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THYROCYTES AS THE TARGET CELLS FOR HHV-6 INFECTION IN PATIENTS WITH AUTOIMMUNE THYROIDITIS

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Human herpesvirus-6 (HHV-6) is a ubiquitous betaherpesvirus with immunomodulating properties that have been suggested to play an important role in the development of several autoimmune disorders. Although the primary targets for HHV-6 replication, both in vitro *and* in vivo*, are CD4+ and CD8+ T lymphocytes, some studies have reported the presence of HHV-6 sequences in different solid organs, including in the thyroid gland, showing possible involvement of this herpesvirus in development of autoimmune thyroid disease. The aim of this study was to determine loads of HHV-6 in thyroid gland tissue in comparison to those in peripheral blood of patients with autoimmune thyroiditis. Seven patients [women mean age 45 (28–65)] with histologically confirmed autoimmune thyroiditis were enrolled in this study. Fluorescence-activated cell sorting was used to distinguish and sort lymphocyte populations from peripheral blood mononuclear cells of patients. HHV-6 load was determined by real-time PCR for peripheral blood and thyroid gland tissue samples. Additionally, all results from molecular analyses were compared with histological results obtained by light microscopy. Viral load was detected only in one (46 viral copies/ 1×10^6cells) blood sample; others were under the detection limit of the used kit. However, in all HHV-6 positive tissue samples viral load was detected in the range of 132–1620 viral copies/10^6 cells. Substantial HHV-6 load in lymphocyte subpopulations was detected in two of seven patients. HHV-6 load was detected in NK and CD95$^+$ cells of two patients. The obtained results show that thyroid gland cells (tyrocytes) act as target cells for HHV-6.*

Key words: *HHV-6, autoimmune thyroiditis, cell sorting, light microscopy.*

INTRODUCTION

Human herpesvirus-6 (HHV-6) is a ubiquitous betaherpesvirus with lymphotropic and neurotropic potential existing in variants A and B (Hall *et al*., 1998; Santoro *et al*., 1999), which were re-classified as separate species (HHV-6A and HHV-6B) in 2012 by the International Committee on Taxonomy of Viruses, based on the different bio characteristics regarding cell tropism and pathological implications (Ablashi *et al*., 2014). Although the primary targets for HHV-6 replication, both *in vitro* and *in vivo*, are CD4+ and CD8+ T lymphocytes (Lusso, 2006), some studies found HHV-6 sequences in different solid organs, including in the thyroid gland (Chen and Hundal, 2006; Alvarez-Lafuente *et al*., 2008). HHV-6 has immunomodulating properties, which may operate in several autoimmune disorders, including autoimmune hemolytic anemia/neutropenia (Yagasaki *et al*., 2011), autoimmune acute hepatitis (Grima *et al*., 2008), and multiple sclerosis (Chapenko, 2003; Tejada-Simon *et al*., 2003; Nora-Krukle *et al*., 2011). Although the potential mechanism of autoimmune pathogenesis by HHV-6 remains unknown, molecular mimicry may play a role in the initiation of self-reactive immune responses (Dagna *et al*., 2013). Recent studies show possible involvement of herpesviruses in development of autoimmune thyroid disease (Thomas *et al*., 2008; Caselli *et al*., 2012).

The aim of this study was to determine loads of HHV-6 in thyroid gland tissue in comparison to those in peripheral blood of patients with autoimmune thyroiditis.

MATERIALS AND METHODS

Seven patients (women mean age 45, range 28 to 65) with histologically confirmed autoimmune thyroiditis were enrolled in this study. Samples from patients were tested for the presence of auto-antibodies to thyroid peroxidase (TPO), thyroglobulin (TG) and thyroid stimulant hormone receptor (TSH).

Patients had the following clinical diagnoses: *Struma nodosa III thyreotoxicum* (n = 3) and *Struma nodosa III euthyreocum* (n = 4). Surgical treatment *Thyreoidectomia totalis* was applied to all patients.

