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Expressions of CD Markers, Anti-NMDAR and Anti-GAD Antibodies, and Serum Levels of Immunoglobulins in Children with Autoimmune Encephalitis

Shiping Wang[1*], Yingzi Zhang[2], Lili Yang[3], Rong Zhu[4], Hong Zhou[5] and Ping Zeng[6]

[1]Department of Pediatrics, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan, 611731, China. E-mail: xcb57851@126.com
[2]Department of Pediatric Cardiology, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan, 611731, China. E-mail: 3iykgx@jczye.com
[3]Department of Pediatric Surgery, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan, 611731, China. E-mail: zkcok0@jczye.com
[4]Pediatric Nephrology Department, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan, 611731, China. E-mail: cctp4z@jczye.com
[5]Neonatology Department, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan, 611731, China. E-mail: zq33bh@jczye.com
[6]PICU, Chengdu Women's and Children's Central Hospital, School of Medicine, University of Electronic Science and Technology of China, Chengdu, Sichuan, 611731, China E-mail: w6e65e@jczye.com

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ABSTRACT This study investigates the levels of expressions of various CD markers, anti-NMDAR, anti-GAD antibodies; and the serum levels of immunoglobulin in children with autoimmune encephalitis (AE), and to find out their relationships with the degree of complications of AE. A total of 82 children were divided into patients (52 patients with AE) and control (30 healthy patients) group. Patients' expressions of anti-NMDAR and anti-GAD antibodies was determined in blood and cerebrospinal fluid using ELISA kits. Levels of CD markers, and the concentrations of IgA, IgG, and IgM were tested in blood sample patients. The levels of CD3 and CD4 patients were significantly lower in the patients' group, but, levels of CD8 and CD54 were similar in the two groups. The CD4/CD8 ratios patients was significantly decreased ($p < 0.05$) in the patients' group. Serum levels of IgA, IgG and IgM in the patients' group were significantly increased ($p < 0.05$), when compared to those of the control group. There were also significant differences ($p < 0.05$) in the levels of IgA, IgG and IgM in the four patients' subgroups. Patients' study found a negative correlation of CD markers levels and a positive correlation of these immunolglobulins with degree of complications of AE.

INTRODUCTION

Autoimmune encephalitis (AE), is a condition which occurs when the immune system makes auto-antibodies that attack healthy brain cells wrongly identifying them as foreign. It is more prevalent among children and teenagers, characterized by cerebral nerve dysfunction and clinical manifestations such as convulsion, delirium, consciousness and cognitive disorder, and abnormal behavior (Chen and Deng 2016). Early skull MRI find focus local lesion in cerebral limic lobes such as hippocampus, hook-like gyrus, callosum, hypothalamus and mesencephalon, while ECG shows multiple forms such as spike wave, slow wave and diffused wave (Sun 2016). Studies have shown that its incidence is

[*]*Address correspondence:*
Shiping Wang,
Department of Pediatrics,
Chengdu Women's and Children's Central Hospital,
School of Medicine, University of Electronic Science and Technology of China, Chengdu,
Sichuan, 611731, China.
Cell: 86-18922964423
E-mail: xcb57851@126.com

related to NMDAR of nerve cell surface, GAD, GABAR and their corresponding antibodies (Wang and Liu 2017). Neuropathological autopsy of patients with AE have revealed a large amount of lymph cell infiltration in cerebral lesions, an indication that AE is closely related to lymph cell-mediated immune reaction (Dale et al. 2017). T-lymphocytes mediate cell immunity *in vivo* by directly killing target cells and stimulating B-cells to produce antibodies (Scheer and John 2016). T-lymphocytes are grouped into auxiliary T cells (CD4), cytotoxic T cells (CD8) and inflammation-related T cells (CD54), based on their different cell surface receptors (Bakkali et al. 2019; Gengzangdajie et al. 2018). B-lymphocytes are proliferated and differentiated into plasmocytes which secrete antibodies such as IgA, IgG and IgM after interaction with antigen-presenting cells (APC) such as T cells and mononuclear macrophages. Studies have shown that the expressions of various CD markers, anti-NMDAR and anti-GAD antibodies; and the serum levels of IgA, IgG and IgM change with the degree of complications of AE (Ren et al. 2016; Cui et al. 2018). At present the pathogenesis of AE is unclear, and severity of AIE vary among individuals which is always a challenge to predict (De Bruijn et al. 2019; Cui et al. 2018). Therefore, it is necessary to find newly differential diagnostic and prognostic markers on these disorders. In this study the levels of few CD markers, anti-NMDAR and anti-GAD antibodies; and the serum levels of different immunoglobulins were determined in children with autoimmune encephalitis (AE), and their association to the degree of complications in AE was studied.

