

Impaired catecholaminergic signalling of B lymphocytes in patients with chronic rheumatic diseases

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Abstract

Objective—To investigate further the influence of the autonomic nervous system on chronic rheumatic diseases.

Methods—The density and affinity of β_2 adrenergic receptors (β_2R) on CD19+ lymphocytes in patients with rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc), as well as intracellular cAMP levels in patients with RA and SLE, were determined. Human peripheral blood mononuclear cells were separated from venous blood of patients and healthy controls by Ficoll-Hypaque density centrifugation. CD19+ lymphocytes were purified by magnetic cell sorting, and β_2R were determined by a radioligand binding assay with [¹²⁵I]iodocyanopindolol. Intracellular cAMP levels and β_2R agonist induced cell death were measured by a radioimmunoassay and flow cytometry using annexin-V binding, respectively. Systemic disease activity of the patients was evaluated using multifactorial scoring systems.

Results—The density of β_2R on peripheral CD19+ lymphocytes was significantly decreased in patients with RA, SLE, and SSc compared with healthy controls. In patients with RA and SSc β_2R density was negatively correlated with systemic disease activity. Furthermore, although basal intracellular cAMP levels were raised in patients with RA and SLE, the increase of cAMP upon stimulation of β_2R was significantly reduced in these patients compared with control subjects. Preliminary data suggest that β_2R agonist induced cell death is diminished in patients with RA exhibiting decreased β_2R densities.

Conclusions—The results of this study show a reduction of β_2R densities on B lymphocytes mirrored by an impaired intracellular cAMP generation in patients with chronic rheumatic diseases, indicating a decreased influence of the autonomic nervous system on B cells in these conditions.

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Although the cause of inflammatory autoimmune diseases like rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), and systemic sclerosis (SSc) is unknown, it is well accepted that cellular and humoral immune responses underlie the distinct pathology of these diseases. A hallmark of chronic rheumatic diseases is the formation of autoantibodies—

for example, rheumatoid factor in RA, antibodies against double stranded DNA (dsDNA) and Smith antigen (Sm) in SLE, and against topoisomerase-II (Scl-70) in SSc. Additionally, immune complexes and hypergammaglobulinaemia are common features, indicating a B cell hyperactivity.¹

A growing body of evidence points towards a modulation of immune responses in vitro as well as in vivo and the inflammatory activity of autoimmune diseases by the autonomous nervous system. It has been shown that lymphocytes and noradrenergic varicosities form synapse-like junctions in lymphoid organs^{2,3} and that β_2 adrenergic receptors (β_2R) are expressed on various lymphocyte subpopulations.^{4,5}

Previous studies of our group showed that in patients with chronic rheumatic diseases the density of β_2R is decreased on peripheral blood mononuclear cells (PBMC). Further investigations showed that β_2R are modulated differentially on lymphocyte subsets because β_2R densities were reduced on CD8+ lymphocytes but not on CD4+ cells.^{6,7} However, little is known about β_2R on B lymphocytes in these disease entities.

Therefore we investigated the characteristics of β_2R on peripheral blood B lymphocytes (CD19+ mononuclear cells) in patients with RA, SLE, and SSc, together with the systemic disease activity, and determined the coupling of β_2R to the intracellular signal transduction cascade.

Methods

PATIENTS AND CONTROL SUBJECTS

Patients with RA (n=24), SLE (n=13), and SSc (n=6) according to classical diagnostic criteria^{8–10} and a group of healthy blood donors (n=16) were included in the study. We excluded patients in whom other factors were supposed to influence β_2R (that is, infectious and atopic diseases, hyperthyroidism/hypothyroidism, hypertension, treatment with sympathomimetic/sympatholytic agents, cancer).¹¹ Patients were examined by taking a history, physical examination, and laboratory findings (erythrocyte sedimentation rate (ESR), C reactive protein (CRP), haemoglobin, packed cell volume, leucocytes, lymphocytes, platelets, autoantibodies, creatinine). Inflammatory disease activity in RA, SLE, and SSc was determined by different multifactorial scoring systems. For patients with RA we used a modified total disease activity score according to Farr *et al*, (TAD)¹² which includes morning stiffness, the Ritchie articular index,¹³ ESR,

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Table 1 Measures used to obtain the disease activity score of patients with systemic sclerosis, called the systemic sclerosis score (SSS). Each variable was graded from 0 to 3, points were then added to the total activity index with a maximum of 78 points

