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Maternal IgG suppresses NMDA-induced spasms in infant rats and inhibits NMDA-mediated neurotoxicity in hippocampal neurons

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Abstract

Maternal immunoglobulin G (IgG) was derived from Wistar rats that just delivered the new offsprings. We examined the effect of this maternal IgG on infantile spasms induced by *N*-methyl-D-aspartate (NMDA) in immature rats. Pup animals were treated subcutaneously with 10 mg/kg/day maternal IgG from day 11 to day 15 after birth followed by a single intraperitoneal dose of NMDA (15 mg/kg). Administration of maternal IgG decreased the severity and increased the number of ACTH immunoreactive cells in the cortex of rats with NMDA-induced spasms. Furthermore, maternal IgG inhibited NMDA-induced intracellular LDH activity in cultured hippocampal neurons in a dose-dependent manner. The results indicate that maternal IgG can attenuate NMDA-induced seizures. In infantile spasms, some factors may during pregnancy negatively affect the transfer of maternal IgG from mother to fetus thereby causing a decrease in the amount of protective maternal IgG.

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Keywords: *N*-methyl-D-aspartate (NMDA); Maternal immunoglobulin G (IgG); Primary neuronal cell culture; Infantile spasms

1. Introduction

West syndrome is an age-specific epileptic syndrome comprised of the triad infantile spasms, hypsarrhythmic EEG pattern and mental retardation (Dulac et al., 2002). Several studies (Riikonen, 2001; Hrachovy and Frost, 1989; Brunson et al., 2002) demonstrated that the adrenocorticotrophic hormone (ACTH) was effective in suppressing infantile spasms. Therefore, it has become a mainstay in West syndrome therapy (Dulac et al., 2002). However, West syndrome belongs to the catastrophic epilepsies of childhood, because in about 25% of cases it is refractory to any therapy including ACTH. Additionally, in some responsive cases when ACTH suppresses the spasms and normalizes EEG, mental retardation and developmental regress still persist. ACTH is part of the hypothalamic–pituitary–adrenocortical (HPA) axis produced by the

adrenohypophysis under control of hypothalamic corticotrophin releasing hormone (CRH) and suppressing CRH release by a negative feedback mechanism (Brunson et al., 2001a,b). Accordingly, one explanation of the clinical response to ACTH could be the suppression of CRH production. In particular, studies of Baram's group argue for a role of low ACTH and high CRH activity in the pathogenesis of West syndrome (Brunson et al., 2001a,b; Baram et al., 1995).

As a neuro-hormone *ACTH* has different effects on the body affecting arousal, memory, temperature regulation and recovery from damage in the central nervous system (CNS). However, the mechanisms of action of successful ACTH therapy of West syndrome remain widely unknown. Several studies showed that the immune system was out-of-balance in patients with infantile spasms as indicated by decreased serum IgG or IgA (Zou et al., 2003), decreased CD4/CD8 ratio (Montelli et al., 2003), and altered serum cytokine levels (Liu et al., 2001) suggesting that ACTH may act via modulation and normalization of the altered immune system.

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It has been suggested that NMDA receptors may play a potential role in the pathogenesis of infantile spasms (Kábová et al., 1999; Mares, 1992; Stafstrom and Sasaki-Adams, 2003). Intraperitoneal injections of NMDA into developing and young adult rats caused hyperflexion (emprosthotonic) seizures, consisting of repetitive tonic, whole body flexion, which are reminiscent of infantile spasms (Kábová et al., 1999; Mares, 1992; Stafstrom and Sasaki-Adams, 2003). In developing rats, the EEG of the seizure consisted of periods of suppression mixed with ictal activity of serrated waves and high-voltage chaotic EEG activity (Kábová et al., 1999; Mares, 1992). In adult rats, NMDA-induced EEG alterations involved spike and spike-and-wave activity (Kábová et al., 1999). NMDA also deteriorated performance of young rats in the open field, rotorod, and elevated plus maze tests (Kábová et al., 1999; Stafstrom and Sasaki-Adams, 2003). It is known that many prenatal factors such as infections and hypoxia are related to infantile spasms (Wong and Trevathan, 2001) and it has also been shown that stress in pregnancy affects the infants' immune system (Tuchscherer et al., 2002; Schwerin et al., 2005). Therefore, we supposed that there may be a correlation between infantile spasms and maternal immune factors. The maternal immunoglobulin G (IgG) is the only Ig capable of penetrating the placenta and it is considered to be an indicator of the humoral immune system. In the present study, we attempt to explore the effect of maternal IgG on NMDA-induced seizures and on ACTH expression in the brain in a rat model of infantile spasms in order to contribute to the elucidation of possible mechanisms of the spasms. Additionally, effects of maternal IgG on NMDA-induced cytotoxicity are tested in hippocampal cell cultures.

