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RELATIONSHIP BETWEEN THE OCCURRENCE OF ANTI-SSA, ANTI-SSB AUTOANTIBODIES AND HLA CLASS II ALLELES FROM THE ASPECT OF *IN VITRO* INHIBITORY EFFECT OF GLUCOCORTICOSTEROID ON THE ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY IN PATIENTS WITH PRIMARY SJÖGREN'S SYNDROME

A. KOVÁCS[1], ÉVA SZEKERES[1], LIVIA BEREK[1], EMŐKE ENDREFFY[2], L. KOVÁCS[3], ÉVA MAKULA[4], J. ELLER[5], GY. POKORNY[3], ILDIKÓ B. PETRI[1]

[1]Blood Transfusion Centre, [2]Department of Pediatrics, [3]First Department of Internal Medicine, [4]Department of Radiology, [5]Department of Medical Informatics, Albert Szent-Györgyi Medical University, P.O. Box 427, H–6701 Szeged, Hungary

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Though at present there is no evidence-based algorithm for the treatment of primary Sjögren's syndrome, it is generally accepted that glucocorticosteroid (GS) therapy must be introduced in cases with severe systemic manifestations. As the side-effects of the GSs are well known, it would be useful to know in advance how the patients will respond to this type of treatment. For this reason we measured the *in vitro* steroid sensitivity of 29 SS patients using inhibition of antibody dependent cellular cytotoxicity (ADCC) test by methylprednisolone compared to that of 28 controls. SS patients proved to be significantly less sensitive to GSs than controls (inhibition of ADCC reaction: 42.4 vs 53.1%; $p < 0.01$). This was especially true in SS patients with anti-SSA and/or SSB autoantibody positivity and with HLA-DR2 and/or -DR3 alleles. Comparing the results of the *in vitro* GS sensitivity and the clinical effectiveness of the previously applied corticosteroid therapy it seems that steroid inhibition of ADCC reaction has a predictive value in determination of *in vivo* sensitivity to GSs. However, in patients with decreased in vitro GS sensitivity a more expressed *in vivo* steroid sensitivity cannot be excluded.

Keywords: HLA class II alleles, ADCC, Sjögren's syndrome

Sjögren's syndrome (SS) is one of the systemic inflammatory autoimmune diseases characterized by lymphocytic infiltration of exocrine glands, first of all the salivary and lacrimal ones. This results in xerostomia and xerophthalmia, which are the obligatory symptoms of the disease. The sicca symptoms can be present alone (primary

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SS), but can join other well-defined autoimmune connective tissue diseases (e.g. Rheumatoid arthritis, Systemic lupus erythematosus, etc.). This latter form is called secondary SS. In primary SS, beside the local symptoms, systemic manifestations (articular, lung, vascular, etc.) can also occur.

In SS patients, focal lymphoid infiltrates (predominantly CD4+ T-cells) are present in the lacrimal and salivary glands. Another important feature is the expression of HLA-DR antigens on the glandular epithelial cells [1]. Clinically, primary SS is characterized by hyperactivity of B cells which is manifested in polyclonal hypergammaglobulinaemia, and overproduction of different autoantibodies such as rheumatoid factor, anti-SSA/Ro and anti-SSB/La antibodies. The latter two antibodies are the most characteristic of the disease, and these are directed against small ribonucleoprotein antigens. SS is a multifactorial disease, and in its pathogenesis not only the immunological changes, but also genetic predisposition and environmental factors, such as viruses, are involved [2, 3].

