benefit afforded bydexamethasone treatment also appears to be the result of increased folate uptake, where the dexamethasone suppresses inflammatory reaction around FRα. The inflammatory response owing to autologous Abs in the FRα autoimmune disorder in humans may vary from that owing to the heterologous Abs we have used in our rat model. Regardless, the Abs appear to cause immune-mediated damage and block folate transport.

These mechanisms of impaired folate transport can potentiallyexplain pregnancy-related disorders, including neural tube defectsin the presence of FRα autoimmune disorder. Daily folate intakeand folate supplementation during pregnancy may minimize theeffect of the autoantibody but may not be sufficient in those withhigher Ab titer. Therefore, identifying women positive for theautoantibody and treating them with high-dose folinic acid alongwith other interventions to lower the Ab titer are effectivestrategies for a favorable outcome. A case that utilized such astrategy showed positive results in preventing pregnancy-relatedcomplications owing to the presence of FRα autoimmune disorder.$^{41}$ Our rat study suggests that steroids and otherimmunosuppressant drugs could decrease placental inflammationinduced by the FRα autoantibody. Consequently, further investiga tion of similar treatment options to prevent pregnancy-related complications owing to FRα autoimmune disorder is warranted.

It should be noted that the methods for the detection of FRα-specific autoantibodies in the serum of patients provide a measure ofautoantibody titer in serum.$^{39}$ The FRα protein is a glycolip phosphatidylinositol-linked peripheral membrane protein andAbs directed against this protein would seek the target antigen onthe membrane.$^{42}$ Therefore, Ab in circulation is likely to represent excess free Ab. As in many autoimmune disorders, fluctuations in Ab titer is a common occurrence,$^{43,44}$ and therefore testing patients multiple times during 2–6 months may be necessary to rule out the autoimmune disorder. The prevalence of autoantibodies in $>$70% of children with autism$^{18}$ and improvements in the core symptoms of autism with folinic acid treatment$^{5,18}$ provides compelling evidence linking the auto immune disorder with autism. Parental FRα autoantibodies witheither mother or father positive have also been linked to autism in children.$^{19}$ Particularly, mothers of children with ASD were found to have a significantly higher prevalence of FRα autoantibodies compared with controls (26% vs 3.3%, $P < 0.01$),$^{19}$ and therefore identifying and treating these mothers could potentially reduce the risk of autism in the offspring.

In conclusion, the findings of this study suggest severebehavioral and cognitive changes mirroring ASD symptomsfollowing gestational Ab exposure in a rat model and protectionafforded by folinic acid and dexamethasone treatment. This hasmajor implications in the treatment of FRα autoimmune disorderin women during pregnancy and reducing the risk of autism in theoffspring.

**CONFLICT OF INTEREST**

Two of the authors (JMS and EVO) are inventors on a US patent for the detection of FRα autoantibodies issued to the Research Foundation of the State University of New York, USA. The other author declares no conflict of interest.

**ACKNOWLEDGMENTS**

Funding for this work was provided by Autism Speaks grant no. 8202.
AUTHOR CONTRIBUTIONS
AD, JMS and EVQ designed the studies. AD and JMS conducted all experiments. AD, JMS and EVQ participated in the writing of the manuscript.

REFERENCES
42 Desai A, Sequeira JM, Quadros EV. The metabolic basis for developmental disorders due to defective folate transport. Biochimie 2016; 126: 31–42.

Supplementary Information accompanies the paper on the Molecular Psychiatry website (http://www.nature.com/mp)