Light microscopy. Tissue samples from thyroid glands of autoimmune thyroiditis (AIT) patients were fixed in 4% glutaraldehyde in phosphate buffer for 24 h and post-fixed in osmium tetraoxide for 24 h. After dehydration in acetone (70–100%), the specimens were processed in acetone-resin Durcupan 1 (without accelerator) mixture for 24 h at 50 °C and embedded in resin Durcupan 2 (with accelerator added). Semi-thin (thick) sections (0.5 um) were cut using a ultramicrotome Reichert-Jung, Austria, collected in a metal loop, transferred to a drop of distilled water on glass slides, dried at 50 °C for 10 min and stained with centrifuged toluidine blue at high temperature. Slides were rinsed with distilled water and dried at room temperature. The observations were carried under immersion by light microscope (Leica DM 500B, Wetzlar, Germany) and images were recorded using a connected digital camera.

The stained materials were used for general morphological analysis to determine the areas of interest for further TEM (transmission electron microscope) observation. All chemicals were purchased from Sigma Aldrich Chemie GmbH, Germany.

HHV-6 genomic sequence detection. Nested polymerase chain reaction (nPCR) was used to detect HHV-6 genomic sequences in DNA isolated from whole blood and thyroid tissue, in accordance with Secchiero *et al.* (1995). A positive control (HHV-6 genomic DNA; Advanced Biotechnologies Inc, Columbia, MD, USA) and a negative control (DNA obtained from practically healthy HHV-6 negative blood donors and a water control) were included in each experiment.

The HHV-6 load in whole blood and tissue samples from patients with HHV-6 persistent infection (DNA sequence in peripheral blood DNA) was determined using a HHV-6 Real-TM Quant Real-TM kit (Sacace, Italy) and Applied Biosystems 7500 Real-time PCR System (Applied Biosystems, Carlsbad, CA, USA), in accordance with the manufacturer's recommendations. Collected data were processed and analyzed with the specialised software ABI 7500 system to calculate viral load as viral copies/10^6 cells.

FACS sorting. BD FACSAria II flow cytometer (USA) and BD FACSDiva software were used for sorting and analysis of peripheral blood mononuclear cells (PBMC). Commercial monoclonal antibodies conjugated with flourochromes (anti- CD3-FITC, CD4-PerCP-Cy7, CD8-PE, CD16-V450, CD19-APC, CD45-V500, CD56-V450 and CD95-PerCP-Cy 5.5) were used to distinguish and sort lymphocyte populations.

All procedures were performed with standard BD protocols for staining and sorting PBMC's. The study was approved by the Ethics Committee of Rīga Stradiņš University, and written consent was obtained from all patients.

RESULTS

Light microscopy. Microscopic examination of parenchyma units of the examined thyroid glands showed different stages of follicular damage and degeneration, expressed in alteration of follicular shape and cell appearance, decreased intensity of colloid staining, extensive lymphoid cell infiltration, coalescence of adjacent follicles and atrophy, and slight fibrosis of the interstitial tissues. In cases diagnosed as macrofollicular colloid struma, thyroid follicles consisted mainly of more or less flattened thyrocytes (follicular epithelium), where some cells had enlarged or overlapping nuclei and a central mass of colloid with tinctorial variations (Fig 1a, b; Fig. 2). In cases of microfollicular colloid struma and follicular adenomas (Fig. 3), we observed small and fewer thyroid follcules with cubical cells in ring-like position. The examined materials also showed morphological features characteristic of cell death of thyrocytes, such as: light-colored and vacuolated cells with polymorphic, fragmented or pyknotic nuclei and focal necroses in some specimens (Fig. 1a, b, Fig. 3). Small and fewer large and lighter staining parafollicular cells (C cells) were seen near the basement lamina in the confines of the residual follicles, excluded from the lumen by follicular epithelium. Lymphoid cell migration (predominantly lymphocytes and plasma cells) was observed as in the follicles – around the parenchyma cells and in the colloid (Fig. 2) as well in the interstitium of the examined glands of AIT patients.

