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Levetiracetam but not valproate inhibits function of CD8$^+$ T lymphocytes

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ABSTRACT

Purpose: To further elucidate possible immune-modulatory effects of valproate (VPA) or levetiracetam (LEV), we investigated their influence on apoptosis and cytotoxic function of CD8$^+$ T lymphocytes in humans.

Methods: In 15 healthy subjects (9 female (60%), 35.7 ± 12.1 years), apoptosis and cytotoxic function of CD8$^+$ T lymphocytes were measured using flow cytometry following *in vitro* exposure to LEV (5 mg/L and 50 mg/L) and VPA (10 mg/L and 100 mg/L). Apoptosis rates were determined after incubation with LEV or VPA for 1 h or 24 h. Cytotoxic function was assessed following 2 h stimulation with mixed virus peptides, using perforin release, CD107a/b expression and proliferation. The presence of synaptic vesicle protein 2A (SV2A) was investigated in human CD8$^+$ T lymphocytes by flow cytometry analysis, Western blot and real time polymerase chain reaction (rtPCR).

Results: High concentration of LEV decreased perforin release of CD8$^+$ T lymphocytes (LEV 50 mg/L vs. CEF only: 21.4% (interquartile range (IQR) 16.5–35.9%) vs. 16.6% (IQR 12–24.9%), $p = 0.002$). LEV had no influence on apoptosis and proliferation ($p > 0.05$). VPA (100 mg/L) slowed apoptosis of CD8$^+$ T lymphocytes after 24 h (VPA 100 mg/L vs. control: 7.3% (IQR 5.4–9.5%) vs. 11.3% (IQR 8.2–15.1%), $p < 0.001$), but had no effects on perforin release ($p > 0.05$). SV2A protein was detected in CD8$^+$ T lymphocytes.

Conclusion: LEV decreased degranulation of CD8$^+$ T lymphocytes which may contribute to the increased incidence of upper respiratory tract infections in LEV treated patients. Inhibition of SV2A may be responsible for this effect.

1. Introduction

Levetiracetam (LEV) is a new generation antiepileptic drug whose efficacy and tolerability in epilepsy treatment is well recognized.[1,2] Synaptic vesicle protein 2A (SV2A), an intracellular protein, is the binding site of levetiracetam in the brain.[3] Several clinical trials reported a clear anticonvulsant effect of LEV but also an increased incidence of pharyngitis and rhinitis in LEV-treated patients.[4–9] The reason for this latter finding remains unknown. It was reported that white blood cell and neutrophil counts were in the normal range in levetiracetam-treated patients who developed infections[8] but were significantly decreased during treatment compared to the pretreatement status.[9] Similarly, we found interictally no clear effect of LEV on B lymphocytes as well as

Abbreviations: CEF, CTL-CEF-Class I peptide pool "Plus"; CFSE, carboxyl fluorescein succinimidyl ester; IFNγ, interferon gamma; IL, interleukin; IQR, interquartile range; LEV, levetiracetam; PBMC, peripheral blood mononuclear cell; rtPCR, real time polymerase chain reaction; SV2A, synaptic vesicle protein 2A; TNFα, tumor necrosis factor alpha; VPA, valproate.

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on CD4+ and CD8+ T lymphocyte counts in the blood of LEV-treated patients.[10]

The primary role of CD8+ T lymphocytes is to protect against viral infections by lysing infected cells via degranulation-dependent perforin release which can be measured by increased expression of CD107a and CD107b on the cell surface[11] and secretion of soluble factors, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFα).[12–15] Treatment with valproate (VPA) was not found to be associated with a higher infection rate.[16] However, VPA is a potent histone deacetylase (HDAC) inhibitor[17–19] and histone modification plays a role in the regulation of the effector functions of memory CD8+ T cells.[20] Moreover, VPA can induce apoptosis in various leukemia cells *in vivo* and *in vitro*.[17–19] VPA also influenced postictal blood levels of TNFα and interleukin (IL)-1β as well as CD4+ T cell counts.[21,22]

In this study, we investigated the influence of LEV and VPA on proliferation, apoptosis, CD107 mobilization and perforin release of CD8+ T lymphocytes *in vitro* in order to better understand their pharmacological effects and adverse events.

2. Materials and methods

2.1. Study population

Fifteen healthy adult volunteers recruited from the staff of the Department of Neurology, University of Marburg (9 female (60%), age: 35.7 ± 12.1 years; range: 18–60 years) were included in the study. None of the volunteers took any medication. Immediately after venous blood drawing (10 mL), the experiments were started. The study was approved by the local ethics committee.

