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INVESTIGATING THE POSSIBLE ROLE OF THE TORQUE TENO VÍRUS (TTV) AS A TRIGGERING FACTOR OF AUTOIMMUNITY IN AUTOIMMUNE BULLOS DISORDERS

PhD thesis

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I. INTRODUCTION

Torque Teno Virus (TTV): a non pathogenic virus?

In 1997-ben Nishizawa et al. isolated a then unknown viral genomic sequence out of posttransfusional hepatitis patients with high ALT levels. Known viral agents of hepatitis (A-E) could not be identified at that time in those patients.

Since then TTV is the subject of intense research. Its prevalence and its possible role in several pathogenetic processes were examined. International data on prevalence revealed that the viral presence in the general population is indeed very high. This extreme prevalence, the stability of the viraemia and the lack of definitive pathogenicity led scientists to hypothesize TTV to be a commensalist or orphan virus. However, it is of interest, that several other viruses bore the term "orphan" – such as several Adenoviruses, Parvovirus B19, EBV and Herpes Viruses 6 and 7 – before their pathogenicity and their role was revealed in their respective diseases.

Heterogeneity of the TTV genome

TTV displays extreme high genomic heterogeneity; genomic sequences taken from two isolates may even differ in more than 50%. This genomic instability feature – most probably caused by the viral replication- of heterogeneity is unique among the DNA viruses. Although distribution of this heterogeneity seems to be almost equal excluding some shorter, more conserved regions which occur mostly in the untranslated genomic region (UTR), but one is found also in the largest, first open reading frame (ORF), which is the N22 termed region.

Viral genomic heterogeneity is further increased by the observed incredible recombination ability – even TTV's with very different genomes are able to undergo viral genomic recombination with each other.

TTV detection, diagnostic methods

In vitro routine detection of TTV presence is still an up to date issue because of following reasons:

1. We don't know exactly what cells are TTV's main targets, although replicative forms of TTV have been shown to be present in several tissues (mostly in combination with bone marrow originated cells).
2. The immune response to TTV is not fully known and understood, we don't know yet what role humoral or cellular elements of the immune system could be involved into the clinical diagnostics.
3. Up to date no routine sensitive and specific (standardized) serological method is known to detect viral antigens or antibodies directed against the viral components of TTV.
4. TTV detection is based on PCR technology from the discovery on: firstly it was directed against the conserved N22 region with nested or seminested methodics, later - after exploring sequences and secondary structures of the UTR - primer systems were designed for that region as well
5. The described N22 PCRs only recognize genotypes 1-6, while recent UTR PCR's are able to detect more than 16 genotypes. This caused the believed medium prevalence data of the normal population to increase from 20 up to 80-90%. Also sensitivity increased, nested and seminested PCR's have a detection limit of at about 10^2-10^3 genomic copies/ml. Using real-time PCR technology sensitivity was upgraded up to a limit of 10^{-3} genomic copies/ml, and involving this technology mad also viral quantification possible.

TTV and its possible linkage to autoimmunity

Recent prevalence data have drawn attention to a possible connection of TTV and autoimmunity. It has been previously observed that the virus replicates in the bone marrow and its cells, also in other lymphoid tissues and in the peripheral mononuclear cells and lymphocytes – although more recent results describe that viral replication is limited to the activated cells only. It is also known, that systemic autoimmune diseases (such as rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE)) display pathogenic lymphocyte behavior and apoptotic processes following lymphocyte activation, which all contribute to the pathogenic autoimmune response causing the diseases above. This could be explained by an unknown viral agent disturbing normal functions.

When examining lymphocytes taken from patients with sclerosis multiplex in order to clarify the roles of triggering factors of the environment, it was found that the CNS infiltrating, neurotropic CD4+ T cells recognize TTV originated peptides. (Sospedra et al, 2005)

96.2% of the recognized peptides were from the 74 AA residue long N-terminal part of TTV ORF1, the above mentioned conserved and arginine-rich N22 region. This region was found to be a T-cell activator and may also be a substrate of the human TLR9, a receptor with an intensively researched possible role in the triggering of pathogenic autoimmunity.

A possible way, by pathogens may induce pathogenic autoimmunity is the so called molecular mimicry. While this evolutionary process occurs, the pathogenic agent becomes more similar to the host proteins – this is most effective by fast turnover viruses, and this can be observed by TTV. Molecular mimicry then leads to loss of tolerance against the „copycatted" proteins.