Lesions varied from initial alterations of parenchyma to fibrosis progress around the residual follicles and layers of the loose connective tissue, containing capillary network (Fig. 1b, Fig. 3).

HHV-6 load in peripheral blood and thyroid tissue samples. Using real-time polymerase chain reaction (RT-PCR), HHV-6 load was determined whole blood and thyroid gland tissue of patients. In patient blood the viral load was detected only in one (46 viral copies/1 × 10^6 cells) sample, others were under the detection limit of the used kit, while in all HHV-6 positive tissue samples viral load was detected within the range 132–1620 copies/10^6 cells. However, in all patients enrolled in this study (7/7; 100%), the HHV-6 genomic sequence in tissue and blood DNA samples was detected using nPCR.

FACS sorting of lymphocytes' subpopulations. The difference in frequency of the HHV-6 genomic sequence in thyroid tissue and peripheral blood samples was studied by

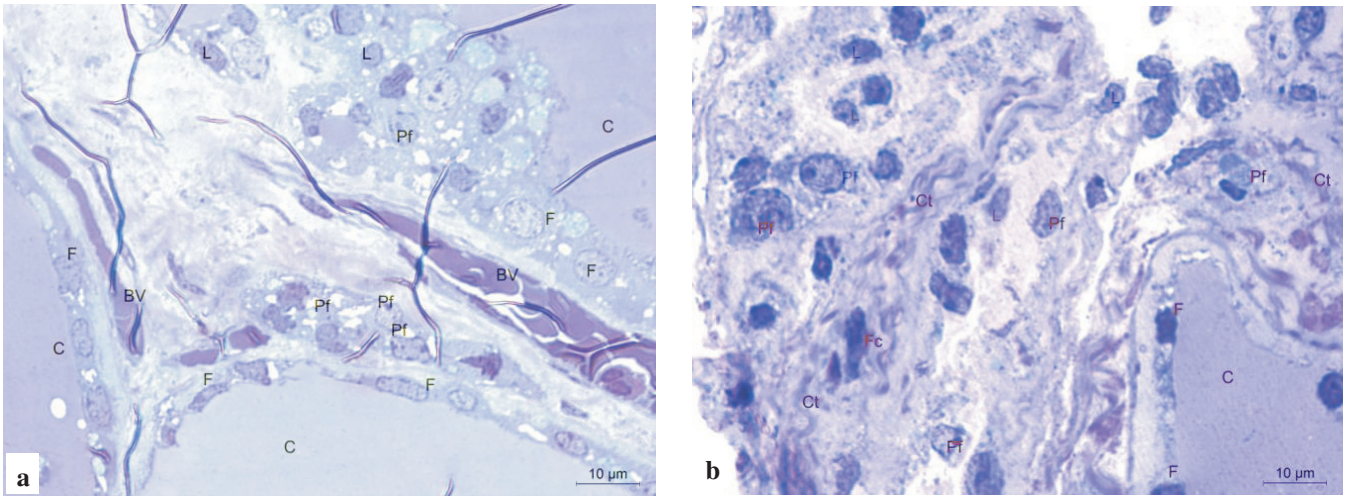

Fig. 1. Different stages of follicular damage and lymphoid infiltration in thyroid glands of patients diagnosed with autoimmune thyroiditis. Dilatation and loss of normal follicular shape, flattened epithelium and decreased intensity of colloid staining in some of the follicles, fibrosis of loose conective tissue, macrofollicular colloid struma (a); coalescence of adjacent follicles, focal necroses and fibrotic bands in the intersticial connective tissue (b); F-follicular cells, Pf- parafollicular cells, L-lymphoid cells, Ct- fibrotic bands in connective tissue, Fc-fibrocyte, C-colloid, Bv-blood vessels; semi-thin sections (0.5 um), immersion, light microscope Leica DM 5000B, ×100, Westzlar, Germany.

