IMMUNOLOGIC ASPECTS OF WEST SYNDROME AND EVIDENCE OF PLASMA INHIBITORY EFFECTS ON T CELL FUNCTION

Terezinha C.B. Montelli¹, Angela M.V.C. Soares², Maria Terezinha S. Peraçoli²

ABSTRACT - Study objective: The purpose of this study was to assess the extent of immune dysfunction in a well-defined group of epileptic patients: children with diagnosis of West syndrome (WS) or with transitions to another age-related EEG patterns, the multifocal independent spikes (MIS), and the slow spike-wave complexes (Lennox-Gastaut syndrome – LGS). Thus, WS was studied at different points of the natural evolutive history of the disease. Method: A group of 50 patients (33 with WS, 10 with LGS and 7 with MIS) and 20 age-matched healthy controls were submitted to enumeration of T lymphocyte subsets: CD1, CD3, CD4, CD8, CD4/CD8 ratio and lymphocyte proliferation assay to phytohaemagglutinin (PHA), in the presence of autologous and AB, homologous plasma. Dinitrochlorobenzene (DNCB) skin test sensitization was performed only in patients. Determinations of IgG, IgA, and IgM serum levels were compared to standard values for Brazilian population in different age ranges. Results: Sensitization to DNCB showed absent or low skin reactions in 76% of the patients. High levels of IgG (45.7%) and IgM (61.4%), and lower levels of IgA (23.9%) were detected in the serum of the patients. Enumeration of lymphocyte subsets in peripheral blood showed: low CD3+ (p<0.05), low CD4+ (p<0.05), high CD8+ (p<0.01) and low CD4+/CD8+ ratio (p<0.001). The proportion of CD1+ cells in the control group was less than 3%, while ranged between 3 and 11% in 18% of the patients. The in vitro PHA-induced T cell proliferation showed significantly low blastogenic indices only when patients' cells were cultured in presence of their own plasma. No differences in blastogenic indices were observed when the cells of patients and controls were cultured with human AB plasma. Conclusion: The immunodeficiency in WS was mainly characterized by anergy, impaired cell-mediated immunity, altered levels of immunoglobulins, presence of immature thymocytes in peripheral blood and functional impairment of T lymphocytes induced by plasma inhibitory factors.

KEY WORDS: dinitrochlorobenzene, West syndrome, Lennox-Gastaut syndrome, multifocal-independent spike syndrome, T-lymphocytes subsets, immunoglobulins, proliferation assay, T cells, CD1, CD3, CD4, CD8.

Aspectos imunológicos da síndrome de West e evidência de efeitos inibitórios do plasma sobre a função de células T

RESUMO - Objetivo: O objetivo deste estudo foi determinar o perfil da deficiência imune em um grupo bem definido de epilepsia: crianças com síndrome de West (SW) e seus padrões EEG de evolução, idade-dependentes, como os complexos onda-aguda- onda lenta generalizadas da síndrome de Lenox-Gastaut (SLG) e as pontas multifocais independentes (PMI). Método: Um grupo de 50 crianças, 33 com SW, 10 com SLG, 7 com PMI e 20 crianças sadias (controle) foram avaliadas em relação aos seguintes parâmetros: determinação de subpopulações de linfócitos T (CD1, CD3, CD4 e CD8), relação CD4/CD8 e resposta proliferativa de linfócitos frente a fitohemaglutinina (PHA), na presença de plasma autólogo ou de plasma AB (homólogo). A prova cutânea de sensibilização ao Dinitroclorobenzene (DNCB) foi realizada apenas nos pacientes. Os níveis séricos de IgG, IgA e IgM foram comparados aos valores normais em crianças Brasileiras, em diferentes faixas etárias. Resultados: A resposta ao DNCB foi ausente ou fracamente reativa em 76% dos pacientes. Níveis séricos elevados de IgG (45,7%) e de IgM (61,4%) e baixos de IgA (23,9%) foram detectados nos pacientes. A determinação das subpopulações de linfócitos T em sangue periférico mostrou: deficiência nas proporções de células CD3+ (p<0,05) e de CD4+ (p<0,05), aumento de CD8+ (p<0,01) e diminuição da relação CD4+/CD8 (p<0,001). A proporção de células CD1+ no grupo controle manteve-se menor que 3%, enquanto que em 18% dos pacientes esses níveis variaram entre 3 e 11%. A resposta proliferativa de linfócitos frente a PHA revelou índices blastogênicos significativamente mais baixos apenas quando células dos pacientes foram cultivadas na presença do próprio plasma (plasma autólogo). Quando estas células foram cultivadas na presença de plasma AB, não