	1 = mild	2 = moderate	3 = severe
General progression	Limited		Diffuse
Weight loss	<10% Body weight		>10% Body weight
Fatigue	No limits on activity		Functional limitation
Sclerodactyly	Oedematous state	Sclerotic state	Atrophic state
Cutaneous expansion	Face or extremity	Face and extremity	Face, extremity, and trunk
Respiratory insufficiency	Partial		Global
Pneumonitis	Dry cough	Shortness of breath with exercise	Shortness of breath at rest
Dysphagia	Present		
Peptic oesophagitis	Gastro-oesophageal reflux		Complications
Raynaud's phenomenon	Present		
Telangiectasia	Present		
Capillary microscopy	Low pattern		Active pattern
Diastolic blood pressure (mm Hg)	90-105	105-115	>115
Heart failure	Compensated		Decompensated
Pericarditis	Signs in ECG	Retrosternal pain	With haemodynamic effect
Cardiomyopathy	Present		
Cardiac arrhythmias	Present		
Myalgia/myositis	Myalgia	Moderate functional impairment	Severe functional impairment
Arthralgia/arthritis	Arthralgia	Inflammation	Functional impairment
Haemoglobin (g/l)	W: 119-110 M: 129-120	W: 109-100 M: 119-110	W: <100 M: <110
Leucocytes (g/l)	3.5-2	1.9-1	<1
Lymphocytes (g/l)	1.5-1	0.9-0.5	<0.5
Thrombocytes (g/l)	150-100	99-50	<50
ESR (mm/1st h)	25-50	51-70	>70
Creatinine ($\mu\text{mol/l}$)	120-180	190-350	>350
Urine sediment	>5 Erythrocytes/leucocytes; <0.5 g/l/day protein	>10 Erythrocytes/leucocytes; 0.5-3.5 g/l/day protein	>25 Erythrocytes/leucocytes; >3.5 g/l/day protein

CRP, and haemoglobin. For patients with SLE the SLE activity measure (SLAM) was used.¹⁴ For the patients with SSc a new scoring system adapted from the SLAM was developed and called the SSc score (SSS, table 1). Table 2 summarises the clinical characteristics of patients and control subjects. Treatment with non-steroidal antirheumatic drugs or steroids up to 7.5 mg prednisolone equivalent a day were allowed in all patient groups (RA 15/24 patients, range 2-7.5 mg prednisolone equivalent a day, SLE 9/13, range 2.5-7.5 mg, SSc 5/6, range 2-7.5 mg). No patient received disease modifying antirheumatic drugs.

Cell death induced by isoprenaline was determined in four patients with RA and five healthy controls.

PURIFICATION OF CD19+ LYMPHOCYTES

PBMC were separated from heparinised venous blood by Ficoll-Hypaque (Seromed, Berlin, Germany) density gradient centrifugation.¹⁵ Cells were then washed three times in phosphate buffered saline supplemented with 0.5% bovine serum albumin (PBS/BSA) and adjusted to a cell concentration of $1 \times 10^8/\text{ml}$. CD19+ lymphocytes were purified by magnetic cell sorting.¹⁶ To separate CD19+ cells, PBMC of patients or control subjects were incubated with a monoclonal antihuman CD19 antibody coupled to supramagnetic

microparticles for 20 minutes in a refrigerator (Miltenyi Biotec, Bergisch-Gladbach, Germany). Cells were then washed in cold PBS/BSA and CD19+ cells were separated by filtration through a magnetised steel wool column (MACS cell separator, MACS column A2 and LS, Miltenyi Biotec). The efficacy of the magnetic separation was evaluated by flow cytometry using a FACScan (Becton Dickinson, Heidelberg, Germany). The purity of the isolated CD19+ lymphocytes averaged 95% in all experiments.

DETERMINATION OF $\beta 2R$

The number of $\beta 2R$ on purified CD19+ cells was determined as previously described.¹⁷ In brief, aliquots of 0.6×10^6 CD19+ cells were incubated for 60 minutes at 37°C with six different concentrations of [¹²⁵I]iodocyanopindolol (ICYP, DuPont, Boston, MA, USA), ranging from 12.5 to 200 pmol/l ICYP. Cells were then harvested immediately on Whatman GF/B filters (Whatman Inc, Clifton, NJ, USA). Filters were washed to remove unbound radioactivity, and radioactivity on wet filters was determined in a multicrystal gammacounter (LB 2103, Berthold, Wildbad, Germany). Unspecific binding of ICYP was measured in parallel by incubating the CD19+ cells in the presence of high concentrations of propranolol (1 $\mu\text{mol/l}$), a competitive antagonist of ICYP.