TTV prevalence was determined in several autoimmune diseases, where viruses have been suggested to play a role in the induction phase as environmental triggering factors – as in RA, osteoarthritis (OA), sclerosis multiplex (MS), autoimmune hepatitis, SLE and idiopathic myopathies (dermatomyositis, polymyositis). The prevalence was found to be significantly in SLE and juvenile dermatomyositis. Based on my previous research results and experiments I myself do not exclude but think that the role of this virus as a triggering factor in multifactor autoimmune diseases is of possibility and needs to be carefully researched further.

Literature data on TTV viraemia and its consequences are great in amount but partly or on some subjects fully contradictory. Even the published prevalence data of the Hungarian population is not of uniform nature and the possible implications and pathogenetic consequences of a TTV infection are also unknown.

II. AIMS

The experiments and examinations carried out in the present thesis have the aim to examine and clarify a possible connection of TTV viraemia and skin-specific autoimmunity by means of complex in silico and in vitro methodics.

Three, well known autoimmune blistering disorders were chosen for this as subjects – bullous pemphigoid (BP), the pemphigus group (pemphigus vulgaris (PV) and foliaceus (PF)) and also dermatitis herpetiformis.

- Our aim was to undertake in silico similarity-research of the most common molecular targets in these diseases and TTV proteins, to identify possible similarities or even exact matches. If such regions are to be found, antigenicity prediction for it will be carried out.
- Prevalence of TTV in the serum samples of patients with the disorders and healthy controls shall be determined by our in vitro PCR examinations. A correlation study of TTV DNA positivity and autoantibody defined subgroups is also to be carried out.
- If a possible relation between disease and TTV can be hypothesized, the response and response specificity against TTV antigens shall be explored by in vitro lymphocyte testing with synthesized peptides.

III. PATIENTS AND METHODS

Patients

Diagnoses of Hungarian patients with autoimmune blistering diseases were established by the physicians of Semmelweis University's Department of Dermatology, Venerology and Dermatooncology.

Serum samples were isolated and frozen for this study from BP (n = 40, medium age 74,3 ± 11.0 yrs, 11/40 M/F), PV (n = 33, m. age 56.8 ± 13.65 yrs, 12/33 M/F), and DH patients (n = 20, m. age 48.3 ± 8.2 yrs, 10/20 M/F), and also from two healthy control groups (K1: n = 95, m. age 41.5 ± 11.2 yrs, 43/95 M/F; K2: n = 50, m. age 70,09 ± 9.65 yrs, 9/50 M/F).

Furthermore 4 BP patients (n=4, (B1-4) m.age: 58 ± 6.2 yrs 1/4 M/F) and 3 control person's (C1-3 m.age: 49.5 ± 11.2 yrs 0/3 M/F) lymphocytes were isolated for further in vitro cell experiments. B3 while displaying clinical signs of BP also has inflammatory bowel disease.

The diagnosis of BP was based on routine skin histology, direct immunofluorescence (IF) and circulating IgG class anti-basement membrane zone antibody studies. The target antigens of these autoantibodies were verified by Western blotting and ELISA.

PV patients were diagnosed by routine histological and direct IF studies, further by detecting intercellular binding of circulating anti-desmosomal antibodies on monkey esophagus.

DH was also verified by routine and IF skin histology, and by circulating anti-endomysial (IIF), anti-gliadin and anti-tissue-transglutaminase IgA autoantibodies (ELISA).

All control sera were obtained from two control groups of healthy voluntary blood donors. The first group consisted of controls age and sex matched to the whole bullous dermatosis group, while the second control group was especially age-matched to the BP patients, because of their higher age at disease onset. For this second control group, older voluntary blood donors preferable over 60 years were selected. All individuals of patient and control groups were informed pre-sample donation, and were asked to sign the consent.

Protein and epitope level similarity studies

Protein query sequences for BLAST research were chosen from previously described antigenic parts of major autoantigens of the bullous dermatoses.

Residue lengths of sequences were chosen to be between 200 and 700. Protein sequences were obtained from public protein sequence resource of the UniprotKB/Swiss-Prot. These sequences were analyzed with the public access to Basic Local Aligment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI) homepage for protein-protein homology and for short nearly exact matches with TTV protein sequences. The observed similarities between BP180 domains and TTV ORF's were tested for antigenic epitopes with the online antigenic epitope predicting software of the Molecular Immunology Foundation.