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West syndrome (WS) is an infantile epileptic encephalopathy, with a devastating clinical course characterized by axial spasms in clusters, hypsarrhythmia and psychomotor delay, beginning in the first year of life. Atypical presentation may include age of onset from the first month to 4 years, modified hypsarrhythmia, and psychomotor development ranging from normal to delayed or deteriorated. Were associated to WS multiple pathologies in Central Nervous System (CNS), such as structural anomalies, prematurity, phakomatoses, and CNS infections. But despite the multiple underlying factors, there are common clinical and electroencephalographic (EEG) patterns of WS that indicate a “final common pathway” for the pathophysiology of WS. Ten to 20% of cases are considered idiopathic, with no evidence of brain lesions. According to the present knowledge about WS, spasms seem to involve subcortical structures, and hypsarrhythmia affects cortical areas, causing psychomotor deterioration. Even though the pathogenesis is still unclear, a disturbance of immune function in the brain has been considered a possible factor.

Infections are frequent and represent serious problems at times, leading to fatal outcome during ACTH treatment. Infectious process have been involved in the etiology of WS, such as citomegalovirus, rubella and herpes simplex virus, meningococcus and pneumococcus. Many cases of WS evolve with age into Lennox-Gastaut syndrome (LGS) or into multifocal independent spike syndrome (MIS). Many clinical and experimental data strongly support the role of immune mechanisms in the pathogenesis of childhood epilepsy. The role of immunity alterations is also clinically supported by the effectiveness of immunomodulatory treatments in children with catastrophic epilepsies and the spontaneous remission of the spasms after viral infections. Our group previously reported immunological disturbances in patients with WS and LGS, consisting of impairment of T cell subclasses, T cell functional tests, in vivo skin sensitization tests and immunoglobulin levels and the demonstration of the presence of antibody to brain extract in the sera of WS and LGS children. Other authors have described similar findings recently.

The aim of this study was to assess the extent of immune dysfunction in WS, and in its evolutive and age-related disorders, LGS and MIS.

METHOD

Patients and controls - A group of 50 children, 23 males and 27 females, with the diagnosis of WS, LGS and MIS, followed at Hospital das Clínicas, Unesp, Botucatu, State University of São Paulo, was included in the study. Inclusion criteria were: 1. they must have been followed in the outpatient clinic with the diagnosis of WS. The immunological study was performed at different points of the natural history of the disease, therefore we had included children with WS, LGS and MIS. 2. they must had had a regular follow-up, to permit a carefully prospective study. The longest follow-up lasted 17 years, and the shortest 11 months, with median age 2 years and 2 months; 3. they must have had good nutritional condition. There were no selective procedures about characteristics of WS, LGS and MIS, neither frequency of infections.

Thirty-three patients with WS (median age 1y 3mo, age ranges from 4 mo to 10 y 2mo), 10 with LGS (median age 8y 1mo, age ranges from 3y 1mo to 18y 5mo) and 7 with MS (median age 4y 1mo, age ranges from 7mo to 15y) were included in the study. The control group included 20 age-matched healthy children (12 males and 8 females).

Immunity was evaluated during infection-free periods, and ACTH-free therapy. Approval from Hospital Ethics Commission to study immunocompetence in children with convulsive disorders and informed consent from the parents of patients and controls were obtained.