Despite progress in understanding the pathogenesis of SS, at present there is no therapeutical modality that is effective towards the overall disease. For the treatment of dry mouth and eyes, the exocrine substitution and drugs, which can increase the lacrimal and salivary output, are the most important means of alleviating the sicca symptoms [4]. In cases with severe, sometimes life-threatening internal (kidney, lung, vascular, etc.) manifestations, GSs are generally used [5]. Taking into account the well-known short and long-term side-effects of steroid therapy, it would be essential to know in advance, whether a patient will or will not respond to GSs. For this reason we measured antibody-dependent cellular cytotoxicity (ADCC) reaction and *in vitro* methylprednisolone inhibition of this reaction in patients with SS by methods described by Petri et al. [6, 7]. We wanted to know whether these *in vitro* examinations may help to determine the *in vivo* steroid sensitivity of SS patients if GS therapy is required. Another question was if HLA class II genotype, autoantibody positivity and clinical manifestations had any influence on GS sensitivity.

Patients and methods

ADCC activity and *in vitro* methylprednisolone inhibition of the reaction were determined in 29 patients with primary Sjögren's syndrome and in 28 age and sex-matched healthy blood donors as controls. All SS patients met the European Diagnostic Criteria (1993) [8].

Of the 29 patients all but one were female. The mean age of patients was 55 years (range: 32–74 years) and the mean duration of the symptoms was 10.3 years (range: 1–20 years).

In the *ADCC reaction* fresh, human "0" Rh (D) positive red blood cells were used as target cells. Human anti-D serum was adsorbed onto the cells [9] labelled with $^{51}Cr/Na_2\ ^{51}CrO_4$; 7–8 Gbq/mg Cr, Amersham. The effector lymphocytes were isolated on Ficoll Uromiro gradient after treatment of the whole blood with colloidal iron powder (GAF, USA). The effector/target cell ratio was adjusted to 10:1. Methylprednisolone was added to the culture medium for a final concentration of 10 μg/ml. The spontaneous activity was given by the count rates for cultures without anti-D antibody. The total activity was calculated as the radioactivity of labelled red blood cells lysed in distilled water. The cells were incubated at 37 °C in a 5% CO_2 thermostat for 18 hours. The cytotoxicity and steroid inhibition were calculated by the next formulas:

$$\text{cytotoxicity \%} = \frac{\text{test supernatant cpm} - \text{spontaneous cpm}}{\text{incorporated total activity cpm}} \times 100$$

$$\text{steroid inhibition (\%)} = \frac{\text{ADCC with steroid (\%)}}{\text{ADCC without steroid (\%)}} \times 100$$

Grade of sensitivity to GSs was given as a percentage of the inhibition of the ADCC reaction due to the steroid. Steroid sensitivity was defined when inhibition of basic ADCC was >30% [6, 7].

Examination of HLA status and autoantibody profile – statistical analysis

HLA II class antigenes, DRB1, DQA1 and DQB1 alleles were investigated in 28 of the 29 SS patients. Genomic DNA was extracted by a standard phenol-chloroform-proteinase-K method [10]. Polymerase chain reaction (PCR) was carried out: 1. for DRB1 using Amplicor TM PCR Diagnostics KIT (Roche Diagnostics Systems), 2. for DQA1 using Ota's PCR-RFLP method [11], 3. for DQB1 using Mercier's PCR-RFLP method [12]. After polyacrylamide gel (12%) electrophoresis the alleles were determined by comparison of estimated fragment size with the predicted size.

Anti-SSA and anti-SSB autoantibody positivity was determined by enzyme-linked immunosorbent assay (Epignost, Leonding/Linz, Austria).

Statistical analysis was made by Dunnett-2-sided *t*-test, and Spearman's correlation coefficients were calculated as well.

Table I

Main clinical manifestations and laboratory variables in patients with primary Sjögren's syndrome (n=29)

Manifestations and laboratory changes	Occurrence in frequency (%)
Parotid enlargement 10/29	(35%)
Articular 29/29	(100%)
Renal 4/29	(14%)
Vascular Raynaud 13/29	(45%)
Purpura 5/29	(17%)
Vasculitis 2/29	(7%)
Upper airway 27/29	(93%)
Lower airway 10/29	(35%)
Hepatomegaly 23/29	(79%)
Splenomegaly 7/29	(24%)
Lymphadenopathy 12/29	(41%)
Anaemia 14/29	(48%)
Leukopenia 15/29	(52%)
Hypergammaglobulinemia 15/29	(52%)
Antibody positivity	
Antinuclear 20/29	(69%)
IgM Rheumatoid factor 23/29	(79%)
Anti-SSA 19/29	(66%)
Anti-SSB 12/29	(41%)
Both Anti-SSA+SSB positive 11/29	(38%)
Both anti-SSA+SSB negative 9/29	(32%)