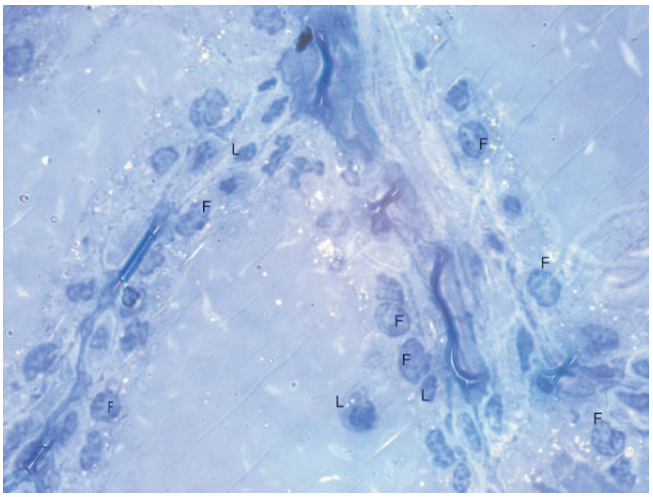

Fig. 2. Thyroid gland of patient diagnosed with autoimmune thyroiditis and macrofollicular colloid struma: thyroid follicles with flattened follicular epithelium and shrunk parenchymal cells with pycnotic or fragmented nuclei. Lymphocyte migration into the follicular colloid; F-follicular cells, L-lymphoid cells; semi-thin sections (0.5 um), immersion, light microscope Leica DM 5000B, ×100, Westzlar, Germany.

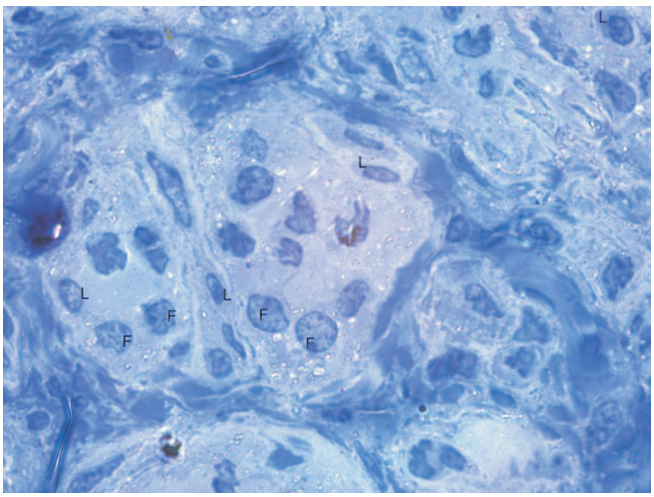

Fig. 3. Thyroid gland of patient diagnosed with autoimmune thyroiditis and follicular adenoma: atrophic and small follicles with abundance of cuboidal to low columnar follicular cells and lymphoid cells, reduction of the colloid, focal necroses and fibrotic bands in the intersticium; F-follicular cells, L-lymphoid cells; semi-thin sections (0.5 um), immersion, light microscope Leica DM 5000B, ×100, Westzlar, Germany.

sorting main lymphocytes subpopulations ($CD4^+$, $CD8^+$, $CD16^+$, $CD19^+$ and $CD95^+$) from PBMC of 7 patients with AIT.

DNA from the main lymphocyte subpopulations was also examined by RT-PCR. Substantial HHV-6 load in lymphocyte subpopulations was detected in two of seven patients. In the first patient, who was positive for the presence of the HHV-6 genomic sequence in whole blood and thyroid gland tissue DNA using nPCR, HHV-6 load was detected in NK and $CD95^+$ cells (24 and 100 viral copies/1 × 10^6 cells, respectively). In this patient also a lower HHV-6 load was detected in whole blood (46 copies/1x10^6 cells) comparing to tissue, where the HHV-6 load was 355 copies/1x10^6 cells (Fig. 4). In the second patient, who was nPCR positive for the presence of HHV-6 genomic sequence only in tissue DNA of the thyroid gland, the HHV-6 load was detected only in $CD95^+$ cells — 40 copies/1 × 10^6 cells (Fig. 4). In the other five patients, HHV-6 load was not detected in lymphocytes' subpopulations and whole blood samples because they were under detection limit of the used kit (< 5 copies per reaction).

