Association of Cytomegalovirus Infection with the Sustenance of Autoimmune Response in Patients with Pemphigus Vulgaris

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ABSTRACT
In genetically vulnerable people, the cytomegalovirus (CMV) could be a major cause of autoimmunity. Emerging evidence reveals that CMV proteins are ubiquitous in autoimmunity-affected tissues and that autoimmune reactions may be sustained by both molecular mimicry and viral antigens. This study looked at how in-vitro CMV antigen stimulation affected T cell immunity and cytokine responsiveness in pemphigus vulgaris (PV) patients. Compared to healthy people, CMV antigen caused an increase in CD4+ T cells, notably the memory subset, and CD8+CD45RO+ T cells in PV patients. The CMV antigen induced an increase in the production of IL-4 and IFN-γ by PV PBMCs. The single nucleotide polymorphism IFN-γ +874 (rs2430561) was found to have a significant correlation with PV illness in our cytokine polymorphism analysis. IFN-γ may over-activate the immune system, resulting in antibody-mediated acantholytic disease via abnormal cell-mediated and humoral immunological responses. CMV infection may be a rare factor that sustains or exacerbates autoimmunity in PV disease in a non-specific way, especially in genetically predisposed hosts.

INTRODUCTION
The target antigens and autoantibodies for pemphigus vulgaris (PV), an autoimmune skin disease, have been extensively identified, however, the origins of the aberrant response have remained elusive. PV is a severe form of blistering disease in which autoantibodies attack keratinocyte desmosomal (desmoglein-3) proteins (Fassihi et al., 2006). Microbial infections and other environmental factors may catalyze the events by inducing an exuberant host immune response, which then attacks identical self-antigens, according to mounting evidence (molecular mimicry). In recent years, the focus of autoimmune disease research has shifted to a multidisciplinary approach to gain a better knowledge of the common mechanisms that underpin disease pathogenesis.

Studies done so far on the link between cytomegalovirus (CMV) and autoimmune diseases indicate a paucity of information in the literature about this frequent herpes virus infection and skin-related autoimmune diseases. CMV could be a major cause of autoimmunity in people who are genetically predisposed to it (Söderberg-Nauclér 2012). CMV infection has been linked to autoimmune diseases such as SLE (Sekigawa et al., 2002), diabetes type 1 and 2 (Filippi and von Herrath 2005; Roberts and Cech 2005), inflammatory bowel diseases (Criscuoli et al., 2006), systemic sclerosis (Shoenfeld et al., 2004; Varani and Landini 2011), and antiphospholipid syndrome (Uthman et al., 1999).
High CMV IgG titers detected in the sera of patients with a variety of autoimmune disorders point to several unanswered questions about the role of CMV infection in these diseases. Despite the lack of evidence tying CMV to the initiation of an autoimmune reaction, an initial inflammatory shock may reactivate it leading to the continuation or worsening of an autoimmune response by promoting the release of type I cytokines through particular mechanisms (Qiu et al., 2008; Slinger et al., 2010; Zhu et al., 2002). Furthermore, the presence of CMV proteins in autoimmunity-affected tissues may maintain the autoimmune response through molecular mimicry.

Furthermore, given the importance of cytokines in immune responses, genes producing them could be candidates for predicting pemphigus susceptibility. Several investigations have discovered elevated cytokine concentrations and expression in PV patients' sera, blister fluids, or afflicted skin, and documented their relationship with disease activity, implying that they play a role in disease pathogenesis (Javor et al., 2010; Keskin et al., 2008; López-Robles et al., 2001; Narbutt et al., 2008). Polymorphisms in the gene sequences of cytokines affect their production or function, at least in part (Bidwell et al., 1999). As a result, it's fair to believe that specific cytokine polymorphisms play a role in PV susceptibility. The study of cytokine polymorphisms may provide crucial information for our understanding of PV etiology and clinical manifestations.

This study investigated the in-vitro response of T cells from PV patients to CMV antigen. The T cell cytokine production was estimated for the Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) responses. The connection of well-known single nucleotide polymorphisms (SNPs) in these cytokines was also studied.