2. Materials and methods

2.1. Animals

Experiments were performed using 4 female Wistar rats (body weight 300–450 g) purchased from the Experimental Animal Center of The Academy of Military Medical Sciences, China, and their respective rat pups as well as 4 wet-nurse rats. We used a total of 51 pups (15–20 g) eleven days old (the day of birth was considered as day 1) including 23 females and 28 males. The pups were separated from their 4 mothers at birth and raised by 4 wet-nurse rats. All rats were kept on a 12 h/12 h-light/dark cycle under controlled temperature and humidity and had free access to food and water.

2.2. Maternal IgG extraction

- (1) Establishing a standard protein curve for protein quantification. Calf serum (1 mg/ml) was added to distilled water and used to produce solutions of serial protein concentrations, which were estimated by an ultraviolet spectrometer. These protein solutions served to estab-

lish a calibration curve with a regression factor R^2 of 0.9997.

- (2) Maternal IgG extraction. Pregnant rats (body weight 300–450 g), specific pathogen-free (SPF) grade; were purchased from the Experimental Animal Center of The Academy of Military Medical Sciences, China. Blood (about 10 ml) was drawn from Wistar rats who had just delivered offsprings. The blood was stored overnight at 4 °C and the serum was extracted the next day. IgG was extracted by ammonium sulfate salt fractionation. Maternal serum was added to equal amounts of 0.9% NaCl. Subsequently, ammonium sulfate was added drop by drop with vortexing after each drop until a concentration of 40% was reached. The solution was incubated for 30 min and centrifuged at 3000 rpm at 4 °C for 15 min. The remaining serum was discarded. 2 ml of 0.9% NaCl solution was added to the pellet and subsequently mixed with ammonium sulfate in the same manner as described above. The solution was again incubated for 30 min and centrifuged at 3000 rpm at 4 °C for 15 min. The supernatant was discarded leaving IgG in the pellet. The protein was added to 0.9% NaCl (approx. 3–4 ml) and placed into an osmosis bag. After 3 h, the bag was placed in bath of 0.0175 M ammonium sulfate buffer (pH 6.3) and left for three days at 4 °C exchanging PBS 4 times per day. IgG was extracted by cellulose DEAE-52 (Whatman) chromatography. The ammonium sulfate protein solution was added to the DEAE-52 column. The solution was eluted at 20 drops/min for 2 h to a volume of 3 ml per tube. 20% salicylic acid was used to test the presence of protein. The OD of the eluents was measured by ultraviolet spectroscopy and used to calculate the amount of protein present in the eluents. The protein stock solution was kept at 4 °C before use at a concentration of 1 g/l. The purity of IgG was assessed by agarose gel electrophoresis.

2.3. Treatment with NMDA and maternal IgG

NMDA was dissolved in normal saline (0.9% NaCl, NS) at a concentration of 1 mg/ml. Maternal IgG was adjusted to a concentration of 1 mg/ml after dialysis with NS. 51 pups were obtained from 4 mothers and randomly sorted into four groups.