Table 2 Clinical characteristics of patients and control subjects studied

Group	n	Sex (M/F)	Age* (years)	Duration of disease* (years)	Autoantibody positive patients (n)	Disease activity index† (points)
Patients with RA	24	4/20	62 (32-76)	13.5 (1-35)	Rheumatoid factor 20	TAI 3-15
Patients with SLE	13	0/13	47 (23-69)	4 (1-15)	Anti-dsDNA (pos) 10	SLAM 3-11
Patients with SSc	6	1/5	53.5 (42-69)	6.5 (1-11)	Anti-Scl-70 (pos) 4	SSS 7-15
Control subjects	16	3/13	43 (27-56)			

*Mean (range).

†TAI = total disease activity score; SLAM = SLE activity measure; SSS = SSc score.

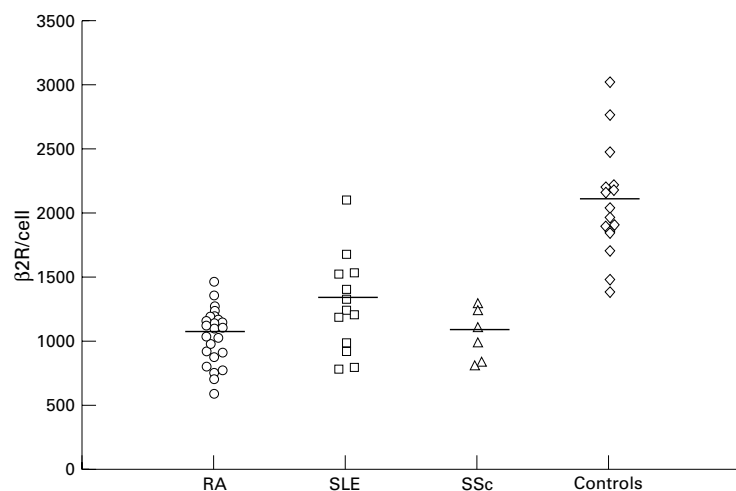


Figure 1 Density of β_2 adrenergic receptors (β_2R) on CD19+ lymphocytes. The density of β_2R on CD19+ cells is significantly reduced in patients with chronic rheumatic diseases (rheumatoid arthritis (RA, $n=24$), systemic lupus erythematosus (SLE, $n=13$), systemic sclerosis (SSc, $n=6$) compared with healthy control subjects (controls, $n=16$) ($p<0.001$; one way analysis of variance followed by Tukey's procedure; RA, SLE, or SSc *v* controls). No significant difference is seen between the three patient groups. Data are presented as plots of the receptor numbers of individual subjects, the bar representing the mean.

Specific binding of ICYP was determined by subtracting unspecific binding from the total binding capacity. The maximum number of ICYP binding sites, representing the number of β_2R , and dissociation constant (KD) for ICYP of β_2R were calculated according to the method of Scatchard.¹⁸

DETERMINATION OF BASAL AND STIMULATED INTRACELLULAR cAMP

The concentration of basal and stimulated levels of cAMP in CD19+ lymphocytes was determined in patients with RA ($n=5$), SLE ($n=4$), and controls ($n=8$) by a radioimmunoassay. Aliquots of 1×10^6 CD19+ cells in PBS/BSA containing 50 μM theophylline and 10 μM ascorbic acid were either incubated for 15 minutes at 37°C to determine basal cAMP levels or incubated with 10 μM (-)-isoprenaline for 15 minutes to determine stimulated cAMP levels.^{19,20} After boiling and centrifugation, cAMP in the supernatant was measured by a [¹²⁵I]cAMP radioimmunoassay (Dianova-Imunotech, Hamburg, Germany) according to the guidelines of the manufacturer. The increase of cAMP upon β_2R stimulation with isoprenaline was calculated by subtraction of basal from stimulated levels.

