

The aim of our study was to analyse a significance of tumour necrosis factor (TNF)- $\alpha$  promoter gene polymorphisms in relation to the HLA-DR locus in genetic predisposition to pemphigus. TNF- $\alpha$  gene polymorphisms in position -238 and -308 were identified using a modified polymerase chain reaction-restriction fragment length polymorphism method in 53 patients with pemphigus (38 with pemphigus vulgaris, 15 with pemphigus foliaceus) and 87 healthy controls. The HLA-DRB1 locus was typed using the polymerase chain reaction SSO method in all the patients and 152 population controls. Carriers of the TNF- $\alpha$  polymorphic -308 A allele were found to be more frequent in the pemphigus foliaceus group in comparison with the control group (odds ratio (OR) = 8.12;  $p$  = 0.0005). A significant association between HLA-DRB1\*04 (OR = 3.86;  $p_{\text{cor}}$  = 0.0001) and DRB1\*14 (OR = 8.4;  $p_{\text{cor}}$  = 0.0001) and pemphigus vulgaris was found. In this group of patients a decreased frequency of HLA-DRB1\*07 (OR = 0.08;  $p_{\text{cor}}$  = 0.006) was also identified.

We have shown for the first time a positive association of TNF- $\alpha$  polymorphism in position -308 with pemphigus foliaceus.

**Key words:** Polymorphism tumour necrosis factor- $\alpha$ , HLA class II, Pemphigus vulgaris, Pemphigus foliaceus, Polish population

## Tumour necrosis factor- $\alpha$ polymorphism as one of the complex inherited factors in pemphigus

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### Introduction

Pemphigus is an autoimmune disease, clinically characterized by bullae and erosions on the skin and mucous membranes. The aetiopathogenesis of pemphigus is not fully elucidated; however, additionally to an autoimmunological background, certain environmental agents such as medications, ultraviolet irradiation, diet, and viral infections are regarded as triggering factors, especially in genetically susceptible individuals. Two major forms of pemphigus, pemphigus vulgaris (PV) and pemphigus foliaceus (PF), based on the clinical picture, histological and immunological examinations are distinguished. In PF patients circulating IgG autoantibodies are directed against desmoglein 1 (Dsg 1) solely, while in PV antibodies are detected against desmoglein 3 (Dsg 3) sometimes in combination with anti-Dsg 1.

Studies on immunogenetic background in pemphigus have so far focused on association between the disease and certain HLA antigens. We have not found any literature data analysing other genetic factors involved in immune response in pemphigus.

Tumour necrosis factor (TNF)- $\alpha$  is a proinflammatory cytokine that plays a key role in the pathogenesis of many infections and inflammatory diseases. The

TNF- $\alpha$  gene is located within the class III region of the human HLA locus and several studies have shown extensive linkage disequilibrium both within the TNF locus and HLA class I and II. Several polymorphisms in the TNF- $\alpha$  gene were found to be associated with various autoimmune disorders. Elevated levels of TNF- $\alpha$  mRNA and protein have been demonstrated in skin and sera of patients with pemphigus vulgaris.<sup>1,2</sup> Additionally, TNF- $\alpha$  receptor-deficient mice demonstrated a decreased susceptibility to pemphigus development after passive transfer of IgG autoantibodies.<sup>1</sup> TNF- $\alpha$  gene polymorphism in pemphigus patients has not so far been studied.

Literature data highlight a strong association between the disease and HLA class II locus. Associations of HLA-DRB1\*0402, DRB1\*1401 and DQB1\*0503 alleles with pemphigus vulgaris were reported in Jewish, non-Jewish and Japanese populations.<sup>3–6</sup> Interestingly, it has been shown that autoreactive T cells from PV patients recognize certain Dsg 3 fragments in restriction to HLA-DRB1\*0402 and DQB1\*0503.<sup>7</sup> Similarly, in PF patients associations with HLA-DRB1\*04, DRB1\*14 and DRB1\*16 were identified,<sup>8</sup> and direct involvement of these HLA molecules in presentation of desmoglein 1 fragments was suggested.<sup>9</sup> There are many reports on HLA alleles profile in pemphigus in various populations

(Spanish, French, Italian, etc.); however, studies including exclusively Polish patients have not so far been performed.

In order to analyse a significance of TNF- $\alpha$  gene polymorphism in genetic predisposition to pemphigus, we designed an association study in a group of patients with PV and PF in Polish population, genotyped for HLA-DRB1.