MATERIAL AND METHODS

Patients and General Data

Ethical approval for the study was obtained from the Ethics Committee of Chengdu Women and Children Central Hospital, China. A total of 82 children were recruited over a period of 3 years in this study. They were divided into two groups: patients group of 52 patients with AE and control of 30 healthy patients. Patients in the patients group were further divided into four subgroups: highly complicated, moderately complicated, general and simple. Patients presenting clinical manifestations such as epilepsy, disturbances of consciousness, abnormal behavior and biochemistry of cerebrospinal fluid, changes in cytology, and edge cerebral lobe injury did not receive any immunotherapy before admission to hospital; and signed written informed consent were included in this study. Patients with systematic organ dysfunctions; with tumor, immunodeficient, with connective tissue diseases and hematological diseases; and who had cerebral diseases such as viral encephalitis and tuberculosis cerebropathy were excluded from the study.

Determination of Anti-NMDAR and Anti-GAD Antibodies

Cerebrospinal fluid (5 ml) was taken from patients in the patients group prior to treatment, while 5 ml of fasting peripheral venous blood was taken from patients in both groups 8 h after admission into hospital. Fasting venous blood 2 h after collection were centrifuged at 3000 rpm for 15 min and the supernatants were used for the determination of the levels of anti-NMDAR, anti-GAD antibodies, and CD markers (CD3, CD4, CD8, CD54), in the two groups were recorded and analyzed using ELISA kits (AG Co., Ltd. (Germany). The serum was used for the determination of the levels of IgA, IgG, and IgM using IMMAGE 800 specific protein analyzer (AG Co., Ltd. (Germany).

Determination of the Levels of Expressions of CD3, CD4, CD8 and CD54

An aliquot (20 ul) of CD3-FITC/CD4-PE double label, CD8-PE and CD54 FITC antibodies (Rongfei Biological Co., Ltd., Beiging China**)** were separately taken and put in three different flow cytometer tubes, followed by the addition of 100 ul of fresh peripheral venous blood, mixing, and incubation at room temperature for 15 min. This was followed by the addition of 200 ml of 10 percent hemolysin, mixing, and incubation at room temperature for 12 min, and centrifugation at 1500 rpm for 5 min. The resultant precipitate was taken and washed twice, and 1ml balanced solution was added to it, and analyzed using a flow cytometer (Merck Millipore; Burlington, Massachusetts, United States).

Statistical Analysis

The statistical analysis was done with SPSS (23.0). Groups were compared using Kruskal-Wallis test. Numerical data were analyzed using Mann-Whitney U rank sum and Kruskal-Wallis rank sum test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Study Background

The patients' group consisted of 27 males and 25 females aged between 3 and 14 years (mean age = 8.49 ± 2.78 years). There were 21 cases of disturbances of consciousness, 23 cases of epilepsy, 19 cases of mental and behavioral disorders, 16 cases with changes in EEG, and 5 cases with changes in skull MRI iconography. Control healthy group consisted 30 patients including 16 males and 14 females with mean age = 8.51 ± 2.16 years. There was no statistical difference in patients based age and sex.

Expressions of Anti-NMDAR and Anti-GAD Antibodies in the Patients Group

All control samples were negative for anti-GAD and anti-NMDAR antibody. In patients' group only two of the 52 patients had positive results anti-NMDAR antibody, while all were negative for anti-GAD antibody.

Levels of Expressions of CD3, CD4, CD8 and CD54 in the Two Groups

The levels of expressions of CD markers in the patients group were significantly lower ($p < 0.05$) than those of the control group, but there were no significant differences ($p > 0.05$) in the levels of expressions of CD8 and CD54 in the two groups (Table 1). The reduction in CD4 and CD8 was highly significant at level of $p<0.0001$ and CD4/CD8 ratio in the patients group was significantly decreased ($p < 0.05$) compared to the control group.

Levels of Expressions of CD3, CD4, CD8 CD54, and CD4/CD8 in the Four Patients Sub-groups

There were significant differences ($p < 0.05$) in the levels of expressions of CD3 and CD4, and CD4/CD8 in the four patients' subgroups, in the order: highly complicated < moderately complicated < general < simple. However, the level of expression of CD54 in the patients group was not significantly different ($p > 0.05$) from that of control. The levels of expressions of CD54 in the four patients' subgroups were also not significantly different (Table 2). CD4/CD8 ratios was lowest in highly complicated group and maximum in simple group. Within sub group CD 54 expression was similar and not significant different among patients in sub-group.