INDUCTION AND DETERMINATION OF CELL DEATH IN ACTIVATED CD19+ LYMPHOCYTES

Cell death induced by stimulation of β_2R in activated CD19+ lymphocytes was determined in patients with RA ($n=4$) and control subjects ($n=5$). Aliquots of 2×10^5 CD19+ cells in serum free RPMI medium supplemented with penicillin/streptomycin, L-glutamine, and 2% TCH defined serum supplement (ICN, Eschwege, Germany) were activated with 1 $\mu g/ml$ plate bound anti-IgM polyclonal antibody (BioSource, Camarillo, CA, USA) for 16 hours in the presence or absence of 10 μM isoprenaline. After washing in binding buffer (150 mM NaCl, 2 mM CaCl₂, 10 mM HEPES) the number of apoptotic and necrotic cells was

evaluated using annexin-V-FITC (Bender Med Systems, Vienna, Austria) and propidium iodide staining. At least 5000 events were counted on a FACScan (Becton Dickinson) using the Cell Quest software (Becton Dickinson). Spontaneous and β_2R agonist induced cell death (apoptotic and necrotic cells) was determined with a histogram and two dimensional dot plots.

STATISTICAL ANALYSIS

Values in tables and figures are given as means and standard errors of the mean (SEM) if not otherwise indicated. Correlations between β_2R characteristics and disease activity scores were calculated by the Pearson product moment correlation. A comparison of independent single variables between the groups was calculated by one way analysis of variance (ANOVA) followed by Tukey's procedure. When normality test failed, Kruskal-Wallis one way ANOVA on ranks was used. A comparison of isoprenaline induced cell death in patients with RA and healthy controls was calculated by a paired *t* test. *p* Values less than 0.05 were considered to be significant.

Results

β_2R DENSITY AND DISSOCIATION CONSTANTS

The number of β_2R on CD19+ cells was significantly decreased in patients with chronic rheumatic diseases compared with control subjects ($p<0.001$). The mean (SEM) number of β_2R was 1041 (44) binding sites (bs)/cell in patients with RA, 1278 (103) bs/cell in patients with SLE, and 1035 (83) bs/cell in patients with SSc, respectively. On CD19+ cells derived from healthy donors 2060 (106) bs/cell were detected (fig 1). No significant difference was found between the β_2R density in the various patient groups.

The KD values of β_2R for ICYP were reduced in the patient groups (RA 5.89 (0.53) pmol/l; SLE 6.36 (0.92) pmol/l; SSc 5.9 (0.76) pmol/l) compared with the control group (9.86 (1.36) pmol/l). However, when the Kruskal-Wallis test was used the difference between patient groups and control subjects did not reach significance ($p=0.113$).

Stratifying patients with RA and SLE according to the use of corticosteroid drugs did not disclose a difference in β_2R characteristics between the respective groups: patients with RA receiving corticosteroid treatment 1036 (62.6) bs/cell (KD 5.9 (0.7) pmol/l; $n=15$) *v* patients with RA without corticosteroid drugs 1048 (56.2) bs/cell (KD 5.8 (0.8) pmol/l; $n=9$); and patients with SLE receiving corticosteroid drugs 1287 (140) bs/cell (KD 6.4 (1.2) pmol/l; $n=9$) *v* patients with SLE without corticosteroids 1273 (144) bs/cell (KD 6.3 (1.5) pmol/l; $n=4$).

CORRELATION OF β_2R STATUS WITH DISEASE

ACTIVITY IN RA, SLE, AND SSC

In patients with RA and SSc, β_2R density on CD19+ lymphocytes showed a significant negative correlation with disease activity. In patients with RA a negative correlation between the β_2R density and the TAI ($r=-0.76$,