Reagents - Monoclonal antibodies directed against human CD1+, CD3+, CD4+ and CD8+ were obtained from Ortho Diagnostics Systems, (Raritan, N.J.) and were employed throughout this study. For the indirect immunofluorescence assay, biotinylated horse anti-mouse IgG and fluorescein-labelled avidin-D (Vector Laboratories, Burlingame, PA) were used.

Enumeration of lymphocyte subsets - Peripheral blood mononuclear cells were isolated from heparinized venous blood by Ficoll-Hypaque density centrifugation and resuspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 10% (v:v) of fetal calf serum. Approximately 2 x 10^6 cells were centrifuged onto a poly-I-lysine-coated coverslip and staining reactions carried out at 4°C as described previously. Briefly, after reacting with the monoclonal antibody, the cells were treated with
biotinylated horse anti-mouse IgG and fluoresceinated avidin-D and fixed in formaldehyde. At least 500 cells per sample were examined and scored under phase and fluorescence microscopy.

Proliferation assay - Lymphocyte cultures were prepared according to Musatti et al. Peripheral heparinized blood was obtained from patients and normal control subjects. After separation of mononuclear cells by Ficoll-Hypaque density centrifugation, the cells were washed twice in RPMI 1640 culture medium, and counted in a hematocytometer and cell viability was determined by 2% Trypan blue exclusion. The cell suspension was then cultured in RPMI 1640 medium to a final concentration of 2 x 10⁶ cells/ml. One hundred microlitres of the cell suspension was cultivated separately in triplicate on flat-bottomed 96-well plates (Corning, Miami, FL, USA), and in the presence of RPMI medium containing 20% homologous AB or autologous heat-inactivated plasma. Control wells did not receive any stimulation and experimental wells were stimulated with 8 ug/ml PHA (Difco Laboratories, Detroit, MI, USA). The cultures were incubated for 72h in 5% CO₂ at 37°C, and were pulsed with 1 mCi/mM Tritiated thymidine (specific activity 6.7 Ci/mM; New England Nuclear, Boston, MA) 18 h before the end of incubation. The cells were harvested using an automatic collector (Cambridge Technology Inc., Cambridge, MA, USA). Isotope incorporation by the cell cultures was counted in a beta scintillation counter (Beckman Instruments, Inc., Los Angeles, CA, USA). The results are expressed as counts of radiation emission per minute (cpm) and defined as “blastogenic index” (BI) which is the ratio: cpm of stimulated tubes/cpm of control tubes. Based on the response of healthy controls, BI > 20 was considered as positive. The employment of 20% homologous AB or autologous heat-inactivated plasma in lymphocyte cultures stimulated with PHA had the aim of detecting the presence of circulating immunosuppressive factors in plasma of patients and controls. The presence of inhibitory activity in the plasmas was determined by the suppression index (SI), obtained by the equation proposed by Park et al.: SI = 1 – [cpm cultures with PHA plus autologous plasma – cpm control cultures / cpm cultures with PHA plus homologous plasma – cpm control] x 100.

Immunoglobulins levels - Quantitative determination of IgG, IgA, and IgM were carried out by single radial immunodiffusion and the results expressed in mg/dl. Commercially prepared immunodiffusion plates with standards “Partigen plates” were purchased from Behringwerke, Marburg Laboratories (USA). The Ig values were compared to standard values for Brazilian population in different age ranges, as described by Naspitz.

**DNCB test** - Sensitization with dinitrochlorobenzene (DNCB) was accomplished according to Mendes et al. Sensitizing doses of 2000 ug of DNCB dissolved in 0.1 mL of acetone were spread and maintained over a 2 cm area of skin, on the back skin of the patients for 48 h. Eighteen or more days later, a patch test was repeated using 100 ug of DNCB in acetone, on a skin site different from the one used for sensitization. Skin reactions consisting of erythema and induration at 48 h, were accepted as evidence of sensitization. This test was not performed in control healthy subjects due to ethical reasons.