MATERIALS AND METHODS

Patients and Samples:

The study enrolled 16 individuals who were clinically diagnosed with PV and supported by well-accepted laboratory investigations/criteria. Expert dermatologists conducted a clinical assessment to establish a baseline index. Relevant tests were used to determine seropositivity. All the study participants were either new cases or had not used any steroids, immunosuppressive medications, or other therapeutic agents in the previous 6 months. Each patient's peripheral venous blood was collected aseptically into EDTA vacutainer tubes and utilized for PBMC isolation, and DNA extraction. For comparisons, 16 healthy age-matched volunteers were also enrolled. Each participant gave written informed consent before being enrolled in the study.

PBMC Isolation:

The blood samples were immediately diluted 1:1 with Hank's Balanced Salt Solution (HBSS) and placed over an equivalent volume of HiSep LSM (Hi-Media Laboratories) in sterile 50 ml conical centrifuge tubes at room temperature (RT). The sample was allowed to run down on its side while the centrifuge tube was kept at 45°. At room temperature, the tubes were centrifuged at 500 x g for 30 minutes. The hazy layer at the interface was aspirated carefully and washed three times with HBSS and once with RPMI 1640. (Hi-Media Laboratories). Centrifugation at 250 x g for 10 minutes at room temperature was used during the washing process. The cells' viability was determined using a hemocytometer-based trypan blue dye exclusion cell quantitation and viability assay, with results routinely exceeding 95%.

Antigen Stimulation of PBMCs:

In a 12-well cell culture plate (Nalgene Nunc, Rochester, NY), PBMCs (1x10⁶ cells/ml) in RPMI 1640 were seeded into an anti-human CD3 antibody precoated wells and treated with CMV antigen (Microbix Biosystems, Inc.,
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Canada). All stimulations/exposures were performed in triplicate, and cells were incubated for 72 hours at 37°C in humidified air containing 5% CO2.

Staining of PBMCs and Fluorescence-Activated Cell Sorting:

The PBMCs were extracted after incubation by centrifugation at 500 x g for 5 minutes, and the cell-free culture supernatants were withdrawn and frozen at -80°C for cytokine quantification through ELISA. Before labeling with different combinations of conjugated monoclonal antibodies (mAbs), harvested PBMCs were washed three times with wash buffer (0.5 percent BSA + 0.1 percent NaN3 in 1X PBS [pH 7.4]). (BD Biosciences). Each mAb was employed at a proper concentration, as determined by the previous titration. The cells were stained on ice for 30-60 minutes in the dark, then washed three times with wash buffer, fixed with 300 μl ice-cold 2 percent paraformaldehyde in 1X PBS, and kept at 4°C until analysis.

PBMCs were stained with FITC anti-human CD4 and APC anti-human CD25 concurrently before being stained with FoxP3. After washing, cells were fixed for 10 minutes at room temperature in the dark using a fixing buffer (containing diethylene glycol and formaldehyde). Cells were washed again after being centrifuged at 500 x g for 5 minutes to remove the fixative. The cells were permeabilized for 30 minutes at room temperature in the dark with permeabilization buffer (containing diethylene glycol, formaldehyde, and sodium azide). Cells were washed twice and stained intracellularly with PE anti-human FoxP3 antibody for 30 minutes at room temperature in the dark. Cells were washed and resuspended in 300 μl of 2% paraformaldehyde in 1X PBS after staining and kept at 4°C until analysis. Flow cytometry was done on a BD FACS Calibur™ system within 18 hours (BD Biosciences). Per condition, a minimum of 20,000-30,000 occurrences were gathered.

WinMDI 2.9 was used to evaluate all of the data. To establish the specificity of antibody binding on an equally gated group of cells, each experiment contained isotype-matched control antibodies.

ELISA for Cytokine Quantification:

BD Biosciences supplied ELISA kits for measuring cytokines such as IL-2, IL-4, IL-10, and IFN-γ were used. Following the manufacturer's instructions, the levels of the cytokines were determined in the culture supernatants of PBMCs stored earlier. Within 30 minutes of terminating the procedure, the absorbance was measured at 450nm. By graphing the standard curves with concentration versus absorbance, the concentrations of IL-2, IL-4, IL-10, and IFN-γ in samples were measured. The concentration of diluted samples was multiplied by the dilution factor. All of the kits have a detection limit ≥ 4 pg/ml.