DISCUSSION

Thyroid gland, as a highly reactive organ, regulating growth and normal metabolism, may be affected by a variety of direct or indirect physical, chemical or biological agents. Since effects on the thyroid may be subtle or related to age and environmental conditions, it is necessary to provide a wide range of diagnostic techniques targeting proper medical decisions and excluding cancer formation stages.

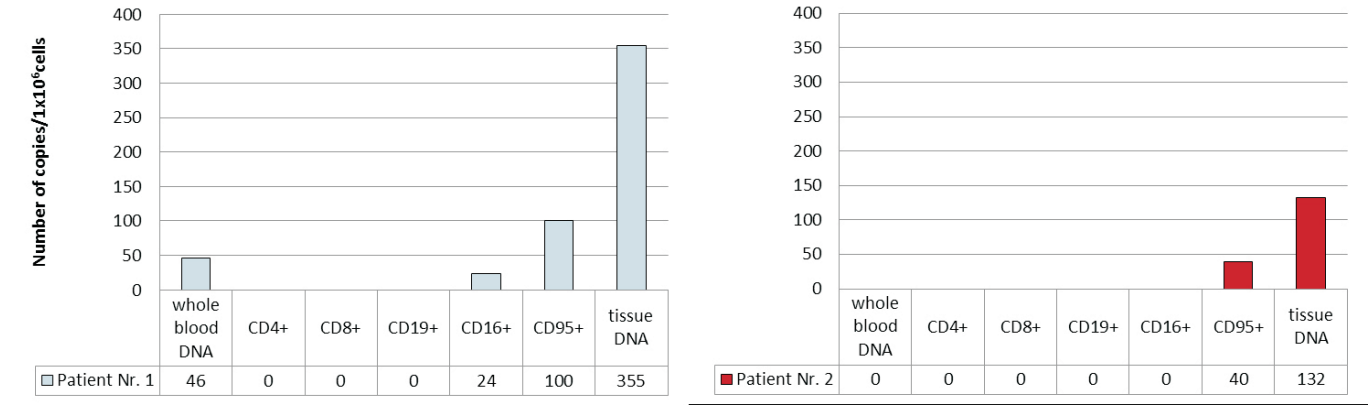

Fig. 4. HHV-6 load in DNA samples of whole blood, tissue and main lymphocyte subpopulations.

Light microscopic examination showed a lymphocytic infiltration as a histological finding, which needs to be supported by high serum titers of anti-thyroid autoantibodies to confirm autoimmune thyroiditis.

Dilatation and loss of normal follicular shape, flattened follicular epithelium are characteristic of hypofunction of the thyroid gland associated with the development of the condition macrofollicular struma nodosa, which could be caused not only by simple lack of iodine, but also by autoimmune problems. We observed microfollicular architecture and cuboidal epithelial cells in specimens of patients diagnosed with microfollicular colloid struma and follicular adenoma. McHenry and Phitayakorn (2011) reported that most patients with a follicular adenoma were clinically and biochemically euthyroid and approximately 1% of follicular adenomas — "toxic adenomas" showed hyperthyroidism. In our study *Struma nodosa III thyreotoxicum* was found in three and *Struma nodosa III euthyreocum* in four of the cases, and two patients had developed follicular adenomas. In the development of benign tumors and progression to cancer of thyroid gland, factors such as gene mutations, gene rearrangement and oncogene activation are suspected (Nikiforova *et al.*, 2003; McHenry and Phitayakorn, 2011). Also, factors including cell receptor transformation are suspected as triggers initiating autoimmune processes affecting the thyroid gland. Viral infections, in particular HHV6 with its immunomodulating properties, could be a facilitator or have direct influence on development of autoimmune processes in the thyroid gland (Dagna *et al*., 2013).

