

1-1-2001

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Recommended Citation

ESEN, NİLÜFER; GÜRBÜZ, YEŞİM; ŞAHİN, DENİZ; and ATEŞ, NURBAY (2001) "Investigation of Some Immune System Parameters and GFAP Immunoreactivity in Convulsive and Non-Convulsive Seizures in Rats," *Turkish Journal of Medical Sciences*: Vol. 31: No. 4, Article 4. Available at: <https://journals.tubitak.gov.tr/medical/vol31/iss4/4>

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Investigation of Some Immune System Parameters and GFAP Immunoreactivity in Convulsive and Non-Convulsive Seizures in Rats

Received: August 11, 2000

Abstract: The aim of this study was to investigate the systemic humoral and cell-mediated immune system parameters, and glial fibrillary acidic protein (GFAP) immunoreactivity in non-convulsive absence epilepsy and pentylenetetrazole (PTZ)-induced generalized tonic-clonic seizures.

Animals were divided into three groups: i. the control group, ii. the genetic absence epilepsy WAG/Rij group, and iii. the chronic generalized tonic-clonic convulsion group injected with PTZ. After the experimental procedure, blood samples were collected intracardially, and CD3+ (T cells), CD4+ (T helper), CD8+ (T cytotoxic), CD19+ (B cells) and CD25+ (IL-2 reseptor, active T cell) cell ratios were determined by indirect immunofluorescence in FACScan, and serum IgG, and IgA, IgM levels were evaluated by using rat radial immunodiffusion plates. After decapitation, the brains were removed and GFAP staining was evaluated in the caudate nucleus, thalamus, hippocampus, amygdala and cerebellum by immunohistochemistry.

The evaluated immunological parameters were found to be significantly increased in the

convulsion group given PTZ when compared with WAG/Rij and normal Wistar albino rats. In WAG/Rij rats, only CD8+ and CD19+ cell ratios were higher than in normal Wistar albino rats. IgM and IgA levels were found to be increased in both the PTZ group and WAG/Rij rats. GFAP+ cells did not differ among the groups except in the caudate nucleus where the GFAP+ cells were lower in WAG/Rij rats than in the other groups.

Our findings indicated that PTZ-induced convulsions activated both humoral and cellular immunity without inducing gliosis. However, in rats with genetic absence epilepsy, humoral immune system parameters in particular were increased, and there were also significant changes in GFAP immunoreactivity. These results suggest that cellular and humoral immunity may contribute to the etiopathogenesis of epilepsy.

Key Words: Absence epilepsy, tonic-clonic seizures, PTZ, immune system, astrocytes, immunohistochemistry.

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Introduction

The immune system plays a role in the pathogenesis of several conditions, and may also be associated with epileptic seizures. Recent studies in epileptic patients have shown that humoral and cell-mediated immunity were changed either by epilepsy itself or by antiepileptic medication. However, many of these studies revealed conflicting results; while some researchers reported immunoglobulin A (IgA) deficiency (1,2) and increased IgM and IgG levels (3,4), others did not find any significant difference in Ig levels (5,6,7). Additionally, some reports indicate that T helper ratio, Th/ Ts ratio and natural killer (NK) activity have been decreased in epileptic patients (8,9).

Since astrocytes play a role in local immunological responses by secreting cytokines and presenting antigens, they would also be affected by systemic immunological changes. In addition, it is now obvious that astrocytes have an important role in the maintenance of extracellular K⁺ ion concentration, and thus in neuronal excitability and related pathologies (10). Previous studies have revealed that a variety of seizures, such as kindling seizures (11), single electroconvulsive seizures (12), and neonatal seizures (13), strongly induced glial fibrillary acidic protein (GFAP) immunoreactivity and expression in astrocytes.

In the present study we investigated whether there was any change in immune system parameters and in

GFAP immunoreactivity in absence seizures and pentylenetetrazole (PTZ) induced generalized tonic-clonic seizures.

Materials and Methods

In this study 6-month-old male WAG/Rij and Wistar albino (WA) rats were used. WAG/Rij rats were donated by Prof. Dr. AML Coenen (University Hospital Nijmegen, The Netherlands). All animals were bred in the animal laboratory of Kocaeli University Medical School. They were housed in groups of 3-4 and fed ad libitum without water restriction and kept at 12-12 hour light-dark conditions. Animals were divided into three groups:

Group 1: The Wistar albino control group (n= 7)

Group 2: The genetic absence epilepsy WAG/ Rij group (n= 7)

Group 3: The generalized tonic-clonic convulsion group given PTZ (n= 7)

PTZ (55mg/kg) (Sigma Chemicals Co., St. Louis, MO, USA) was dissolved in isotonic saline and injected on alternative days for a total of 5 doses. To eliminate the effect of the drug, the animals were sacrificed one day after the last dose. All animals in this group showed generalized tonic-clonic convulsions. On the day of the experiment, all rats were anesthetized with ether and 4-5 ml blood was collected intracardially. Rats were perfused with physiological saline through the carotis artery for 15 minutes, and then the brains were removed, weighed, and frozen at -80°C until immunohistochemical staining.