Extraction of Genomic DNA:

Genomic DNA was isolated from PV patient blood samples in order to analyze 9 known SNPs in the cytokine genes of IL-2, IFN-γ, IL-4, and IL-10. DNA was also extracted from the blood samples of an even number of healthy subjects for cytokine genotyping comparisons. Following the manufacturer's instructions, genomic DNA was isolated from EDTA anticoagulated peripheral blood using the HiPurA™ blood genomic DNA extraction kit (HiMedia Laboratories). The DNA samples were kept at -20°C until they were needed. In order to genotype cytokines, DNA samples were treated to a specialized PCR reaction using sequence-specific primers.

Cytokine Genotyping Assay:

Using a commercially available Cytokine Genotyping Kit, cytokine genotyping was performed from genomic DNA using polymerase chain reaction with sequence-specific primers (Invitrogen Corporation, USA). This PCR-based kit was used to analyze SNPs (IL-2 –330 T/G and +166 G/T; IFN-γ +874 A/T; IL-4 –1098 T/G, –590 T/C, and −33 T/C; IL-10 –
1082 G/A, –819 C/T, and –592 C/A) in all PV patients and healthy subjects. Amplification was performed using the following thermal cycler profile: denaturation for 2 minutes at 94°C; 10 cycles of denaturation for 15 seconds at 94°C and annealing for 60 seconds at 65°C; 20 cycles of denaturation for 15 seconds at 94°C, annealing for 50 seconds at 61°C, and extension for 30 seconds at 72°C; and finally, hold at 4°C. For electrophoresis, the PCR products were put onto a 2% agarose gel in a specified order and ran at 150 volts for 20-25 minutes to separate the DNA. The ethidium bromide-stained gel was photographed after electrophoresis and evaluated for specific amplification patterns using the worksheet that came with the kit. Each lane was checked for the presence of a control band. An 89-bp snippet of the β-globin gene served as an internal control in wells recognizing the IL-2, IL-4, and IL-10 cytokines. As an internal control, wells detecting IFN-γ included a 440 bp snippet of the human C-reactive protein gene.

Statistical Analysis:

The mean (±SD) is used to present the data. The two-way analysis of variance (ANOVA), followed by a Tukey’s test, was used to examine differences in proportions of T-lymphocyte subpopulations as well as cytokine levels. The p-values of less than 0.05 were considered significant. The allele, genotype, and haplotype frequencies of patients and controls were compared using a two-sided Fisher’s exact test. The relative hazards associated with rare alleles, genotypes, and haplotypes were evaluated as odds ratios (ORs) with 95 percent confidence intervals (95% CIs), with a significance level of <0.05. A goodness-of-fit Chi-square test was used to compare the observed genotype frequencies with the anticipated frequencies among PV patients and healthy subjects to evaluate the divergence from Hardy-Weinberg equilibrium (HWE). If the polymorphisms diverged from HWE, they were ruled out. SPSS 16.0 was used to conduct all statistical analyses (SPSS Inc).

RESULTS

Patients and Controls:

In this study, 16 adult PV patients and an equal number of adult healthy control subjects (HCs) were enrolled. Pemphigus was diagnosed based on the clinical profile, histological evidence of intra-epidermal vesicles with suprabasilar acantholysis, and direct immunofluorescence of immunoglobulin G (IgG) deposition on the keratinocyte cell surface. Patients with severe disease had mucocutaneous involvement in 85% of cases, while patients with mild disease had minor mucosal involvement in 15% of cases. A total of 16 age and sex-matched normal human volunteers with no known autoimmune disease served as controls.