This study was designed to evaluate the relationship of the mentioned conditions to HHV-6 infection.

As HHV-6 is a lypmhotrophic virus, we expected to see its dominance in whole blood samples and especially in CD4 and CD8 T-lymphocytes populations, which was not supported by molecular findings. HHV-6 load was detected only in one patient's whole blood sample but others were under detection limit of the kit (< 5 viral copies per reaction). Also, none of the CD4 and CD8 T-lymphocytes subpopulations of patients showed HHV-6 load, and a very low HHV-6 load was found only in CD95+ cells (100 and 40 copies/10^6 cells) of two patients' and in NK cells (24 copies/10^6 cells) of one patient

A low HHV-6 load in peripheral blood immunocompetent cells and in overall blood samples, in comparison to thyroid tissue, demonstrates that the persistency site for HHV-6 could be the thyroid gland. In addition, the provided light microscopic examination suggested signs of impaired thyroid follicular structure and particularly, thyrocytes, which supports the idea that peripheral blood lymphocytes are target cells for HHV-6 persistency in patients with autoimmune thyroiditis.

In addition, presence of the HHV-6 genomic sequence in some immunocompetent cell populations indicates that the virus can infect thyroid gland during inflammation in the autoimmune process. In that scenario, HHV-6 more likely acts as a facilitator, but more detailed investigation with a larger patient group is required.

The presented results logically lead to further investigations, including electron microscopic observation to detect viral particles or assembled viruses in thyroid gland tissue.

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TIREOCĪTI KĀ CILVĒKA HERPESVĪRUSA-6 INFEKCIJAS MĒRĶŠŪNAS PACIENTIEM AR AUTOIMŪNO TIREOIDĪTU

Cilvēka herpesvīruss seši (HHV-6) ir izplatīts imūnmodulējošs betaherpesvīruss, kas tiek asociēts ar vairāku autoimūno slimību attīstību. HHV-6 replicējas CD4+ un CD8+ šūnās *in vitro* un *in vivo*, taču ir pētījumi, kur HHV-6 genoma sekvenci atrod ne tikai T limfocītos, bet arī dažādu orgānu audos, tai skaitā vairogdziedziera audos. Tādēļ šie pētījumi liecina par herpesvīrusa iespējamo iesaisti autoimūno vairogdziedzera slimību attīstībā. Šī pētījuma mērķis bija noskaidrot HHV-6 atšķirīgās klātbūtnes nozīmīgumu audos un asinīs pacientiem ar autoimūno tireoidītu. Šajā pētījumā tika iekļauti septiņi pacienti (sievietes ar vidējo vecumu 45 gadi (28–65)), kuriem histoloģiski bija apstiprināts autoimūns tireoidīts. Fluorescences aktivizēta šūnu sortēšana (FACS) tika izmantota, lai no pacientu perifēro asiņu mononukleārajām šūnām atšķirtu un sortētu limfocītu populācijas. Tika salīdzināta HHV-6 slodze vairogdziedzera audu un perifēro asiņu paraugos. Turklāt visi molekulāro pētījumu rezultāti tika salīdzināti un analizēti ar gaismas mikroskopijas rezultātiem. HHV-6 slodze tika konstatēta viena pacienta asins paraugā (46 vīrusa kopijas/1 × 10^6 šūnu) un pārējo pacientu asins paraugos slodze bija zemāka par detektējamo robežu, tikmēr HHV-6 slodze vairogdziedzera audu paraugos tika konstatēta visos paraugos (132 – 1620 vīrusa kopijas/1 × 10^6 šūnu). Diviem no septiņiem pacientiem tika konstatēta būtiska HHV-6 slodze limfocītu subpopulācijās. HHV-6 slodze tika konstatēta NK un CD95 + šūnās diviem pacientiem.