Serum was separated from half of the blood and kept for immunoglobulin assessment. The other half was used for lymphocyte analysis. Total leukocyte count was determined by an automatic cell counter (Cell-Dyne 3500, Abbott Lab., USA).

Lymphocyte Subpopulations: Heparinized blood was first lysed with erythrocyte lysing buffer (8.29 mg/L NH_4Cl , 1 g/L KHCO_3 and 37 mg EDTA) and incubated for 10 min in the dark at room temperature. Then the tubes were centrifuged for 10 min at 800 g. After discarding the supernatant, the cells were washed with phosphate buffer solution (PBS) and centrifuged again for 10 min at 1500 g. Cells were then incubated for 30 min at $+4^{\circ}\text{C}$ with rat specific purified monoclonal antibodies (MoA) (Caltag Lab., Burlingame, CA, USA). The CD3, CD19, CD4, CD8, CD25 MoA were used for total T cells, B cells,

T helper cells, T cytotoxic cells and IL-2 receptor expressing active T cells, respectively. After incubation they were washed with PBS and 5 μl GAM-FITC (Goat-antimouse 1g FITC) (Caltag Lab.), added to each tube and incubated for another 30 min at $+4^{\circ}\text{C}$. Cells were then washed with PBS and analyzed by flowcytometer (Becton Dickinson FACScan, Grenoble, France).

Serum Ig Levels: The IgA, IgG, IgM levels in the serum were determined by radial immunodiffusion (RID) plates (The Binding Site Ltd., Birmingham, UK), which contained anti-serum specific to the antigen. The recommended amount of serum was put into the wells of plates and incubated for 72-96 hours at room temperature. The diameter of the precipitation ring was then measured and the concentrations of Igs were determined by using standard nomograms.

Immunohistochemistry

8 μm frozen brain sections were taken by cryotome (Shandon Scientific Ltd., Cheshire, England) at -20°C . From each brain, 3 sections at the level of the caudate nucleus, thalamus-hippocampus-amygdala, and cerebellum, according to a rat brain atlas (14), were placed on glass slides. The slides were fixed in acetone for 2-3 hours. Then the staining procedure was started following the instructions of Histostat-SAP kit (AP-Red) (Zymed Lab. Inc. San Francisco, CA, USA). As a primary antibody, rabbit polyclonal antibody for GFAP (Zymed Lab. Inc.) was used in 1: 50 dilution. Slides were evaluated under light microscope with x200 magnification. In each section GFAP+ cells were counted in five different areas and the mean was calculated, which was considered as the mean of the area of that animal.

Statistics

All results were compared between the groups by the Mann-Whitney U test in the SPSS program.

Results

Body weights of animals were not significantly different between the groups. The total leukocyte counts were in the normal range (3500- 10000/ mm^3) and not significantly different between the groups.

Lymphocyte Subpopulation

Most of the flowcytometric parameters evaluated (CD4, CD8, CD19, CD25) were significantly higher in the

PTZ group than in both 6-mo- old WAG/Rij rats and WA rats ($p < 0.001$) (Figure 1). In WAG/Rij rats, only CD8+ and CD19+ cell ratios were found to be significantly higher than in nonepileptic WA rats ($p < 0.01$) (Figure 1).

Serum Immunoglobulins

IgM levels were significantly higher in the group given PTZ than in WAG/Rij and WA rats ($p < 0.01$ and 0.001 , respectively). The IgM level of WAG/Rij rats was also higher than that of WA rats ($p < 0.05$) (Table). On the other hand, the IgA levels in the group given PTZ were significantly lower than in the 6-mo- old WAG/Rij rats ($p < 0.05$), but still higher than in the 6-mo- old naive WA rats ($p < 0.001$) (Table).

Immunohistochemistry

It has been found that GFAP immunoreactivity in the investigated areas, when compared to that of the control group, was not affected prominently by PTZ induced generalized tonic-clonic convulsions. In contrast, GFAP

immunoreactivity in caudate nucleus sections of WAG/Rij rats was lower than that of both the group given PTZ and the non epileptic group ($p < 0.05$) (Figure 2).