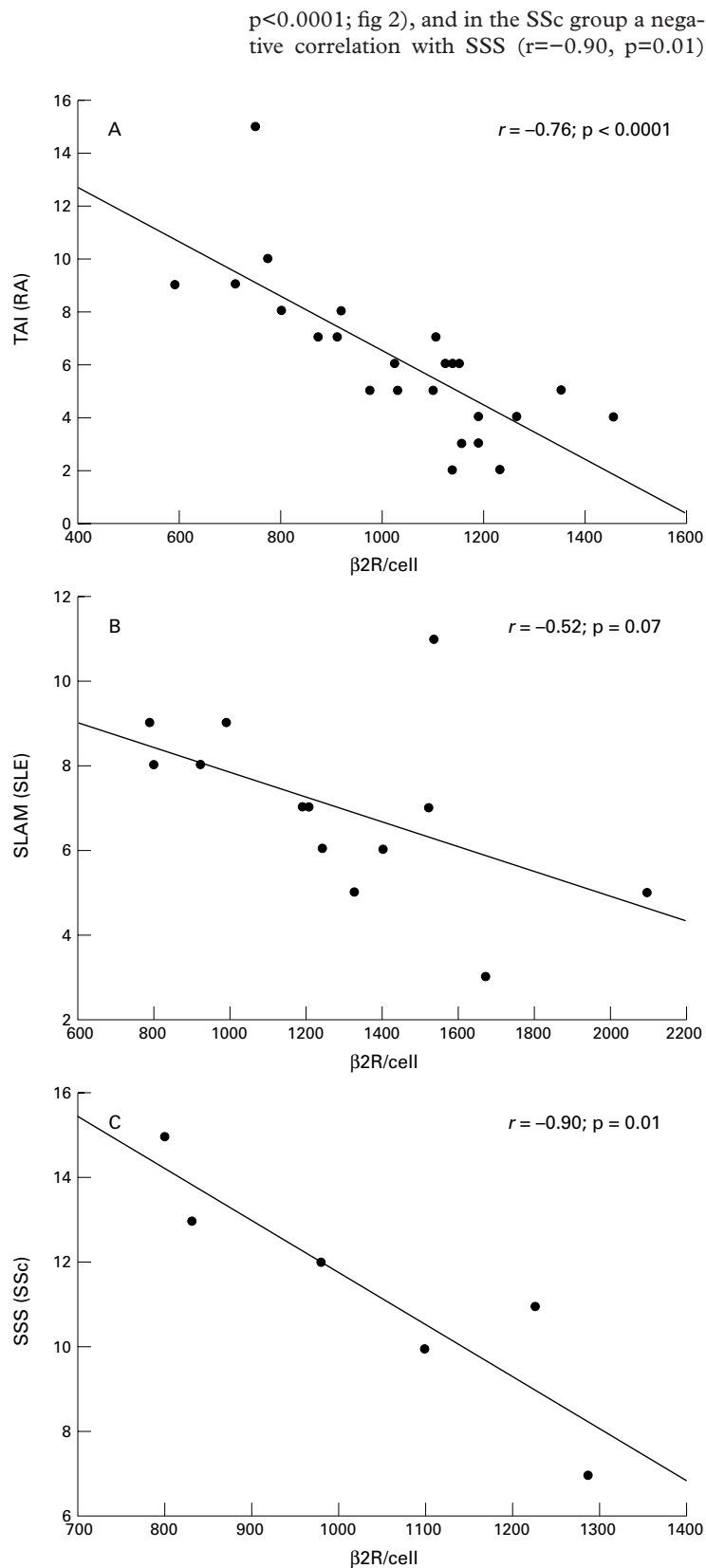


Figure 2 Correlation of the density of β_2 adrenergic receptors (β_2R) with disease activity. In patients with rheumatoid arthritis (RA) and systemic sclerosis (SSc) a significant negative correlation could be seen between systemic activity scores and the density of β_2R on CD19+ lymphocytes. Correlation between β_2R and disease activity just missed the significance level in the group with SLE: (A) β_2R v the total disease activity score (TAI) in patients with RA; (B) β_2R v the SLE activity measure (SLAM) in patients with SLE; (C) β_2R v the SSc score (SSS) in patients with SSc. Correlation between β_2R and the values obtained by the different scoring systems used in this study (TAI, SLAM, SSS) was calculated by the Pearson product moment correlation test.

was found. In the SLE group β_2R density on CD19+ lymphocytes was negatively correlated with SLAM, but the correlation was not significant ($r=-0.52$, $p=0.07$). This was because one patient had high disease activity as well as high β_2R density (fig 2). When this patient was omitted a significant negative correlation between β_2R density and disease activity ($r=-0.79$, $p=0.002$) was found. No significant correlation was found between the KD values of the various patient groups and the disease activity ($p>0.05$).