In silico analysis was performed on following subjects:

BP

- BP180: NC16 region (aa 1 – 566),
- BP180: NC16A region (aa 489-566)
- BP180: C-terminal region (aa 1252-1532);
- BP230: C-terminal domain B and C subdomains (aa 1946 – 2649);

PV and PF

- Dsg3 (PV): extracellular domains (EC1-4 parts) (aa 87-566);
- Dsg1 (PV and PF): extracellular domains (EC1-4 parts) (aa 84-569)

DH

- whole TG3

In silico sequence analysis was carried out for known self-antigen targets of skin autoimmunity: BP180 – NC16 region (aa 1 – 566), the C-terminal region (aa 1252-1532) and BP230 B and C subdomains of the C-terminal part (aa 1946 – 2649), Dsg3 (aa 87-566) for PV and the whole TG3 for DH. The protein sequences were probed for alignment with TTV ORF sequences for shorter, exactly matching (SEM) regions, and longer but less exact protein-level matching regions (PPB). Expected values (E) of BLAST for similarity were considered high if E value was below 10, general similarity was accepted if E was below 25.

Detection of TTV DNA

Serum samples were collected from patients and controls, and were kept at -80 °C. All samples were assayed for viral nucleic acid preparation parallel. 200 µl of serum was used with the Roche High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany). TTV DNA was amplified by means of a nested polymerase chain reaction (PCR), in which the second PCR step was carried out with SYBR Green I-based PCR in an ABI 7500 Sequence Detection System (Applied Biosystems, US, California) and the product was later detected by melting-curve analysis as described. Reactions were carried out in triplicate. Sequences of the products specific for TTV were randomly tested by cycle-sequencing.

Peptide design

We used the peptide sequence H-RRRRRWRRWR-OH from the conserved TTV genome region; we termed it peptide ORF1/N22. This sequence was previously described and used by Sospedra et. al. The peptide was synthesized and purified by high pressure liquid chromatography to have a purity scale >95% (BACHEM, Germany).

Lymphoblast transformation assay (LTT)

Patient and control lymphocytes were harvested from 10 ml citrate anticoagulated fresh blood by Phicoll gradient centrifugation. LTT test was followed by thymidine incorporation assay and was performed as previously described by Pichler et al with the following modifications:

1. The thymidine incorporation was detected in a LKB RackBeta 1215 Liquid Scincillation Counter (LKB Instruments, Rockville, US).
2. Peptide concentration was set to 10, 25 and 50 ugs/well. Lymphocyte viability at all peptide concentrations was checked after 48 h-s by tripane-blue staining.
3. Assays were set up in 96 standard well systems, where 6 wells were used to study spontaneous proliferation, and 3 wells each were used to inspect reaction to pokeweed (PMW) and concavalin A (CON-A) mitogens.

4. Counts per minute (CPM) ratio of peptide provoked and spontaneous proliferation was used to objectively express the reaction of lymphocytes and was given as the stimulation index (SI). A SI above a 2.5 value was accepted as a reaction and as recognition of the peptide.

IV. RESULTS

Autoantigen and TTV-sequence likeness analysis

Protein-protein BLAST for large scale analysis showed no significant TTV similarities for PV and DH antigens, however, similarities to all probed BP antigens were detected. Short exact matches were detected for all probed proteins. Analysis of the BP180-NC16 region resulted in 16 hits, in four major regions matching, E values were between 0.29 – 7.9, with an average S score of 24.25 bits. Sequences were mostly matching for TTV ORF3 and ORF1 originated proteins. SEM E values were ranging from 0.9 to 9.0 of high similarity; 13 - 23 for lower similarities. Most sequence similarity for the SEM was detected for ORF1 and ORF2.

BP180 NC16A (aa 489-566) when subjected to SEM BLAST showed several overlaps with previously described B and T cell epitopes in this region. Likeness and overlaps were detected mostly for ORF1.

BP180 C-terminal large scale matching analysis resulted in identification of one region of about 200 amino acids, it was similar to database ORF1 sequences with E = 7,5, S = 21,9 bit. SEM similarities were detected mainly with the ORF2.

PPB of the BP230 C-terminal subdomain sequence showed 4 longer matching regions in the last 100 amino acids for TTV ORF1 sequences, E values ranging from 5,8 - 8.4, by an average s score of 23,5. SEM matches were identified for ORF1. E values were 5.0-8.9 for highly similar and 12 – 22 for lesser similar sequences, by an average s score of 24.2 bits.