Changes in T-cells with CMV Antigen Stimulation:

To stimulate PBMCs from PV patients and HCs, CMV lysate was inactivated by sonication and employed at a 1/100 dilution of 3.4 mg/ml concentration. PV patients had a much higher CD4+ population and a significantly lower CD8+ population of T cells than HCs (Fig. 1). To define the cell membrane phenotype of CD4+ and CD8+ T-cells, CMV-stimulated PBMCs were stained for surface expression of CD45RA. In the majority of PV patients, a small but considerable CD4+CD45RA+ and CD8+CD45RA+ T-cell population could be seen. The low expression of CD45RA on both CD4+ and CD8+ T cells suggests that the phenotype of these T-cells in PV patients is more CD45RO (about 75%) than in HCs (approximately 40%). These findings point to PV having an effector but revertant memory phenotype, indicating that they had previously been exposed to CMV. Before and after CMV stimulation of PBMCs in-vitro, no significant changes in CD4+CD25+ T-cell population were observed.
Cytokine Secretion by CMV Stimulated PBMCs:
When compared to HCs, the levels of IL-2 in unstimulated PV cells were much lower. Although CMV antigen stimulation elevated IL-2 levels significantly, they remained lower than in the control group. The levels of IFN-γ in unstimulated cells in HCs were much greater than in PV patients. With CMV antigen stimulation, patient PBMCs showed a large increase in IFN-γ levels, whereas HC PBMCs showed a significant decrease.

PV cells that had not been activated had larger amounts of IL-4 than controls. The CMV antigen significantly boosted IL-4 levels in the control group, but not in the PV group. The amount of IL-10 in unstimulated cells of PV patients was significantly higher than in HCs. The CMV antigen stimulation caused IL-10 levels in PV patients to increase, but more so in HC cells (Table 1).

Table 1: Levels (pg/ml) of IL-2, IL-4, IL-10, and IFN-γ in pemphigus vulgaris (PV) patients and healthy control subjects (HCs) after stimulation with cytomegalovirus (CMV) antigen in-vitro.

<table>
<thead>
<tr>
<th>Antigen/Stimulant</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not stimulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HCs</td>
<td>340.7±27.6</td>
<td>848.6±194.6</td>
<td>5.6±3.7</td>
<td>105.2±17.3</td>
</tr>
<tr>
<td>PV</td>
<td>127.3±16.8</td>
<td>43.1±20.9</td>
<td>20.6±5.4</td>
<td>156.1±29.4</td>
</tr>
<tr>
<td>p-value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

| CMV stimulated    |        |        |        |        |
| HCs               | 303.3±45.7 | 645.2±182.4 | 7.6±1.3 | 322.3±32.4 |
| PV                | 265.4±55.1 | 361.4±71.2 | 15.4±4.5 | 284.2±21.2 |
| p-value           | 0.049<sup>∗</sup> | <0.001<sup>∗</sup> | <0.001<sup>∗</sup> | 0.065<sup>∗</sup> |

Values are presented in terms of mean (±SD); <sup>∗</sup>The mean difference is significant at the indicated p-value.
Cytokine Gene Polymorphism:

By cytokine genotyping with sequence-specific primers, 9 SNPs in four cytokine genes (IL-2, IFN-γ, IL-4, and IL-10) were studied in all PV patients and HCs. All cytokine SNP genotype distributions matched the Hardy-Weinberg distribution with no divergence from HWE (p > 0.05). No significant changes between the two groups were detected in SNPs of IL-2 –330 T/G (rs2069762) and +166 G/T (rs2069763); IL-4 –1098 T/G (rs2243248), –590 T/C (rs2243250), and –33 T/C (rs2070874); and IL-10 –1082 G/A (rs1800896), –819 C/T (rs1800871), and –592 C/A (rs1800872) (data not shown). In the IFN-γ +874 A/T (rs2430561) gene polymorphism, however, significant changes in allelic distribution were detected. PV patients had a 0.34 odds ratio due to the reduced presence of the IFN-γ +874 T allele (p = 0.0388) (95 % CI 0.12 to 0.96) (Table 2).

Table 2: Allele and genotype frequencies of IFN-γ (+874 A/T) gene polymorphism in PV patients and healthy control subjects (HCs).