BASAL AND STIMULATED INTRACELLULAR cAMP LEVELS IN RA, SLE, AND CONTROL SUBJECTS

Intracellular cAMP levels were investigated in patients with RA ($n=5$) and SLE ($n=4$) in comparison with healthy controls ($n=8$). Basal cAMP levels were significantly raised in patients with SLE (3.88 (0.54) pmol/ 10^6 cells) compared with control subjects (1.77 (0.17) pmol/ 10^6 cells, $p<0.001$). In patients with RA basal cAMP levels were slightly raised (2.17 (0.19) pmol/ 10^6 cells), with no significant difference from healthy controls.

The relative increase of cAMP above basal levels after isoprenaline stimulation was markedly decreased in RA (3.51 (1.86)%) and SLE (1.69 (0.51)%) in comparison with the control group (9.43 (1.30)%, $p<0.05$, fig 3). No significant correlation was found between cAMP levels and β_2R characteristics or disease activity scores, respectively.

β_2R AGONIST INDUCED CELL DEATH

Cell death induced by the incubation of activated CD19+ lymphocytes with 10 μM isoprenaline was investigated in patients with RA ($n=4$) in comparison with healthy controls ($n=5$). The proportion of dead cells (defined as apoptotic and necrotic cells based upon staining with annexin-V and propidium iodide, respectively) among the CD19+ cells activated with plate bound polyclonal anti-IgM antibody for 16 hours was raised in patients with RA (30.0 (3.6)%, table 3) compared with control subjects (18.9 (3.7)%). Stimulation of β_2R with 10 μM isoprenaline slightly increased the number of dead cells in patients with RA (36.1 (6.7)%, relative change 18.1 (9.7)%, NS). In contrast, the number of dead cells significantly increased upon activation of β_2R with isoprenaline in the control subjects (32.9 (7.5)%; $p<0.05$, relative change 79.7 (20.5)%; $p<0.05$, table 3).

Discussion

The results of our study demonstrate a profound modulation of β_2R on B lymphocytes (CD19+ lymphocytes) in chronic rheumatic diseases. The density of β_2R on B lymphocytes was decreased in patients with RA, SLE, and SSc compared with healthy control subjects, and a negative correlation between the β_2R density on B cells and systemic disease activity scores was found in all patient groups studied except the SLE group. In addition, inducible intracellular cAMP levels in response to stimulation of β_2R were reduced in

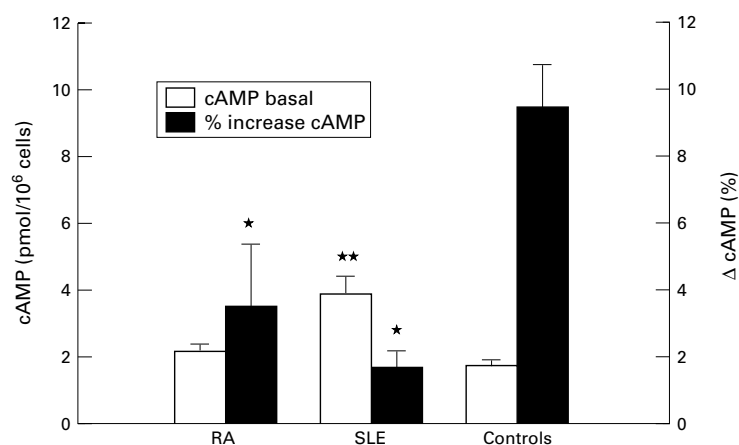


Figure 3 Intracellular cAMP levels. Basal cAMP levels (cAMP basal) were significantly raised in patients with systemic lupus erythematosus (SLE) compared with control subjects. The relative increase of cAMP (% increase cAMP) above basal levels after isoprenaline stimulation was markedly decreased in both patient groups compared with the control group (* $p < 0.05$, ** $p < 0.001$; one way analysis of variance followed by Tukey's procedure; RA, SLE v control).

Table 3 Isoprenaline induced cell death in CD19+ lymphocytes of patients with rheumatoid arthritis (RA) compared with controls

	% Dead cells medium	% Dead cells 10 μ M isoprenaline	% Increase
RA (n=4)	30.0 (3.6)	36.1 (6.7)	18.1 (9.7)
Controls (n=5)	18.9 (3.7)	32.9 (7.5)*	79.7 (20.5)**

* $p < 0.05$ compared with medium.

** $p < 0.05$ compared with patients with RA.

patients with chronic rheumatic diseases, thus demonstrating the functional significance of β 2R modulation.