2.2. Antibodies, reagents and peptides

We used the following antibodies, reagents and peptides: CD3-APC, CD8-PerCP, perforin-PE, IgG2b-PE isotype control, CD107a/b-FITC, Perm/Wash Buffer™, Cytofix/Cytoperm™ solution, BD GolgiStop™ (all BD Bioscience), recombinant human interleukin-2 (rhIL-2) (ProSpec), SV2A-FITC (ByOrbit), IgG-FITC isotype control (antibodies-online GmbH), Polyclonal antibody to SV2A-Aff-Purified (Acris Antibodies GmbH), IgG-HRP (Santa Cruz Biotechnology) Annexin V-FITC apoptosis detection kit (Mountain View), carboxyl fluorescein succinimidyl ester (CFSE) (Invitrogen), pooled human AB serum (3H Biomedical AB), trypan blue solution (0.4%) (GIBCO), RPMI 1640 and 1% penicillin–streptomycin (Sigma) and CTL-CEF-Class I peptide pool "Plus" (Cell Technolgy Ltd.).

2.3. Anticonvulsant drugs

Levetiracetam (Keppra®, UCB Pharma S.A., Brussels, Belgium) and sodium valproate (Ergenyl® vial, Sanofi Aventis, Frankfurt, Germany) were dissolved in sterile physiological saline to produce fresh solutions as required. When treating cells, the drugs were further diluted in the culture medium to the final concentrations of 5 mg/L or 50 mg/L for LEV (serum reference range in adults for drug fasting levels: 12–46 mg/L) and 10 mg/L or 100 mg/L for VPA (serum reference range in adults for drug fasting levels: 50–100 mg/L), respectively.[23]

2.4. Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll–Biocoll Separating Solution by density gradient centrifugation (1500 rpm, 20 °C, 30 min, without brake) of heparinized blood obtained by venipuncture of the healthy volunteers. Viability of PBMCs obtained was always >95%, as determined by 0.4% trypan blue staining. After double washing in cold PBS, PBMCs were cultured with RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum and 1% penicillin–streptomycin.

2.5. Activation of CD8+ T lymphocytes

In the functional assays, CD8+ T lymphocytes were activated with CTL-CEF-Class I peptide pool "Plus", which contains 32 peptides, each corresponding to a defined HLA class I restricted T-cell epitope from cytomegalovirus, Epstein–Barr virus and influenza virus. Most humans have been previously exposed to one or more of these pathogens, respectively. One hundred microliter PBMCs ($2–3 \times 10^6$ cells/mL) were incubated with 64 μg/mL of CEF peptide (100 μL) at 37 °C in a humidified, 5% CO_2 incubator for 2 h. Costimulatory antibodies (BD FastImmune™ CD28/CD49d costimulatory reagent) were added according to the manufacturer's protocol. A negative control (only anti-CD28/CD49d) was included in every experiment.

2.6. Perforin release

Perforin release was measured as previously described.[24] After activation of CD8+ T lymphocytes for 2 h either in the presence or absence of anticonvulsant drugs, cells were first stained with CD3-APC and CD8-PerCP for 30 min and were then resuspended in BD Cytofix/Cytoperm solution for 20 min at 4 °C. After washing and centrifugation (1200 rpm, 20 °C, 4 min), cells were labeled with perforin-PE in Perm/Wash buffer solution. IgG2b-PE isotype control was used for negative control. Perforin expression was measured by flow cytometry. The baseline of perforin expression was measured in unstimulated cells immediately after isolation. A reduction in degranulation was indicated by a higher percentage of perforin+ cells remaining after stimulation.

2.7. CD107 mobilization

CD8+ T lymphocyte degranulation can also be measured by increased expression of surface CD107a and CD107b.[11,25] CD107 mobilization was measured as previously described.[30] Briefly, CD107a/b-FITC and monensin (BD GolgiStop, used according to manufacturer's instructions) were added before activation of CD8+ T lymphocytes in the presence or absence of anticonvulsant drugs. After incubation for 2 h, cells were washed and stained with CD3-APC- and CD8-PerCP-antibodies as described above followed by characterization of cells in the flow cytometer. The baseline of CD107a/b expression was measured in unstimulated cells immediately after isolation.

2.8. Analysis of CD8+ T lymphocyte proliferation by CFSE labeling

PBMCs were labeled with 0.4 μM CFSE at 37 °C for 10 min in the dark. Free CFSE was quenched with ice-cold culture medium for 5 min. Following two washes, the labeled cells were resuspended in the culture medium. Costimulatory antibodies (CD28/CD49d) and 100 μL of CEF peptides were added into 100 μL of CFSE-labeled PBMCs ($2–3 \times 10^6$ cells/mL) in the presence or absence of anticonvulsant drugs. Recombinant human IL-2 (40 IU/mL) was added at the time of peptide stimulation. The negative control consisted of cultures of CFSE-labeled, unstimulated cells. After incubation in a 5% CO_2 incubator at 37 °C for 5 days, cells were washed with culture medium and cell surfaces were stained with CD3-APC and CD8-PerCP. Cells were analyzed by flow cytometry. The definition for low CFSE cells was defined according to the distribution of CSFE in unstimulated cells.