PV antigen Dsg3 showed similarities on the SEM level mostly with ORF1, although ORF3 and ORF4 overlaps were also seen. E values were 8.1 for high, and 11-20 for lesser similarities, with average s score of 23.5 bits.

TG3 SEM BLAST resulted in identifying similarities to TTV ORF1 sequences. No high similarities were detected; E values were ranging from 16 – 21, with an s score average of 23.5

Predicted antigenicity of TTV-similar sequences

TTV similar sequences of the studied self antigens were analyzed for possible antigenic epitopes. These results were highlighted and compared to both PPB and SEM BLAST results.

BP180 NC16 sequence-analysis resulted in 18 identified possible antigenic determinants. The predicted antigenic propensity for this 566 AA long sequence is 1.0072. BP results matched 8 of the possibly immunogenic sequences overlapped with SEM identified similarities – where 7 had exact sequence matches. PPB, SEM and immunogenic peptide overlaps were seen on 5 different sites.

SEM BLAST results of the NC16A region alone (AA 489-566) were also compared with T- and B cell epitope sites described previously in the literature where several overlaps with TTV analog sequences were detected.

BP180 C-terminal domain had 7 possibly antigenic parts predicted. The 245 AA residue long sequence has an average antigenic propensity of 0.9960 SEM BLAST matching and antigenic prediction showed one major overlap.

BP230 C-terminal B and C subdomains antigenic prediction showed 30 results. This sequence of 704 AA residues has an antigenic propensity of 1.0312 predicted. The analyzed 479 residue part of Dsg3 has an average antigenic propensity of 1.0275. 21 possible determinants were detected, none of them matching the SEM BLAST results. The 692 protein sequence of TG3 showed an antigenic propensity of 1.0197. 28 possibly antigenic determinants were deduced of the sequence, none overlapping with SEM BLAST resulted TTV similar sites.

Detection of TTV DNA in patients' and controls' sera

TTV DNA was detected in 72/93 (77.4 %) patients with bullous dermatoses, in 64/95 (67.4 %) of control group 1 and in 31/50 (65.3 %; $p>0.05$) control group 2. Both control groups' involved healthy individuals. Age, gender and duration of disease had no influence on TTV positivity in either examined group.

No associations or tendencies were seen comparing PV patient and control groups for TTV positivity (19/33, 57.5% $p>0.05$). DH patients (17/20, 85% $p>0.05$) showed a slightly elevated prevalence of the virus, further experiments with larger sample number may reveal more significant results.

The TTV positivity in patients with BP was significantly higher than in patients with PV and DH, and then in the control groups (36/40 vs. 31/50, $p < 0,032$).

Association study on serum TTV DNA and BP autoantibodies

TTV DNA positivity was statistically analyzed for possible correlations with the presence of one or more autoantibodies against the following BP antigens: BP180-NC16a, BP180 C-terminal domain and BP230 detected with both ELISA and immunoblotting.

28 out of 40 BP patients were positive for antibodies against the NC16a domain of BP180, and 25 out of 28 were TTV DNA positive. 11 from 12 the NC16a negative BP patients 11 were TTV positive (χ^2, $p > 0.8380$). Immunoblotting against the BP180 C-terminal showed positive reactivity in 8 out of 40 BP patients, 6 out of 8 were also TTV DNA positive. 30 out of 32 BP180 C-terminal negative patients were TTV DNA positive (Fisher, $p > 0.3564$). The 40 tested BP samples were shown to be positive in 29 cases for either of BP180 NC16a or for the C-terminal regions analyzed by immunoblotting. Out of these 29, 25 were TTV positive. All the 11 BP patients negative for BP180 immunoblotting were TTV DNA positive (Fisher, $p > 0.5602$).

In all 6 BP cases positive for antibodies against both BP180 regions TTV positivity could be also demonstrated (Fisher, $p > 0.4648$). 26/40 BP patients were positive on BP180 ELISA, out of these 23 were TTV DNA positive (Fisher, $p > 0.6585$).

The 6 BP patients who were shown to be positive for both BP180 immunoblotts were all positive for ELISA and TTV DNA as well.