**Statistical analysis** - Data were analyzed statistically using the INStat software (Graph Pad Software, San Diego, CA, USA). The results of T cell subsets from patients and control groups were analyzed by Student’s t test. Differences in the lymphocyte proliferative responses between patients and controls were compared by the non-parametric Mann-Whitney U method, and blastogenic indices from lymphocytes cultured in the presence of autologous or homologous plasma in patients and control groups were analyzed by paired non-parametric Wilcoxon sign rank test. Significant values are considered to occur at p < 0.05.

**RESULTS**

Forty children had secondary WS and 10 were included in the primary or criptogenic group. Infections were frequent in patients of both groups: 12 had meningitis, 20 had one or more episodes of pneumonia, 13 had recurrent mucocutaneous candidiasis and 10 had very frequent rhynopharingitis and otitis.

Table 1 shows the percentage of total T cells (CD3+), helper inducer (CD4+), cytotoxic/suppressor (CD8+) T-cell subsets and the CD4:CD8 ratio detected

**Table 1. Percentages of T cell subsets in patients and in healthy control group.**

<table>
<thead>
<tr>
<th>CD+ cell subsets</th>
<th>Patients (n = 31)</th>
<th>Controls (n = 14)</th>
<th>Significance*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3+</td>
<td>53.3 ± 12.9</td>
<td>62.2 ± 9.2</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>CD4+</td>
<td>32.5 ± 11.6</td>
<td>40.2 ± 4.2</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>CD8+</td>
<td>31.7 ± 12.6</td>
<td>22.4 ± 1.8</td>
<td>p &lt; 0.01</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>1.16 ± 0.1</td>
<td>1.80 ± 0.2</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation. *Student’s t test.
in peripheral blood of patients and healthy controls. A significant decrease in proportion of CD3+, CD4+ T cells as well as an increase in the CD8+ cells were detected in the patients in comparison to the control group. Besides, a significant reduction in CD4:CD8 ratio was observed. In 81% (25/31) of the patients the CD4:CD8 values were below the mean ± 2 SD of the value detected in the control group. An elevation of CD1+ cells was observed in some patients. While in the control group the proportion of CD1+ cells was less than 3%, in 18% (4/22) of the patients these levels ranged between 6 and 11%.

The in vitro lymphocyte proliferation assay in response to PHA was employed to evaluate the cell-mediated immune response of patients with WS, LGS and MIS and the control group and the results were expressed as blastogenic index (Table 2). A significant impairment in the response to PHA, demonstrated by the low blastogenic indices was described, when the cells of the patients were cultivated in presence of their own plasma (autologous plasma), but not occurring in cultures with human AB plasma (homologous plasma). The comparison between patients and controls by Mann-Whitney U test revealed significant differences in relation to cell cultures in the presence of autologous plasma. No significant difference between patients and controls was detected when blastogenic indices were compared in cultures supplemented with homologous plasma. Based on the response of healthy controls, only 8 of 28 patients (28.7%) exhibited blastogenic index below 20 (considered as positive).

Plasma from healthy controls had an inhibitory effect on T-cell proliferation assay with PHA ranging from 0 to 35%, while this range was 0 to 97.7% in the patient group (Table 2). Thus, patient plasma with SI higher than 35% were considered as having an inhibitory effect on the cellular immune response to PHA. The median percentage of SI presented by the patients was significantly higher than that of controls. In 15 of 28 (53.6%) patient plasmas, the presence of inhibitory effects was observed. These results suggest the presence of an inhibitory effect of the patient’s plasma on their lymphocyte function.