- (1) Control group (Saline+Saline): 9 pups (4 females and 5 males) received daily subcutaneous injections of NS at 9 a.m. from day 11 to day 15 after birth. At day 15 an additional intraperitoneal injection of NS was given 1 h after the subcutaneous NS injection.
- (2) Sham treated group (Saline+NMDA): 12 pups (5 females and 7 males) received daily subcutaneous injections of NS at 9 a.m. from day 11 to day 15 after birth. At day 15 an additional intraperitoneal injection of NMDA (15 mg/kg) was given 1 h after the subcutaneous NS injection.

- (3) Normal rat IgG treated group (Normal IgG+NMDA): 15 pups (7 females and 8 males) received daily subcutaneous injections of normal rat IgG (10 mg/kg, DrGenes Biologics Ltd) at 9 a.m. from day 11 to day 15 after birth. At day 15 an additional intraperitoneal injection of NMDA (15 mg/kg) was given 1 h after the subcutaneous IgG injection.
- (4) Maternal IgG group (Maternal IgG+NMDA): 15 pups (7 females and 8 males) received daily subcutaneous injections of maternal IgG (10 mg/kg) at 9 a.m. from day 11 to day 15 after birth. At day 15 an additional intraperitoneal injection of NMDA (15 mg/kg) was given 1 h after the subcutaneous IgG injection.

2.4. Evaluation of NMDA-induced status epilepticus

The number of seizures was counted after NMDA had been injected. Rats were observed for seizure activity and latencies to the various seizure stages were recorded for 3 h. Seizures were evaluated as described by Kábová et al. (1999). Latencies to two NMDA-dependent stereotypical behaviors were noted: tail twisting and exaggerated tonic whole-body flexions with a loss of righting reflex resembling a curled ball (emprosthotonus seizures) indicated the status epilepticus. Furthermore, head and spinal flexion including forelimb flexion and hindlimb hip-joint flexion was involved in the status epilepticus.

2.5. ACTH immunohistochemistry

Sixteen brains (4 animals per 4 groups) were obtained 3 h after subcutaneous injections of NMDA or NS, cut into 50 µm thick sections, rinsed with 0.01 M phosphate buffered saline (PBS) three times and treated with 1 N hydrochloric acid for 30 min. After another rinsing with double distilled water (DDW) sections were incubated with 3% H₂O₂ in PBS for 30 min at room temperature and rinsed again with PBS. Sections were blocked with 5% normal goat serum for 30 min and incubated with the primary rabbit anti-ACTH antibody (Beijing Biosynthesis Biotechnology, China) (diluted 1:1000 with PBS) for 48 h at room temperature. After rinsing with PBS, sections were incubated with the secondary antibody (diluted 1:300 with PBS). Biotinylated goat anti-rabbit IgG was used as secondary antibody (Beijing Biosynthesis Biotechnology) and applied for 3 h at room temperature and overnight at 4 °C. After another PBS rinse, sections were incubated with streptavidin-horseradish peroxidase (diluted 1:300 with PBS) for 3 h at room temperature. Positive reactions were visualized in DAB solution (Beijing Biosynthesis Biotechnology) containing 0.1% H₂O₂ at room temperature. No ACTH immunoreactivity was detected in controls omitting the primary antibody. Numbers of ACTH immunoreactive cells in the vertex cortex, temporal cortex were measured by image analysis. Images were captured using a Leica DCRE microscope with Leica DC500 camera and Leica IM50 Image Manager software

(Leica Microsystems, Wetzlar, Germany). Numbers of cells were counted at ×20 magnification in the entire section areas.