## Materials and methods

Our study comprised 53 patients with pemphigus (38 with PV, 15 with PF), 26 males and 27 females, with a mean age of 53.9 years. In all the patients the diagnosis was confirmed by histological and immunological examinations (direct and indirect immunofluorescence tests).

Genomic DNA was extracted from peripheral blood cells using the 'Easy Blood DNA Prep' (A&A Biotechnology, Gdynia, Poland). TNF- $\alpha$  gene polymorphisms in positions -238 and -308 were identified using the modified polymerase chain reaction (PCR)-restriction fragment length polymorphism-amplification created restriction sites method<sup>10</sup> in the patients and in 87 unrelated healthy individuals from the members of the organ donors' families. The 117 base pair PCR fragment was amplified using Taq Polymerase (Rapidozym, Berlin, Germany) and modified primers introducing the *Nco*I restriction site for G in position -308 and the *Ava*II restriction site for G in position -238. After digestion the obtained fragments were detected in a 4% agarose gel, stained with ethidium bromide and visualized under ultraviolet illumination.

Molecular typing of groups of HLA-DRB alleles at a low-resolution level in the patients and 152 healthy unrelated individuals was carried out using the Dynal RELI SSO HLA-DRB Test (Dynal Biotech A.S.A., Oslo, Norway) set of 36 molecular probes. Polymorphic fragments (second exon) of genes DRB1, DRB3, DRB4 and DRB5 were amplified in PCR reaction: 35 cycles of 15 sec at 95°C, 45 sec at 60°C, 15 sec at 72°C and 5 min at 72°C, using Taq polymerase and PCR buffer (Rapidozym, Berlin, Germany). After denaturation the PCR products were hybridized in SSPE buffer at 50°C with a set of specific molecular probes coating nylon stripes. The reaction results were read using the system of horseradish streptavidin-peroxidase. The results of typing were analysed using the Dynal RELI SSO Pattern Matching Program.

The study protocol was approved by the Ethics Committee of the Lodz Medical University. All subjects gave written informed consent before entering the study.

## Statistical analysis

The frequencies of HLA-DRB and TNF- $\alpha$  polymorphic alleles in patients and controls were compared using the two-sided Fisher's exact test. The significance in the genotype frequency differences between patients and controls was evaluated using the  $\chi^2$  (two degrees of freedom) test. Values of  $p < 0.05$  was considered statistically significant. To correct for incidental significance, the  $p$  value was multiplied by the number of alleles compared ( $p_{cor}$ ). An odds ratio (OR) was calculated as the cross product in a  $2 \times 2$  table within 95% confidence intervals (95% CI).

## Results

The distribution of polymorphism of TNF- $\alpha$  in positions -238 and -308 in PV and PF is presented in Table 1. Carriers of allele A in position -308 were found to be more frequent in patients with PF in comparison with the control group ( $p = 0.0005$ , OR = 8.12, 95% CI = 2.34–28.1). The A-allele frequency was also higher in PF patients (40%) versus controls (13.2%) ( $p = 0.001$ , OR = 4.79, 95% CI = 2.02–11.34). No significant differences in -308 TNF- $\alpha$  polymorphism distribution between the PV and the control groups were observed. TNF- $\alpha$  polymorphism in position -238 was not significantly associated with either PV or PF.

The results of HLA-DR analysis in PV and PF patients are presented in Table 2.

PV patients showed a statistically significant association with HLA-DRB1\*04 (OR = 3.86,  $p_{cor} = 0.0001$ ) and DRB1\*14 (OR = 8.4,  $p_{cor} = 0.0001$ ). Also in this group we found a statistically significant decreased frequency of HLA-DRB1\*07 (OR = 0.08,  $p_{cor} = 0.006$ ) when compared with the control group (Table 2). No statistically significant differences between frequencies of HLA-DR alleles in patients with PF and the control group were identified.