Figures 1, 2 and 3 show respectively IgG, IgA and IgM serum levels detected in the patients studied. The continuous line and the area between the broken lines represents the mean and the standard deviation of healthy control immunoglobulin values respectively, in accordance with standard values for Brazilian population in different age ranges, as described by

![Fig 1. Serum levels of IgG in patients with West and Lennox syndromes and epilepsy with multifocal independent spikes with different ages. The continuous line and the shaded area represents respectively the mean and the normal range (+ SD) of the control healthy brazilian people, according to Naspitz, 1992 [25]. m, months; y, years.](image-url)
We observed few cases of depressed IgG (15.2% = 7/46) and IgM (2.1% = 1/47) but more patients presented high levels of IgG 45.7% (21/46) and IgM 61.7% (29/47). On the other hand, 23.9% (11/46) of patients had lower levels of IgA and only 8.7% (4/46) showed values exceeding the upper normal range of controls in different age groups.

Skin reaction to DNCB were reduced in 76% of the patients. Erythema and induration occurred only in 24 % (8/37) of the patients. Such low or absent skin sensitization could be considered as anergic responses.

**DISCUSSION**

This study demonstrated that patients with WS, LGS and MIS exhibited abnormalities in humoral and cell-mediated immune response. The analysis of T cell subsets showed significant decrease in proportion of CD3+ and CD4+ T cells and an increase in CD8+ cells, leading to a consequent low CD4/CD8 ratio in more than 80% of the patients. These results confirm previous studies in epileptic patients, showing similar alterations in T cell subsets. An initial deficiency of B lymphocyte activity and a progressive dysfunction of T lymphocyte were reported in patients with epilepsy. Other alterations such as impaired natural killer cell activity, low CD4+ and high CD8+ lymphocytes, reduction in the CD4+/CD8+ ratio, and decreased C4 complement were also described in epilepsy. Impairment in humoral immune response to antigens was reported in children with LGS. All authors agree about the presence of a combined disorder of the immune and the nervous systems. Thus, evidence of an immune dysregulation associated with epilepsy has been regarded as being accounted for an increased prevalence of autoantibodies, and immunodeficiency.

In the present study it was also observed an elevation in proportion of CD1+ blood cells in 18% of the patients. The reason for the presence of these cells in the peripheral blood of the patients is unclear, since CD1+ cells are found in the thymic tissue as a cortical thymocyte antigens but not in peripheral blood from healthy individuals.

Significant increase of CD1+ cells in the peripheral blood was detected in patients after acute marrow suppression, in mothers of stillborn infants with neural tube defects, and in patients with acquired immunodeficiency syndrome. The presence of thymocytes in the peripheral blood could be interpreted as the result of immature T cells released to the periphery, and further studies are need for the clinical relevance knowledge of this finding. The results of lymphocyte proliferation in response to PHA stimulus showed a significant impairment of cell-mediated immunity when lymphocytes of patients were stimulated *in vitro* with the mitogen, in the presence of their own plasma, as detected by the low blastogenic index. These results as well as the reports obtained in other pathologies such as, febrile seizures and infectious disease are compatible with the presence of inhibitory factors in patient plasma. The nature of these inhibitory factors has not been clarified yet, but some evidence indicate that antibodies, immune complexes, serum protein as alpha2-macroglobulin and parasite antigens may...
exert in vitro an immunosuppressive effect\textsuperscript{37-40}. Autoantibodies against brain cell-antigens were detected in epileptic patients\textsuperscript{31-43} and in children with WS and LGS\textsuperscript{16}. Since it was not observed the inhibitory activity on lymphocyte proliferative response in our patients when their cells were cultivated in the presence of AB plasma, it is possible that antibodies to brain tissue, present in autologous plasma, might play an inhibitory role on the cell-mediated immune response in vivo. This effect could be related to the immunodeficiency observed in patients with WS, LGS, and MIS.

Low or absent skin sensitization to DNBCB was observed in patients with WS, LGS and MIS. Skin reactions were detected only in 24 \% (8/37) of the patients. This may be interpreted as an inability of these children in developing an immune response to newly encountered antigens, as previously suggested in patients with selective IgA deficiency\textsuperscript{44}. DNBCB sensitization has been reported as positive in 87\% of normal children of 1-5 years of age\textsuperscript{46} and in 92\% of children aged between 1 - 17 years\textsuperscript{46}. In previous papers we detected anergy to DNBCB in children with WS and LGS\textsuperscript{13,14}. This anergy might be related to decreased ability to mount an inflammatory response or to any basic immunological defect.