2.6. Hippocampal cell culture

Primary hippocampal cell cultures were prepared according to the method described by Sanfeliu et al. (1990). The hippocampi were isolated from neonatal offsprings of Wistar rats (less than 24 h old of either sex, 5–6 g, SPF grade; purchased from the Experimental Animal Centre of War Academy of Science, China). The tissue was transferred to 15 ml tissue culture tubes and the volume was adjusted to 1–2 ml with PBS (pH 7.4). The hippocampi were mechanically dissociated by sucking through a Pasteur pipette (20 times) followed by sucking through a reduced-bore Pasteur pipette (20 times). The cells were pelleted by centrifugation at 1000 rpm for 5 min, resuspended in Dulbecco's modified Eagle's medium (DMEM)/F-12 Medium (Gibco-BRL, USA) and plated into 35 mm dishes or 96-well plates precoated with 0.1% poly-L-lysine at densities of 1×10^6 /ml (2 ml/dish and 100 µl/well, respectively). On the third day, 10 µmol/l cytosine arabinoside (Ara-C) was added to inhibit replication of non-neural cells. Two days later, fresh culture solution substituted the Ara-C-containing solution. Experiments were performed after day 8 of culturing. For that purpose, cultures were randomized into four groups ($N=12$ per group): (1) control, (2) NMDA (50 µmol/l NMDA), (3) NMDA+maternal IgG 10 mg/l (50 µmol/l NMDA+10 mg/l maternal IgG), (4) NMDA+maternal IgG 100 mg/l (50 µmol/l NMDA+100 mg/l maternal IgG). Culture medium was replaced by Mg²⁺-free Earles solution supplemented with 10 µmol/l glycine and 50 µmol/l NMDA only or additional 10 mg/l and 100 mg/l maternal IgG, respectively. Controls received medium only instead of NMDA. Lactic dehydrogenase (LDH) activity was assayed after 24 h of treatment.

2.7. Lactic dehydrogenase (LDH) assay

LDH assay was provided by Nanjing Jiancheng Biological Laboratory, China, and the method was performed according to the manufacturer's instruction. Briefly, 4-dinitrophenyl-hydrazine was added to the hippocampal cell cultures for the last 15 min of culture at 37 °C. The absorbance of the reaction mixture was recorded at 440 nm and the LDH activity was calculated from the slope of the linear absorbance curve. The LDH activity is expressed in conventional units/g protein (U/g prot).

2.8. Statistical analysis

All data are expressed as means±standard deviations (SD) and analyzed for significant differences between groups by non-parametric Kruskal–Wallis test (for clinical data) and by two-way ANOVA of repeated measures (for

histochemistry and LDH data). *P*-value less than 0.05 was considered as statistically significant.

3. Results

3.1. Effect of maternal IgG in vivo

3.1.1. Clinical observations

Intraperitoneal injection of NMDA (15 mg/kg) into 15 day old rat pups elicited epilepsy-like symptoms. Rats were observed for seizure activity and latencies to the various stages. Specific characteristics of the curling status epilepticus were the following: spine curling, head meets the tail, paws curl against the body, entire body forms ball-like shape, seizures occur singularly or continuously, some pups undergo tonic seizures or even death. We evaluated the number of seizures in each group and found no significant differences between males and females. For the whole groups, the difference of numbers of seizures was significant between the sham treated group and the maternal IgG treated group ($p < 0.05$), but not between the sham treated group and the normal rat IgG treated group (Table 1). Thus only the maternal IgG treated group reduced the number of NMDA-induced seizures significantly.

3.1.2. ACTH immunohistochemistry

ACTH immunoreactive cells were mainly found in the cortex with dominance of the temporal cortex and vertex cortex. Positive cells were elliptical or irregularly shaped and the cytoplasm appeared dark brown. Differences between sections derived from male and female animals were not statistically significant. When comparing the data from the whole groups, the ACTH expressing cells in the vertex cortex were significantly higher in both the maternal IgG treated group ($p < 0.01$) and the normal rat IgG treated group ($p < 0.05$) compared to the sham treated group (Table 2). The

Table 1
Clinical observations

Groups	Number of seizures (mean±SD)	Rats without seizures (N)	Deaths (N)
(1) Control group (saline+saline), N=9	0	9	0
(2) Sham treated group (saline+NMDA), N=12	31.2±20.8	2	3
(3) Normal IgG treated group (normal rat IgG+NMDA), N=15	15.6±5.4	2	2
(4) Maternal IgG group (maternal IgG+NMDA), N=15	2.5±1.3	3	1

Rats received saline, normal rat IgG and maternal IgG, respectively, from day 11 to day 15 after birth by subcutaneous injection. Rats received saline or NMDA, respectively, at day 15 by intraperitoneal injection. The difference of numbers of seizures was significant according to the Kruskal–Wallis test between the sham treated group and the maternal IgG treated group ($p < 0.05$), but it was not significant between the sham treated group and the normal rat IgG treated group.