## Discussion

TNF- $\alpha$  is one of the mediators involved in inflammatory processes. Its production can be regulated at the transcriptional, post-transcriptional and translational levels. Variability in the promoter and coding regions of the TNF- $\alpha$  gene may modulate the magnitude of its secretory response. The results of the *in vivo* studies demonstrated that PV-IgG autoantibodies induced TNF- $\alpha$  mRNA in the skin. Some authors found over expression of this cytokine in the lesions in the pemphigus patients and the correlation between its serum level and the disease activity.<sup>2</sup> These observations suggest the role of this mediator in the devel-

**Table 1.** TNF- $\alpha$  polymorphism in patients with pemphigus vulgaris and foliaceus compared with the control group

	Pemphigus vulgaris (n = 38)		Pemphigus foliaceus (n = 15)		Controls (n = 87)		Statistical analysis	
	n	%	n	%	n	%	PV versus controls	PF versus controls
-238 genotypes								
GG	37	97.4	13	86.5	83	95.4	NS	NS
GA	1	2.6	1	6.75	4	4.5		
AA	0	0	1	6.75	0	0		
G carriers	37	97.4	13	86.5	83	95.4	NS	NS
A carriers	1	2.6	2	13.5	0	0	NS	NS
-238 alleles	n = 76		n = 30		n = 174			
G	75	98.7	27	90	170	97.7	NS	NS
A	1	1.3	3	10	4	2.3	NS	NS
-308 genotypes								
GG	33	86.9	4	26.7	65	74.7	NS	p = 0.002
GA	4	10.5	10	66.7	21	24.1		
AA	1	2.6	1	6.6	1	1.2		
G carriers	33	86.9	4	26.7	65	74.7	NS	p = 0.0005
A carriers	5	13.1	11	73.3	22	25.3	NS	p = 0.0005*, OR = 8.12, 95% CI = 2.34–28.1
-308 alleles	n = 76		n = 30		n = 174			
G	70	92.1	18	60	151	86.8	NS	p = 0.001
A	6	7.9	12	40	23	13.2	NS	p = 0.001*, OR = 4.79, 95% CI = 2.02–11.34

\* Statistically significant. NS, non-statistically significant.

opment of pemphigus lesions by increasing epithelial damage.

We found a significant association of the TNF- $\alpha$  -308 A allele with PF and, interestingly, no such a relation in a group of patients with PV was observed. There was no significant association of pemphigus phenotypes with the polymorphism in position -238 as well. The group of PF patients is rather small, but most of our PF patients (11/15, 73%) were carriers of at least one -308 A allele. At this stage the relevance of this association is difficult to explain. It might result from strong linkage disequilibrium between HLA and TNF- $\alpha$  loci. It has been reported that the TNF- $\alpha$  -308 A allele is in linkage with the HLA-A1/B8/DR3 haplotype,<sup>11</sup> but no such HLA association with PF

has yet been identified, which makes this hypothesis less probable. Another possibility may be related to direct involvement of TNF- $\alpha$  in pathomechanism of PF. The TNF- $\alpha$  -308 polymorphism has been shown to alter function and has been associated with a six-fold to seven-fold higher level of transcription of TNF- $\alpha$ .<sup>12</sup> Other studies, however, have failed to show any functional change associated with this polymorphism.<sup>13</sup> *In vivo* studies have also shown conflicting results, with Chen *et al.*<sup>14</sup> and Juszczynski *et al.*<sup>15</sup> finding high levels of TNF- $\alpha$  associated with the -308 G/A polymorphism, while others reported no significant association between -308 polymorphism and TNF- $\alpha$  levels.<sup>16</sup> Thus, our preliminary observation of positive association between the TNF- $\alpha$  locus

**Table 2.** Frequency of HLA-DRB1 alleles in patients with pemphigus vulgaris and foliaceus compared with the control group

DRB1	Pemphigus vulgaris (n = 76)		Pemphigus foliaceus (n = 30)		Controls (n = 304)		Statistical analysis	
	n	%	n	%	n	%	PV versus controls	PF versus controls
*01	8	10.5	7	23.3	31	10.2	NS	NS
*15/16	5	6.6	2	6.7	51	16.8	NS	NS
*03	3	3.9	8	26.7	31	10.2	NS	p < 0.01, p <sub>cor</sub> = NS
*04	27	35.5	5	16.7	38	12.5	p < 0.0001*, p <sub>cor</sub> = 0.0001, OR = 3.86, 95% CI = 2.16–6.89	NS
*07	1	1.3	3	10	44	14.5	p = 0.0005*, p <sub>cor</sub> = 0.006, OR = 0.08, 95% CI = 0.01–0.58	NS
*08	2	2.6	1	3.3	7	2.3	NS	NS
*09	2	2.6	0		3	1	NS	NS
*10	0		0		7	2.3	NS	NS
*11	11	14.5	3	10	42	13.8	NS	NS
*12	0		0		14	4.6	NS	NS
*13	6	7.9	1	3.3	30	9.9	NS	NS
*14	11	14.5	0		6	2	p < 0.0001*, p <sub>cor</sub> = 0.0001, OR = 8.4, 95% CI = 2.99–23.5	NS