We detected high levels of IgG and IgM in 45.7\% and 61.7\% of the patients, respectively, while the levels of IgA were low in the serum of 23.9\% of the patients with WS, LGS and MIS. Although the patients did not presented any infection at the time of the immunity evaluation, the elevated levels of IgG and IgM might be associated to the past frequent episodes of infections in these children. On the other hand it is possible that some of these immunoglobulins might represent an immune response against brain tissue, as described in our previous study\textsuperscript{13,16}. High concentration of serum IgG and IgM in children with WS, LGS and other forms of epilepsy were reported by other authors\textsuperscript{30,47}. Low IgA levels were found in 5 of 12 patients with myoclonic epilepsy by Nieto et al.\textsuperscript{48}. Eriksson et al.\textsuperscript{3} also referred low IgA levels in up to 25\% of epileptic patients. Hrachovy et al.\textsuperscript{18} concluded that B cells were increased in number and activated in a study of 7 patients with WS.

Our group related the presence of precipitating antibodies to a saline extract of brain tissue in the sera of 24 children with WS and LGS\textsuperscript{16}. This finding was later confirmed by increasing the number of patients studied\textsuperscript{13}. Several authors also referred the presence of autoantibodies to brain-cell-antigens in epileptic patients\textsuperscript{5,11,41-43,49-53}. There were described self antibodies to glial brain specific proteins of the group S-100 and undifferentiated proteins\textsuperscript{51}, IgG subunits to cerebrocyte antigens of human embryo\textsuperscript{3}, acetylcholine receptor and anti-synaptic membrane antibodies\textsuperscript{52}, anti-GM1 ganglioside antibodies\textsuperscript{41}, antibodies to proteins p16, p30 and S-100\textsuperscript{54}, to glutamate receptor subunits\textsuperscript{49}, to phospholipids\textsuperscript{9,42}, and cardiolipin\textsuperscript{53}.

Experimental epilepsy studies with anti-brain antibodies demonstrated that epileptiform discharges can be obtained with anti-neural IgG\textsuperscript{55}, antiserum to the synaptic membrane fraction\textsuperscript{56}, and to brain ganglioside GM1\textsuperscript{57-59}. The demonstration of autoantibodies to GluR3 in Rasmussen encephalitis, that have agonist properties at the receptor, provides a pathogenic model for hyperexcitability leading to epilepsy\textsuperscript{50,61}. On the other hand, there are examples of autoantibody production without autoimmune disease\textsuperscript{50,62}. Such responses are not always harmful and had an important role in regulating the immune response\textsuperscript{62,63}. In a remarkable study, During et al.\textsuperscript{64} reported that a vaccine to an adeno-associated virus generated autoantibodies that targeted a specific brain protein, the NR1 subunit of the N-methyl-D-aspartate receptor. These autoantibodies had strong anti-epileptic activity. However, to establish that a disease has an autoimmune etiology, it is necessary to demonstrate the autoantibody or cell-mediated immunity, the corresponding antigen must be identified, the autoimmune response be obtained in experimental animals and a similar disease must develop. Finally, the direct transfer of autoantibodies or sensitized cells must induce the disease in animals. These conditions were only accomplished with Rasmussen encephalitis\textsuperscript{65}.

Concluding, the immunodeficiency in WS was mainly characterized by disturbances of cell-mediated immunity and in the levels of immunoglobulins, anergy, presence of immature thymocytes in peripheral blood and functional impairment of T lymphocytes induced by inhibitory plasma factors. These findings must have implications in medical care of patients, in order to prevent and treat infections.

The immune mechanisms involved in the pathophysiology of these epileptic syndromes could be better understood by determination of cytokine patterns produced by lymphocytes. Immunomodulatory treatment may improve the course of the disease and perhaps become the next aspect to be seriously investigated.
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