Table 2
ACTH positive cells in the temporal and vertex cortex

Cortex's region	Temporal cortex	Vertex cortex
Normal control group (saline+saline)	35.75±10.5	33.5±21.8
Sham treated NMDA group (saline+NMDA)	118.7±19.8	84.0±35.0
Normal IgG treated NMDA group (normal IgG+NMDA)	129.5±13.5	112.2±14.1
Maternal IgG treated NMDA group (maternal IgG+NMDA)	226.5±20.1	161.5±24.6

Rats received saline, normal rat IgG and maternal IgG, respectively, from day 11 to day 15 after birth by subcutaneous injection. Rats received saline or NMDA, respectively, at day 15 by intraperitoneal injection. The sixteen brains were obtained 3 h after subcutaneous injections of NMDA or saline. Numbers (mean±SD) of ACTH positive cells in the temporal and vertex cortex of Wistar rat pups with different treatment as assessed by immunohistochemistry are given. Statistical evaluation by two-sided ANOVA for repeated measures revealed the following significant differences: in temporal cortex: maternal IgG treated vs sham treated ($p < 0.05$); in vertex cortex: maternal IgG treated vs sham treated ($p < 0.01$), normal rat IgG treated vs sham treated ($p < 0.05$), maternal IgG treated vs normal rat IgG treated ($p < 0.05$). Comparisons with the normal control group were all significant ($p < 0.01$).

difference between the maternal IgG treated group and the normal rat IgG treated group was also significant ($p < 0.05$). In the temporal cortex, the difference of ACTH immunoreactive cells was significant between the sham treated group and the maternal IgG treated group ($p < 0.05$), but not between the sham treated group and the normal rat IgG treated group. All comparisons with the normal control group were significant ($p < 0.01$).

3.2. Effect of maternal IgG in vitro

NMDA treatment resulted in an increase of LDH release from hippocampal neurons ($p < 0.01$ for comparison of NMDA treated neurons versus untreated neurons) (Table 3). Maternal IgG at two different concentrations (10 mg/l and 100 mg/l) inhibited the NMDA-induced LDH release

Table 3
LDH assay of hippocampal neurons

Group	IgG (mg/l)	LDH (U/g prot)	
		Before treatment	After treatment
Normal control		150.20±13.93	167.82±15.36
NMDA		144.82±9.60	338.60±17.73* ^Δ
NMDA+maternal IgG	10	155.67±17.57	293.70±20.19* ^Δ
NMDA+maternal IgG	100	154.74±12.79	269.63±17.16* ^Δ ♦

Neuronal hippocampal cell cultures derived from newborn Wistar rats were grown for 8 days in vitro and subsequently exposed to NMDA with and without maternal IgG for another 24 h ($N=12$ for each group). Culture medium was replaced by Mg^{2+} -free Earles solution supplemented with 10 μ mol/l glycine and 50 μ mol/l NMDA only or with additional 10 mg/l and 100 mg/l maternal IgG, respectively. Normal controls received medium instead of NMDA. LDH release was assayed after a 24 h treatment. Statistical evaluation by two-sided ANOVA for repeated measures revealed the following significant differences: * $p < 0.01$ vs normal control; ^Δ $p < 0.01$ vs NMDA; ^Δ $p < 0.01$ vs before treatment; [♦] $p < 0.05$ vs NMDA+maternal IgG (10 mg/l).

significantly, whereby the higher concentration was more effective ($p < 0.01$) than the lower concentration ($p < 0.05$).