\* Statistically significant. NS, non-statistically significant.

and PF may suggest that TNF- $\alpha$  may be a cytokine directly involved in the pathomechanism of PF or may be related to differential clinical manifestation of various types of pemphigus. A similar role of the TNF- $\alpha$  polymorphism has been suggested in other autoimmune diseases like rheumatoid arthritis or systemic lupus erythematosus.<sup>17,18</sup> Based on the presented data we conclude that TNF- $\alpha$  polymorphism is part of the complex inherited factors in pemphigus.

The role of the genetic background, including mainly HLA class II genes is strongly stressed in the pathogenesis of pemphigus. Many authors have shown that HLA-DR\*04 and DR\*14 confer strong susceptibility to PV in different ethnic groups: Jewish<sup>3</sup>, non-Jewish<sup>4</sup> and Japanese.<sup>5,6</sup> Our studies conducted in Polish pemphigus patients confirmed these observations. The frequency of both HLA-DRB1\*04 and DRB1\*14 alleles were significantly higher in patients with PV than in an ethnically matched control group. Current evidence indicates that Dsg 3 is the dominant initial autoantigen in PV and that anti-Dsg 3 antibodies produced in restriction to HLA-DRB1\*0402 and DQB1\*0503 (in strong linkage disequilibrium with DRB1\*1401) induce lesion formation. It has been shown that only antigen presenting cells expressing HLA-DRB1\*0402 and DQB1\*0503 were capable of presenting Dsg 3 to autoreactive Th1 and Th2 clones, stressing a direct involvement of particular HLA antigens in the pathomechanism of PV.<sup>7</sup>

The relationship between PF and HLA class II locus is less clear. Lombardi *et al.*<sup>19</sup> and Miyagawa *et al.*<sup>20</sup> indicated that HLA-DRB1\*04 and HLA-DRB1\*14 are also associated with PF but in our study group we did not find such an association. In the case of the endemic form of PF (fogo selvagem), it has been shown that the autoimmune proliferative T-cell response is directed to external fragment of Dsg 1 in restriction to certain HLA alleles, mainly DRB1\*0404, DRB1\*1402, DRB1\*1406 and DRB1\*1602, which share the same sequence (LLEQR-RAA) in the DRB hypervariable domain from amino acids 67 to 74.<sup>9</sup> The study performed by Moraes *et al.*<sup>8</sup> in three different Brazilian populations with FS showed positive association with HLA-DRB1\*01, DRB1\*04 and DRB1\*14, which suggests that a similar pathomechanism as in PV may apply to fogo selvagem and possibly to PF as well. The fact that presentation of causative autoantigens in PF may be restricted by several HLA alleles might explain lack of significant positive associations found in our study. Further studies on bigger groups with PF are required to demonstrate such associations with the HLA locus.

In accordance with an idea of direct presentation of pemphigus autoantigens by certain HLA alleles, a negative association of HLA with pemphigus may occur, related to insufficient presentation of these

antigens in restriction to some other HLA alleles. The significantly decreased frequency of HLA-DRB1\*07 observed in our patients, similar to the results of HLA analysis in Italian and Brazilian patients with PV and PF,<sup>19,21</sup> suggest its protective role against pemphigus development. Other studies have also shown that certain HLA alleles play a protective role; for example, in Spanish patients with PV (DRB1\*13)<sup>22</sup> and in Turkish patients (DR 11, DQ 7 and DQ 2).<sup>23</sup>

In conclusion, we have shown a positive association of TNF- $\alpha$  polymorphism in position -308 with PF for the first time. We also show that the same association of HLA-DR\*04 and DR\*14 alleles with PV, as reported before in other ethnic groups, is also present in Polish pemphigus patients. Although TNF- $\alpha$  and HLA-DR genes may play a role in pemphigus development, the genetic association of TNF- $\alpha$  polymorphism with pemphigus seems to be independent of the HLA class II antigens.

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