Table 1: Levels of expressions of CD3, CD4, CD8 and CD54 in the two groups (n, %)

Group	*n*	*CD3*	*CD4*	*CD8*	*CD4/CD8*	*CD54*
Control	30	66.89 ± 13.64	40.25 ± 10.97	23.54 ± 6.98	1.71 ± 0.57	6.45 ± 2.78
Patients	52	55.34 ± 20.75	28.12 ± 7.84	22.26 ± 7.12	1.26 ± 0.46	6.25 ± 1.33
t		3.354	7.415	0.790	5.877	0.578
p		0.001	0.000	0.432	0.000	0.565

Table 2: Levels of expressions of CD3, CD4, CD8, CD54, and CD4/CD8 in the four patients subgroups

Group	*n*	*CD3*	*CD4*	*CD8*	*CD4/CD8*	*CD54*
Highly complicated	11	35.13 ± 10.13	22.11 ± 3.23	20.86 ± 4.97	1.06 ± 0.11	6.17 ± 0.97
Moderately complicated	9	48.07 ± 9.89	26.96 ± 4.01	22.09 ± 4.52	1.22 ± 0.19	6.27 ± 0.69
General	19	59.23 ± 15.67	30.03 ± 4.17	21.76 ± 5.13	1.38 ± 0.17	6.25 ± 1.07
Simple	13	70.93 ± 11.25	35.14 ± 2.83	22.97 ± 4.76	1.53 ± 0.13	6.30 ± 0.72
f		17.38	26.65	0.38	20.83	0.04
p		0.000	0.00020	0.768	0.000	0.988

Serum Levels of IgA, IgG and IgM in the Two Groups

Serum levels of IgA, IgG and IgM in the patients group were significantly increased ($p < 0.05$), relative to those of the control group (Table 3). The increase in IgM was highly significant with p value less than 0.0001 and t value of -3.759.

Serum Levels of IgA, IgG and IgM in the Four Patients Sub-groups

The value of all tested immunoglobulin were least in simple group, while high in highly complicated group. There were significant differences ($p < 0.001$) in the serum levels of IgA, IgG and IgM in the four patients' sub-groups, in the order: highly complicated> moderately complicated > general > simple (Table 4). Level of these immunoglobulin was almost more than double in highly complicated group than simple group.

DISCUSSION

The clinical manifestations of AE are numerous, which include epilepsy, depression, illusion, sleep dysfunction, and amnesia (Huang and Hao 2017; Shao et al. 2017). Anti-NMDAR antibody is the receptor of excitatory amino acids such as glycine and glutamic acid (Ho et al. 2018), and NMDAR consists of two subunits, NR1 and NR2. Anti-NMDAR antibody is expressed mainly on cell membrane of axoneuron. Some authors have speculated that the mechanism of action of anti-NMDAR antibody in the neurons of the frontal cortex and thalamus involves the extrusion of γ-aminobutyric acid (GABA) (De Bruijn et al. 2019), thereby reducing the rate of transfer of glutamate across the postsynaptic membrane, and elevating the concentration of glutamate. The glutamic acid then stimulates an abnormal activation of NMDAR, creating an overload of extracellular Ca^{2+} and inducing the death of nerve cells (Zeng and Pan 2017). Anti-NMDAR-encephalitis is the most common autoimmune encephalitis in which IgG antibodies against the GluN1 subunit of the NMDA receptor are present. It can affect all age groups, but is more common in children and young adults' females (Hara et al. 2018). In children with AE, the level of anti-NMDAR antibody decreases with the tumor, and the onset is characterized by abnormal psychological behaviors such as paranoid, illusion and delusion or epilepsy accompanied with abnormal biochemistry of cerebrospinal fluid, and changes in cytology (Bakkali et al. 2019; Cui et al. 2018). In the present study, only two patients in the patients group had positive expressions of anti-NMDAR antibody. Within the synapses of ectocinerea nerve cells, NMDAR can strip off the carboxyl group of glutamic acid, transforming it into γ-amino butyric acid (Tang et al. 2017). It is known that GAD is encoded by two genes, GAD-1 and GAD-2, and GAD antibody is expressed in cells of the pancreas and cerebral lobe, which are associated with type-1 diabetes, AE and ataxia. Patients with GAD antibody-mediated AE have been found to exhibit constant state of frontal lobe-related epilepsy (Yang et al. 2016). In this

Table 3: Serum levels of IgA, IgG and IgM in the two groups

Group	*N*	*IgA (g/l)*	*IgG (g/l)*	*IgM (g/l)*
Control	30	(0.39, 0.54)	7.49 ± 2.01	0.93 ± 0.30
Patients	52	(0.78, 0.85)	8.74 ± 2.61	1.12 ± 0.33
t		-3.759	2.263	2.594
p		0.000	0.026	0.011

Table 4: Serum levels of IgA, IgG and IgM in the four patients subgroups

Group	*N*	*IgA (g/l)*	*IgG (g/l)*	*IgM (g/l)*
Highly complicated	11	1.23, 0.47	10.36 ± 0.82	1.38 ± 0.17
Moderately complicated	9	0.98, 0.38	9.12 ± 1.02	1.21 ± 0.11
General	19	0.76, 0.53	8.03 ± 1.26	1.08 ± 0.21
Simple	13	0.46, 0.39	6.91 ± 1.23	0.82 ± 0.13
f		29.64	20.29	23.53
p		0.000	0.000	0.000

study the researchers did not see presence of anti-GAD in patients as well in control group. At present, it is generally believed that the incidence of AE is related to abnormal exposure of normal antigens in the body to abnormal antibody production.