<table>
<thead>
<tr>
<th>Single Nucleotide Polymorphism (SNP)</th>
<th>Alleles</th>
<th>PV patients</th>
<th>HCs</th>
<th>p-value</th>
<th>Odds Ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ +874 (rs2430561)</td>
<td>A</td>
<td>24 (75.0)</td>
<td>16 (50.0)</td>
<td>0.0388*</td>
<td>3.00</td>
<td>1.04 - 8.65</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>8 (25.0)</td>
<td>16 (50.0)</td>
<td></td>
<td>0.34</td>
<td>0.12 - 0.96</td>
</tr>
<tr>
<td>Genotypes</td>
<td>AA</td>
<td>10 (62.5)</td>
<td>6 (37.5)</td>
<td>0.0544</td>
<td>10.00</td>
<td>0.96 - 104.49</td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td>5 (31.25)</td>
<td>4 (25.0)</td>
<td>0.7340</td>
<td>1.34</td>
<td>0.25 - 7.00</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>1 (6.25)</td>
<td>6 (37.5)</td>
<td>0.1130</td>
<td>0.14</td>
<td>0.01 - 1.61</td>
</tr>
</tbody>
</table>

Frequencies of alleles and genotypes are presented as absolute numbers with percentages in parentheses; rs - refSNP cluster ID number; 95% CI - 95% confidence interval; *The mean difference is significant at the indicated p-value.

DISCUSSION

Pemphigus can be induced or exacerbated by viral infectious agents and vaccinations, which work by stimulating the cellular immune system (Ahmed and Rosen 1989; Mignogna and Ruocco 2000; Ruocco et al., 1996; Tufano et al., 1999). The viruses of the herpetoviridae family, such as herpes simplex, Epstein–Barr virus, CMV, and others, are the most often implicated infectious pathogens. In this work, stimulating patient PBMCs with CMV antigen resulted in an increase in CD4+ T cells and a decrease in CD8+ T cells when compared to healthy subjects. CD45RO expression was also found to be high on both CD4+ and CD8+ T cells. These findings point to an effector but revertant memory pattern in individuals, implying that they have previously been exposed to CMV.

In patients, stimulation with CMV antigen resulted in an increase in IFN-γ production from baseline, but the levels were much lower than those produced by control PBMCs. Patients, on the other hand, produced considerably more IL-4 in response to CMV stimulation than controls. This suggests that, like control cells, cells from patients have a tendency to create larger levels of IFN-γ, but it's possible that the inhibitory effect of high IL-4 in patients inhibits its optimal induction. IFN-γ is already known to be suppressed by IL-4. The excessive generation of cytokines in response to CMV antigen in pemphigus may overactivate the immune system, resulting in antibody-mediated acantholytic disease in these patients due to abnormal cell-mediated and humoral immune responses.

A wide range of cytokine polymorphism variations has been linked to the development of autoimmune disorders. Studies on their relationship with pemphigus have been sparse thus far, owing to the disease's low prevalence across people. PV and a polymorphism in
the gene IFN-γ at position +874 T/A in the first intron was discovered to be linked in our study. When compared to AA and AT, the lower frequency of IFN-γ (+874) TT genotype imparted an odds ratio of 0.14 and a 95 percent CI of 0.01 to 1.61 (p=0.1130). The ability to create large quantities of IFN-γ is related to the homozygous TT genotype, the heterozygous TA genotype with moderate levels, and the homozygous AA genotype with low levels (Pravica et al., 1999; Pravica et al., 2000).

PV patients had a somewhat higher frequency of the genotype associated with poor IFN-γ production (AA) than controls (62.5 percent vs. 37.5 percent; p=0.0544). PV patients may produce fewer IFN-γ, according to this finding. The timing and concentration of pro-inflammatory cytokines produced during the inflammation process seem to be important factors in reducing T-cell responses. IFN-γ may exert its anti-inflammatory effects by causing T-cell death. Several regulatory T-cell populations, on the other hand, appear to require IFN-γ secretion in order to decrease autoreactive T cells.

**Conclusion:**

Infection with CMV may be an occasional factor sustaining the outburst or worsening of the autoimmune illness in genetically vulnerable hosts in a non-specific way, as herpes virus infections are discovered inconsistently in pemphigus patients. Despite the confusing clinical similarities between viral diseases and pemphigus, as well as the different outcomes of the two conditions, it is critical to diagnose viral infection in a PV patient and begin antiviral therapy as soon as possible, often as an adjunct to immunosuppressive therapy, to prevent the viral antigens from wreaking havoc.

**Conflict of interest** There is no conflict of interest declared by the author.

**Ethical Approval:** The experiments were approved by the Institutional Board Review and Ethics Committee of the Faculty of Applied Medical Sciences, Al-Baha University.

**REFERENCES**