Discussion

The present study attempts to clarify the etiopathological differences between absence and PTZ induced generalized tonic-clonic seizures. Immunologically, our results showed that CD8+ and CD19+ cells were higher in WAG/Rij rats than controls. However, in the PTZ group all parameters were higher than in controls. Among the immunoglobulins evaluated, IgM was higher in both the PTZ and absence epilepsy groups than in the controls. On the other hand, PTZ induced generalized tonic-clonic convulsions did not change astrocytic immunoreactivity, while a significant decrease was found in the region of the caudate nucleus in the genetic absence epilepsy group when compared to the control group.

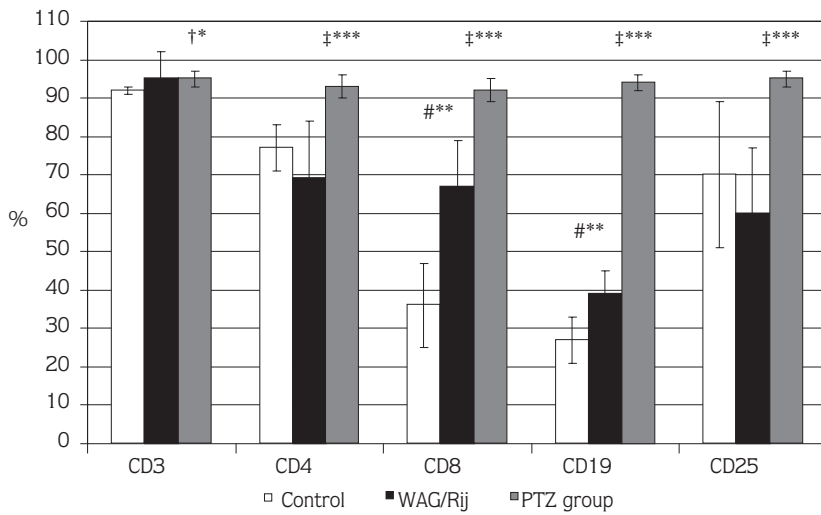


Figure 1. CD3+, CD4+, CD8+, CD19+ and CD25+ cell ratios among the groups.

†* Significantly higher in PTZ group than in control group ($p < 0.05$).

Significantly higher in WAG/Rij rats than in control group ($p < 0.01$).

‡*** Significantly higher in PTZ group than both control and WAG/Rij rats ($p < 0.001$).

	Control	WAG/Rij	PTZ Group
IgG (mg/ L)	22457 ± 7561	20867 ± 6956	27725 ± 2712
IgM (mg/ L)	1273 ± 93	1513 ± 73 #*	1633 ± 65 ‡***
IgA (mg/ L)	45 ± 10	249 ± 77 #***	126 ± 78 †***

Table: Serum immunoglobulin levels in the groups (Mean ± SD).