In this study, the serum levels of IgA, IgG and IgM in the patients group significantly increased compared to control, and the more complicated the clinical manifestations, the higher the serum levels of these antibodies. These results suggest that there is a positive correlation between high serum levels of IgA, IgG and IgM, and the degree of complications of AE. B-lymphocytes are the only cells capable of antibody production *in vivo* (Zhang et al. 2016; Gengzangdajie et al. 2018). Upon activation by antigens, B-cells can proliferate and produce antibodies against the antigens. These antibodies, based on their structures and functions, include IgA, IgG and IgM; IgA accounts for approximately 15 percent of local immune defense (Vinke et al. 2018; Gengzangdajie et al. 2018). When an external antigen enters the organs or tissues, IgA rapidly binds to it and inhibits its invasion and reproduction, thus providing a first line of defense against the antigen. Immunoglobulin G (IgG) is the major antibody *in vivo* and accounts for more than 70 percent of local immune defense (Dubey et al. 2016). Not only does it neutralize viruses by binding to them, it also neutralizes bacterial toxins and promotes immune response of other immune cells (Dubey et al. 2016). Although IgM accounts for only 10 percent of the total concentration of antibodies, it has strong sterilization, pro-agglutination and phagocytic effects due to its huge molecular structure and timely production (Luo et al. 2016).

In the present study, the levels of expressions of CD3 and CD4, and CD4/CD8 in the patients group were significantly lower than those of the control group, and the more complicated the clinical manifestations, the lower the levels of expressions. There was a negative correlation between the levels of expressions of CD3, CD4, CD4 / CD8 and the degree of complications of AE. The CD54 combines with CD3 to participate in T cell activation and proliferation. T-lymphocytes are closely related to the onset of AE, and are the chief components of cell-mediated immunity (Liu et al. 2017). The CD3 are expressed on the surfaces of T cells, and bind to T-cell receptors (TCRs) to present antigens thereby activating the T-cells. Thus, it is important in the early stage of cell-mediated immunity. The CD4 exists on the surfaces of auxiliary T cells. It participates in, and mediates the secretion of cytokines such as tumor necrosis factor β (TNF-β), interleukins (ILs) and interferon-γ (IFN-γ) by helper T cells, thereby playing a role in B-cell activation, proliferation and antibody production (Liu et al. 2017; Gengzangdajie et al. 2018). The CD8 are expressed on the surfaces of cytotoxic or inhibitive T cells, and can identify other specific antigens on the surfaces of antigen presenting cells (APC) by combining with major histocompatibility complex II (MHC-II). Inhibitive T cells inhibit cytotoxic cells and terminate T cell-mediated injury, thus exerting the function of inhibiting cell immunity (Li et al. 2017). Under normal physiological conditions, CD4 and CD8 have opposite effects, and are mutually constrained, thereby creating a balance in the immunity of the human body. An increase in the serum levels of CD4 and CD8 indicates hyper-function of the body's immunity, while a decrease in CD4/CD8 is an indication of a deficient body immunity. Studies have shown that a decrease in CD4/CD8 can stimulate B-cell to synthesize antibodies, activate alexins and mediate the production of immune diseases (De Bruijn et al. 2019; Hara et al. 2018). In this study, the level of expression of CD54 in the patients group was not significantly different from that of control. The levels of expressions of CD54 in the four patients subgroups were also not significantly different, an indication that the degree of complication of AE may not be related to the expression of CD54.

CONCLUSION

Immune mediated encephalitic syndromes form an important group of modifiable neurological disorders. These are being increasingly recognized due to availability of serum makers and have not been characterized in the recent years. These disorders have various patterns of clinical presentations which correlate with antibodies.

RECOMMENDATIONS

Clinical suspicion and knowledge of these syndromes is important to the clinician as these are amenable to immunotherapy. Therefore, cli-

nicians need to be able to recognize these clinical presentations because early diagnosis and optimal treatment may improve the outcome.

STUDY LIMITATIONS

The sample size used in this study was small and the available technology did not allow for the evaluation of the expression of anti-GAD antibody.

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ABBREVIATIONS

Autoimmune encephalitis (AE),
Auxiliary T cells (CD4),
Cytotoxic T cells (CD8)
Inflammation-related T cells (CD54)
Antigen-presenting cells (APC)

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