4. Discussion

Immunological mechanisms have previously been suspected to play a role in the pathogenesis of infantile spasms (Liu et al., 2001). Beneficial effects of IgG treatment in epilepsy were first observed by Pechadre et al. (1977). Since the initial report, IgG has also been employed in several trials of treatment of intractable epilepsy (Echenne et al., 1991). The regimens used in these trials included the application of 400 mg/kg IgG daily for 5 days, called high-dose intravenous IgG treatment (Van Engelen et al., 1994). It was useful for the treatment of cryptogenic West syndrome and for inhibiting brain deterioration caused by epileptic encephalopathy (Duse et al., 1996). The present study shows that treatment with maternal IgG at a dose of only 10 mg/kg daily for 5 days reduces the intensity of spasms in the NMDA-induced seizure model of rat pups, which mimics major features of human infantile spasms. Pretreatment with maternal IgG reduces curling seizures. Additionally, maternal IgG-treated rats show increased numbers of ACTH immunoreactive cells, especially in the temporal cortex.

An efficient humoral immune defense of newborn offsprings depends on the passage of a sufficient amount of maternal IgG through the placenta, since the fetus does not produce its own IgG and IgG is the only can successfully pass through the placenta. We conclude that maternal IgG has not only anti-infective effects, but does also protect infantile offsprings against NMDA-induced spasms probably by increasing the ACTH concentration.

NMDA is an active toxin for the CNS, especially in developing organisms (Stafstrom and Sasaki-Adams, 2003). It causes a breakdown of the blood–brain barrier (Miller et al., 1996). Our results show that the brains of NMDA-treated pups display significant increased numbers of ACTH immunoreactive cells. It has also been shown that the CNS contains melanocortin receptor (MCR) (Brunson et al., 2001b, 2002). ACTH can combine with MCR to induce its physiological effects. Bardgett et al. (1992) have shown that NMDA-treated pups displayed an increase of the ACTH plasma concentration. The increase of cortical ACTH immunoreactive cells in the NMDA-treated animals in our study suggests that ACTH may have a counteracting protective effect on the CNS preventing a further escalation of NMDA-induced neurotoxicity and spasms.

Our investigation reveals that subcutaneous application of maternal IgG at a dose of only 10 mg/kg causes a partial resistance to the toxicity NMDA. In addition, we measured the effect of maternal IgG on NMDA-induced neurotoxicity on hippocampal neurons in vitro by the LDH assay. Measurement of LDH activity in extracellular culture media has been found to be a simple and quantitative method for assessing glutamate mediated central neuronal cell injury. Extracellular LDH is both chemically and biologically sta-

ble. The magnitude of LDH efflux in the cultures correlates in a linear fashion with the number of neurons damaged by glutamate exposure (Koh and Choi, 1987). Our study shows that maternal IgG at concentrations between 10 mg/l dose-dependently inhibits NMDA-induced LDH release. Thus, maternal IgG may interact directly or indirectly with receptors for excitatory amino acids. Likewise the *in vivo* protective effect of maternal IgG against the toxicity of excitatory amino acids could be mediated both directly or indirectly via increase of ACTH.

The group of Tuchscherer et al. (2002) found that stress during pregnancy in pigs decreased the serum IgG level of the offsprings suggesting that gestational stress affects the ontogeny of the fetal immune system with consequences to immune response and susceptibility to diseases of the offsprings. We conclude that some factors may negatively affect the transfer of maternal IgG from mother to fetus in infantile spasms causing a decrease in the amount of protective maternal IgG in the infant's serum. This decrease could influence the body's regulation of ACTH leading to a loss of the normal effectiveness of maternal IgG for indirect protection of the CNS thereby facilitating infantile spasms. It is suggested that maternal IgG, through certain as yet unknown mechanisms can increase the amount of ACTH in the brain and lower the intensity of spasms. The specific mechanisms of this relationship will be further investigated.

In summary: (1) Pretreatment with maternal IgG reduces NMDA-induced spasms in a rat model of infantile spasms. (2) Systemic NMDA administration increases ACTH expression in the cortex of rat pups. (3) Maternal IgG pretreatment increases the number of ACTH immunoreactive cells in the brain of rat pups further, suggesting that maternal IgG protects the CNS at least partly by elevating the ACTH concentration in the brain. (4) Maternal IgG protects primary rat hippocampal neurons against injury by NMDA in a dose-dependent manner.

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