* $p < 0.05$

*** $p < 0.001$

†* Significantly higher in PTZ group than in control group

Significantly higher in WAG/Rij rats than in control group

‡*** Significantly higher in PTZ group than both control and WAG/Rij rats

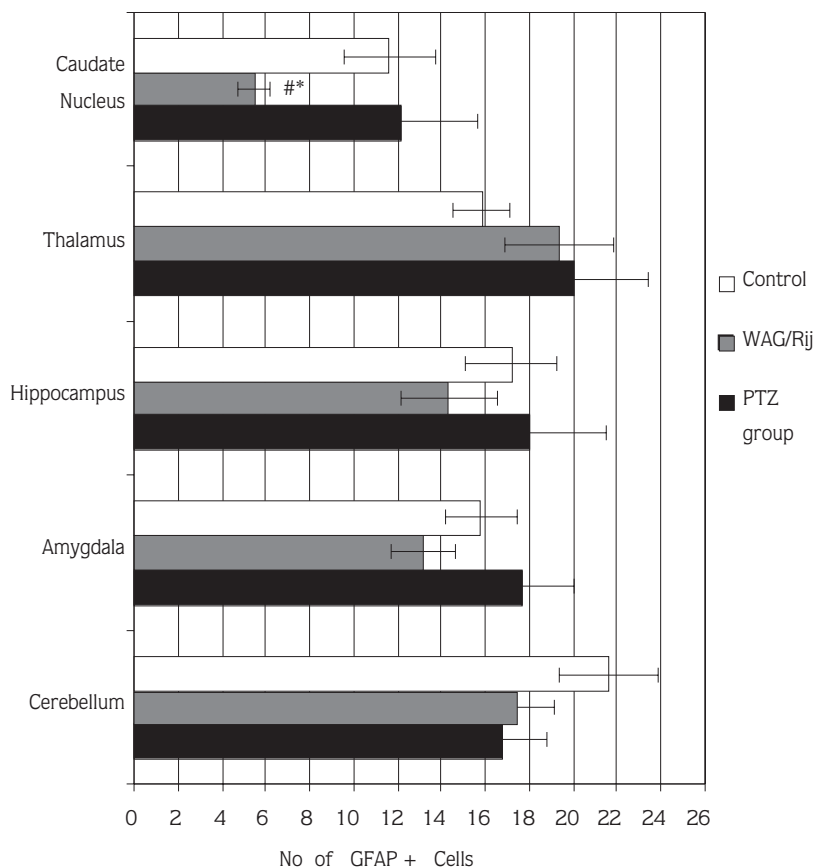


Figure 2. GFAP+ astrocyte distribution in the brain sections of groups.

* Significantly low in WAG/Rij rats when compared with both PTZ given and control rats ($p < 0.05$).

WAG/Rij rats are accepted as an adequate model of human absence epilepsy, because they show spontaneous spike-wave discharges (SWD) in EEG with 7-11 Hz frequency accompanied by the behavioral pattern of absence seizures (15). In the literature there are very few studies investigating the relationship between epilepsy and immunity in genetic and experimental models. It has only been reported that IgG treatment decreases the SWD appearance on EEG of WAG/Rij rats (16) and amygdaloid kindled cats (17). It has also been reported that in 50% of patients with intractable childhood epilepsy, IgG2 deficiency was evident (18) and high dose Ig treatment decreases the clinical seizures and SWD (19). However, in the present study we did not find any difference in IgG levels among the groups. Thus it can be speculated that increased CD19+ cells and IgM levels in WAG/Rij rats are associated with SWD appearance.

Chronic injections of PTZ decreased the severity of seizures (unshown data) and activated both humoral and cellular immunity in Wistar albino rats. These results suggest that PTZ induced seizures may become less severe by

activating the immune system. This suggestion is supported by the study of Asanuma and colleagues (20), who reported that chronic administration of an immunosuppressant, cyclosporin A, aggravated PTZ induced seizures after the 2nd injection of PTZ and showed the presence of some immune responses after convulsions. In contrast some other studies have shown that PTZ induced seizures did not suppress T cells (21) and systemically given PTZ increased IL-1 beta gene expression in the brain (22). In addition to these studies that pointed out the relationship between PTZ and immunity, further studies are needed to clarify this relationship.

Absence epilepsy and tonic-clonic epilepsy are different in terms of pathophysiology and clinical symptomatology. First of all, the increased effectiveness of GABA activity aggravates absence seizures but suppresses PTZ induced tonic-clonic seizures. Secondly, the thalamus is an important area for the occurrence of absence seizures, while the hippocampus, amygdala, brain stem structures and cerebellum are more important brain regions for the generation of tonic-clonic seizures. Additionally, gliosis,

explained as a reaction to fill the areas of dead neurons, is a prominent feature of generalized tonic-clonic seizures, but it has not been detected in absence epilepsy. Stringer et al. (23) have suggested that at least 9 seizures are needed to induce reactive gliosis. This may explain the insignificant changes in GFAP immunoreactivity after the 5 doses of PTZ given in the present study. On the other hand, in absence seizures, instead of gliosis we found a significant decrease in GFAP immunoreactivity in the caudate nucleus, which is an important area in the pathogenesis of absence epilepsy. Meis et al. (24) have shown an increase in inwardly rectifying K⁺ currents in the reticular thalamic neurons of genetically absence epilepsy prone rats. Regarding the role of astrocytes in maintaining the extracellular K⁺ ion concentrations, the observed decrease in astrocytic activity in our study may explain the contribution of the caudate and thalamic region in the pathogenesis of absence epilepsy.

In conclusion, PTZ induced generalized tonic-clonic convulsions activated both humoral and cellular immunity. However, 5 doses of PTZ were not sufficient for induction of gliosis. On the other hand, in absence epileptic WAG/Rij rats, the IgM level and the number of B

(CD19⁺) cells were both significantly higher than in control rats. Thus we suggest that in WAG/Rij rats humoral immunity was affected prominently and astrocytic activity might be more important for the generation of absence seizures. These results suggest that changes in humoral and cellular immune parameters and GFAP immunoreactivity may contribute to the etiopathogenesis of epilepsy. To support this suggestion further and more detailed studies investigating the immunological responses in the central nervous system are needed.

Acknowledgments

This study is a part of a project sponsored by the Scientific and Technological Research Council of Turkey (TÜBİTAK) (project number SBAG- 1218).

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