Results

The clinical manifestations and laboratory changes are summarized in Table I. Articular involvement (arthritis and arthralgia) occurred in all patients, followed by involvement of the upper airways (93%), anaemia and/or leukopenia (66%), Raynaud's phenomenon (45%), parotid enlargement (35%), lower airway disease (35%), purpura (17%), kidney involvement (14%). Examining the autoantibody profile of the patients, IgM rheumatoid factor positivity occurred in 79%, whereas ANA-positivity and anti-SSA and/or anti-SSB was detected in 69% of the patients.

Analyzing the results of ADCC reaction, there was a tendency for elevated ADCC reaction in SS patients (48.3 ± 15.7%), but the difference did not reach the level of significance comparing to that of controls (41.4 ± 14.1%).

In contrast, the *in vitro* GS inhibition of the ADCC reaction was significantly lower ($p < 0.01$) in SS patients (42.4 ± 15.8%) than that in controls (53.1 ± 13.1%). The range of steroid inhibition varied between 39.9% and 66.2% in the healthy blood donors' group and between 0 and 74% in SS patients. Twenty-three of 29 patients (79.3%) proved to be methylprednisolone sensitive, whereas 6 patients (20.7%) showed a decreased sensitivity to GSs. This rate of sensitivity did not differ from that of the controls. We analyzed the results of *in vitro* methylprednisolone inhibition of ADCC in SS patients on the basis of antibody profile and HLA status. The results are shown in Table II and III. The sensitivity to GSs decreased significantly in patients with anti-SSA and/or anti-SSB antibody positivity as compared to the controls. The difference proved also to be significant between the results of anti-SSB negative SS patients and controls. However, there were not any significant differences when the results of SS patients were compared with or without antibody positivity (Table II).

Table II

Steroid inhibition of ADCC reaction in primary Sjögren's syndrome (SS) patients (n=29) with or without anti-SSA and/or SSB antibody positivity comparing to controls (n=28)

	Degree of inhibition (%)	
Controls:	53.1±13.1	
All SS patients	42.4±15.8*	p<0.01
SS patients with		
anti-SSA+ (n=19)	39.6±16.4*	p<0.005
anti-SSA– (n=10)	47.7±16.0	NS
anti-SSB+ (n=12)	40.1±16.6*	p<0.05
anti-SSB– (n=17)	43.9±15.5*	p<0.05
anti-SSA and SSB+ (n=11)	39.6±17.3*	p<0.02
anti-SSA and SSB– (n=9)	47.9±14.7	NS

+: patients with antibody positivity
–: patients with antibody negativity
*: statistically significant differences as compared to controls
NS: not significant

Table III

Steroid inhibition of ADCC reaction in Sjögren's syndrome (SS) patients (n=28•) with or without HLA DR2 and/or DR3 positivity as comparing to controls (n=28)

	Degree of inhibition (%)	
Controls:	53.1±13.16	
All SS patients	42.4±15.8*	
SS patients with HLA phenotype of		
DR2 positivity (n=15)	37.9±20.8*	p<0.005
DR2 negativity (n=13)	45.8±10.6	NS
DR3 positivity (n=16)	40.3±15.6*	p<0.005
DR3 negativity (n=12)	44.7±17.0	NS
DR2 and DR3 positivity (n=6)	36.6±15.4**	p<0.005
DR2 and DR3 negativity (n=5)	52.6±16.2	NS

**: significant difference as compared to controls and to DR2 and DR3 negative patients
•: HLA phenotyping was carried out in 28 of the 29 SS patients
*: significant difference as compared to controls
NS: not significant

In HLA-DR2 and/or -DR3 positive patients the GS sensitivity was significantly lower than in controls. Though the steroid sensitivity was lower in SS patients with DR2 or DR3 positivity than in DR2 or DR3 negative patients, the differences were not statistically significant. In contrast, in cases with DR2/3 heterozygosity the difference proved to be statistically significant not only in comparison to controls, but also to SS patients carrying neither DR2 nor DR3.