ELISA against the BP230 antigen resulted as positive in 20/40 BP patients, and 18 out of 20 positive sera were also TTV DNA positive. Similarly 18 out of 20 BP230 ELISA negative patients were TTV positive (Fisher, $p > 1.390$). No significant difference was found comparing the TTV DNA positive and negative groups of BP patients – and the statistics showed an almost equal viral distribution ($p<1.000$) in all autoantibody-defined subgroups.

LTT-peptide assay results

Control lymphocyte isolates showed no reaction to the TTV ORF1/N22 peptide at concentrations of 50 or 25 ugs, and only one TTV DNA positive healthy control person (C1) reacted to the TTV peptide at 10 ug, and this resulted in a low but elevated proliferation rate.

Otherwise no significant differences were seen when the spontaneous and peptide stimulated proliferation rates where compared ($p>0.05$).

All lymphocyte batches isolated from 4 TTV DNA positive BP patients have shown high rate of proliferation as response to being exposed to the ORF1/N22 peptide in the assays and differences between spontaneous and peptide stimulated rates were deduced as significant ($p<0.01$). We concluded that a positive lymphocyte reaction to this TTV peptide was clearly demonstrated.

V. DISCUSSION

In my present work I wanted to investigate the TTV's possible role in triggering skin specific autoimmunity, on model autoimmune blistering diseases BP, PV-PF and DH.

For this known autoantigen sequences for each disorder respectively and TTV protein sequences were carefully examined and compared by online alignment analysis for protein level (PPB) and shorter but more exact matching (SEM) seeking algorithms of BLAST.

While Dsg3 and TGe proteins showed only limited likeness, BP autoantigens presented with significant similarity for both algorithms. In the case of the BP180-NC16 the resulting TTV matching sites were detected to be overlapping with immunogenic regions.

Parallel, we carried out in vitro SYBR-GREEN I based melting curve analysis PCR's in autoimmune patients' and controls' sera to determine TTV DNA prevalence. Specificity of this method was routinely checked by cycle-sequencing random positive samples.

With this method the cohort created from summarizing the three disease-groups showed no significant difference in prevalence when compared to the control group of healthy donors. PV and DH group TTV DNA prevalence was also comparable to control prevalence data. The virus' prevalence in the BP group however was significantly elevated (BP vs. K2: $p < 0{,}032$).

The association study examining a possible connection between prevalence data and autoantibody defined subgroups was unsuccessful in showing any relations. However one must be aware of the limitations of techniques and note that both applied methodics (ELISA and blotting) are based on denatured antigen recognition and so conformation sensitive autoantibodies are partly not recognized by them.

Our in vitro cell assay showed, that TTV originated sequences are recognized by BP lymphocytes. The conserved Arg-rich sequence was recognized by all 4 BP patient lymphocyte cultures, and it induced a strong proliferation response.

In the present work and thesis we used in silico data and a descriptive approach to examine whether TTV could have any pathologic role in autoimmune bullous dermatoses. The significantly high incidence of TTV DNA among BP patients and the significant in silico similarity of BP180 antigen and the viral proteins together with the results of the in vitro cell response to the TTV ORF1/N22 peptide let us hypothesize a possible role of this virus in the patomechanism of the disease in some BP patients and render a base for further studies.

VI. CONCLUSIONS

Our results with the above given methodics let us believe, that a possible role of TTV in the pathogenesis of blistering autoimmune diseases PV and DH is not of considerable possibility:

- The in silico analysis results showed only minor matches of PV and DH autoantigens and TTV sequences
- TTV DNA prevalence was in both the PV and DH patient groups, as in the cohort group containing all three blistering diseases comparable to the values found in the examined healthy population values.

Our TTV in vitro experiments with samples taken from BP patients showed significant differences when compared to the healthy control samples, also the in silico BLAST analyses indicated a higher degree of overlaps between BP autoantigens and TTV sequences:

- TTV sequence matching's of higher level and direct overlaps with antigenic sites were observed in the case of the BP autoantigens. Similarity with viral sequences was seen highest in the immunogenic NC16 and NC16A domains.
- TTV prevalence in the BP patient group was significantly higher than in the control (K2) group. (36/40 vs. K2: 31/50, $p<0,032$)
- Lymphocytes isolated from BP patients showed strong reaction toward the TTV ORF1/N22 conserved sequence peptide in an in vitro lymphocyte activation assay. Stimulation indexes were significantly higher than lymphocytes obtained from healthy controls.

Our combined in silico and in vitro PCR and cell assay data lets us hypothetize, that a long and persistent TTV viraemia may contribute to the pathogenesis of BP.

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