Previous studies showed that β 2R density was decreased on PBMC in patients with chronic rheumatic diseases.^{7, 17, 21} Recently, it was shown that in patients with RA, β 2R were differentially regulated in lymphocyte subpopulations because β 2R on CD8+ lymphocytes were down regulated in contrast with unchanged β 2R numbers on CD4+ lymphocytes.⁷ When the results of these studies are taken together the picture emerges that in chronic rheumatic diseases the expression of β 2R on T and B lymphocytes is a complex modulated process rather than simply down regulation.

Various mechanisms contributing to the observed differential modulation of β 2R expression are possible. A long term increase of catecholamines decreases β 2R density through the stimulation of the β adrenoceptor kinase.^{22, 23} However, the differential regulation of β 2R on CD4+ and CD8+ lymphocyte subsets in patients with RA argues against a simple down regulation of β 2R due to an increase of systemic catecholamine concentrations,⁷ as can be seen in patients with pheochromocytoma.²⁴ Moreover, a correlation with plasma catecholamine levels could not be shown either for systemic disease activity or for β 2R density on PBMC in patients with RA.⁶

The affinity of β 2R in human neutrophils and the transcription of β 2R mRNA in human lung cells have been shown to be increased by corticosteroids.²⁵ In RA and SLE a decreased cortisol plasma concentration due to alterations in cortisol metabolism in SLE^{26, 27} and a

disturbed adrenal secretion of cortisol in RA²⁸ have been proposed. Therefore, decreased cortisol concentrations are a possible mechanism for the down regulation of β 2R in rheumatic diseases. However, low dose corticosteroids did not influence β 2R characteristics in previous investigations, nor in the study presented here. Thus, at least in patients with chronic rheumatic diseases, low dose corticosteroids do not influence β 2R characteristics of immune cells.^{17, 29}

Other factors, such as a differential regulation of high and low affinity β 2R and a defect in intrinsic β 2R regulation, may also contribute to the disturbed β 2R expression.^{30, 31} Because it has been shown in this study and in previous studies that there was a striking negative correlation between β 2R density and the disease activity,^{6, 7} it is tempting to speculate that the density of β 2R on PBMC is influenced by the inflammatory process. In particular, cytokines and eicosanoids like prostaglandin E₂ are known to modulate β 2R density in vitro.^{32, 33} Furthermore, investigations of our group showed that interleukin 2 may be a key player in modulating β 2R expression differentially in CD4+ and CD8+ cells.³⁴

The generation of cAMP after stimulation of β 2R with isoprenaline was impaired in B lymphocytes of patients with RA and SLE. As has been shown previously, the increase of stimulated cAMP rather than basal levels is crucial for the intracellular signal transduction.³⁵ It seems unlikely that changes in intracellular cAMP levels were induced by preparation of CD19 positive cells, as engagement of CD19 does not interfere with cAMP production in B cells.³⁶

As a functional consequence of β 2R stimulation, cell death induced by isoprenaline was determined in CD19+ cells activated by B cell receptors. An increase of intracellular cAMP influences B cell function at different stages and has been shown to induce apoptotic cell death in resting B cells as well as in B cells activated by the B cell receptor.³⁷⁻⁴¹ As our preliminary data show, the isoprenaline induced cell death of B cells is diminished in patients with RA, underlining the functional consequence of decreased β 2R densities on immune cells of patients with RA.

Hence, our results indicate that the influence of the sympathetic nervous system on B lymphocytes is impaired in chronic rheumatic diseases. Together with interleukin 1 the increase of intracellular cAMP is an essential signal for the primary immune response and the differentiation of B cell precursors into IgM secreting cells through inactivation of protein kinase C.⁴²⁻⁴⁴ In contrast, T helper cell function and the secondary immune response are inhibited by activation of β 2R and an increase of intracellular cAMP.⁴²⁻⁴⁶ An inverse effect on T cell function could be seen after propranolol treatment and sympathectomy.^{47, 48} Therefore, an impaired cAMP response may have a role in the B cell dysregulation in chronic rheumatic diseases.

In conclusion, our results show that the density of β 2R and the agonist induced cAMP

production are impaired in B lymphocytes of patients with chronic rheumatic diseases. However, it is not clear whether this phenomenon occurs in response to the inflammatory process or precedes exacerbations of chronic rheumatic diseases. In every case, changes in the regulation of β 2R density and coupling to the intracellular signalling machine may be an accelerating factor for B cell dysregulation and predispose to the development of rheumatic diseases.

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