Moreover we also evaluated the influence of combinations of both HLA haplotype (DR2, DR3), anti-SSA and SSB antibody positivity on the values of *in vitro* GS sensitivity (Table IV). We observed a tendency that SS patients with at least one of these alleles plus one of these autoantibodies exhibited lower sensitivity to methylprednisolone than SS patients who possessed none or only one of the above-mentioned immunological and genetic markers. However, the difference proved to be significant only between DR2 plus anti-SSA positive and DR2 plus anti-SSA negative subgroups ($p < 0.05$).

Eight of 29 SS patients needed GS therapy because of systemic manifestations during the course of the disease. Six of the 8 steroid treated patients proved to be GS sensitive according to the *in vitro* examinations. This was in concordance with the clinical improvement after the administration of GSs. In contrast, 2 of the 8 steroid

treated patients showed a decreased methylprednisolone sensitivity on the basis of the ADCC inhibition. When they had to be treated with high dose of GS due to severe liver and lung involvements, they responded, however, well to this therapy.

Table IV

Steroid inhibition of ADCC reaction in Sjögren's syndrome (SS) patients (n=28) concerning both autoantibody and HLA status and in controls (n=28)*

	Degree of inhibition (%)			
SS patients	DR2+	DR2–	DR3+	DR3–
Anti-SSA+	n=7	n=11	n=12	n=6
	32.1±22.2%*	43.5±10.7%	37.6±16.8%	42.2±17.6%
Anti-SSA–	n=6	n=4	n=4	n=6
	44.7±16.6%	52.3±8.4%*	48.5±7.7%	47.2±17.6%
Anti-SSB+	n=3	n=8	n=9	n=2
	34.7±31.6%	38.7±10.7%	36.9±18.0%	51.0±7.7%
Anti-SSB–	n=10	n=7	n=7	n=10
	38.9±17.6%	51.1±8.5%	44.7±11.7%	43.4±18.3%
Anti-SSA+,SSB+	n=4	n=7	n=8	n=2
	34.7±31.6%	40.6±11.3%	35.7±18.9%	51.0±7.1%
Anti-SSA–,SSB–	n=6	n=3	n=3	n=6
	44.7±16.6%	54.3±9.0%	49.3±9.2%	47.2±17.6%
Controls (n=28)	53.1±13.2			
All SS patients (n=28)	42.4±15.8			

+: positivity
–: negativity
*: HLA phenotyping was carried out in 28 of the 29 SS patients
*: significant difference between the two subgroups

Discussion

As the ethiology of SS is unknown, a causal treatment is not available at present. Since there are considerable evidences for its immunological background, therefore immune-regulating drugs may be of therapeutic value. In spite of this fact, there is no evidence-based algorithm for the treatment. While the severe extraglandular manifestations (e.g. lung, kidney, etc.) can be decreased by administration of

immunosuppressive treatment, local symptoms may fundamentally not be altered by this type of therapy [13].

Among the immunoregulatory modalities the GSs are used most frequently. For this reason it would be desirable to know in advance the therapeutic response to this type of drugs in patients with SS. This fact made us to investigate the ADCC reaction and *in vitro* methylprednisolone inhibition of this reaction in our SS patients.

The role of RNP, Sm and SSA-specific antisera from patients with lupus erythematosus in inducing ADCC of target cells coated with nonhistone nuclear antigens was investigated by Norris et al. [14]. In SLE patients with high titer anti-RNP sera, a significantly increased ADCC was seen using monocytes, T-lymphocytes, and low-density lymphocyte effector. Using monocyte effectors, a significantly increased ADCC reaction was detected in patients with anti-RNP, anti-Sm, and anti-SSA autoantibodies. In contrast, neutrophils as effectors were ineffective in any tested nuclear antigen-antibody system.

Petri et al. [7] published a significant correlation between the clinical effect of prednisolone treatment, i.e. the prednisolone sensitivity, and the degree of GS sensitivity based on the percentage of ADCC inhibition in children with nephrotic syndrome.

Since mainly the IgG Fc-receptor bearing lymphocytes take part in the ADCC reaction, it is conceivable that the differences in GS sensitivity may be correlated with a shift in the proportion of different T-lymphocyte subpopulations. Crabtree et al. [15] presented considerable evidence that GSs inhibit T-cell proliferation by blocking of production of T-cell Growth Factor. Parillo and Fauci [16] found that GSs suppressed NK-cell activity in humans.

Anti-SSA autoantibodies have been shown to increase ADCC *in vitro* [14]. As a decreased GS sensitivity was observed in patients with anti-SSA and/or anti-SSB antibody positivity, it is conceivable that at certain genetic background these autoantibodies can influence the GS sensitivity, however, the exact mechanism *in vivo* is unknown. In the literature, only the beneficial effect of corticosteroids on circulating anti-ds-DNA titre has been proven [17].

Methylprednisolone inhibition of the ADCC reaction was significantly decreased in pediatric autoimmune patients suffering from juvenile chronic arthritis or idiopathic thrombocytopenia [18].

Our working group [19] used the test of ADCC to investigate the *in vitro* GS sensitivity in patients with chronic uremia and in patients who underwent renal transplantation. While a positive correlation was detected between the HLA-DR6 positivity and steroid sensitivity, a negative association was found in HLA-B8 carriers with the steroid sensitivity.

In our SS patients, the effector peripheral lymphocytes demonstrated an increased ADCC reaction on the target red blood cells compared to those of controls, however, this was only a tendency. In SS patients and controls the rate of *in vitro* steroid sensitivity/resistance did not differ significantly, using 30% inhibition of ADCC reaction as a borderline. In contrast, the average GS inhibition showed a significant decrease in SS patients as compared to controls. This difference was more expressed in SS patients with HLA-DR2 or -DR3 haplotype than in patients not having these haplotypes. When these two alleles occurred together – in cases of DR2/DR3 heterozygotes – the decrease of GS sensitivity was significant comparing not only to controls, but to SS patients without these two alleles.

We observed a similar correlation between the grade of sensitivity to GSs and anti-SSA and SSB autoantibody positivity. Our results suggest that anti-SSA antibody positivity has a greater impact on GS sensitivity.

Furthermore the tendency of being less sensitive to GSs was more expressed in any cases when both type of predisposing factors (genetic and immunological) occurred together. This is especially true for joint occurrence of HLA-DR2 and anti-SSA positivity. Comparing the results of the *in vitro* steroid sensitivity and the clinical effectiveness of the previously applied GS therapy, it seems that *in vitro* methylprednisolone inhibition of ADCC reaction has a predictive value in determination of *in vivo* steroid sensitivity. However, in patients who exhibited a greatly decreased *in vitro* GS sensitivity, the *in vivo* steroid sensitivity cannot be excluded. For this reason, in severe cases with life-threatening manifestations GSs remain the main factors of therapy [20].

We conclude that sensitivity to GSs in patients with SS is influenced by both genetic haplotype and anti-SSA/anti-SSB seropositivity. Co-existence of these two factors may cause a decreased sensitivity to GSs. *In vitro* ADCC steroid inhibition test of the peripheral lymphocytes, coupled with the analysis of HLA class II allele polymorphism and autoantibody profile, was found to be a useful method for the detection of the grade of GS sensitivity in SS. This method may have a predictive value for making a decision of introducing of chronic GS therapy in other systemic autoimmune diseases, as well.

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