Glucocorticosteroid dependent decrease in the activity of calcineurin in the peripheral blood mononuclear cells of patients with systemic lupus erythematosus

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Abstract

Objective—To compare the activity of calcineurin in the peripheral blood mononuclear cells (PBMC) of 32 patients with systemic lupus erythematosus (SLE) and 35 healthy controls.

Methods—The activity of calcineurin was assayed in the supernatants of sonicated mononuclear cells.

Results—There was no significant difference in the calcineurin activity of patients with SLE not taking glucocorticosteroids (GCS) compared with the healthy controls. On the other hand, the activity of calcineurin was reduced in patients with SLE taking GCS, correlating negatively with the dose of GCS. The stimulation of PBMC by phorbol ester and calcium ionophore decreased the calcineurin activity both in patients with SLE and in healthy controls. GCS could also reduce calcineurin activity in the mononuclear cells of healthy subjects in vitro.

Conclusions—In patients with SLE the decrease in the calcineurin activity of PBMC depended on the dose of GCS used for treatment, and it was not a disease specific alteration. The higher the dose of GCS, the greater the inhibition of calcineurin activity. The reduction of calcineurin activity is a new element in the immunosuppressive effects of GCS during the treatment of patients with SLE.

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Large scale defects of the intracellular signal transduction processes were seen in the mononuclear cells of patients with systemic lupus erythematosus (SLE).¹ Various forms of T cell defects were found, including decreased protein kinase C activity,² diminished levels of protein kinase A RI α and RI β transcripts, and proteins leading to disturbance of cAMP metabolism,^{3–5} abnormal NF- κ B, and decreased p65-RelA protein expression.⁶ The abnormalities described in the monocytes can affect both the function^{7–9} and the release of mediators.^{10–12} Recently, oestrogen dependent, increased expression of calcineurin mRNA was found in the T cells of female patients with SLE.^{13 14}

Calcineurin is a Ca²⁺/calmodulin dependent protein phosphatase, decreasing the level of phosphorylation in the transcriptional factor

NF-AT (nuclear factor of activated T cells) required for the production of a series of cytokines (interleukin 2 (IL2), IL4, etc), cell surface molecules (CD40, ICAM, etc), and Fas ligand.^{15 16}

In this study we show that in patients with SLE not taking any glucocorticosteroids (GCS) the calcineurin activity of peripheral blood mononuclear cells (PBMC) does not differ from that of healthy controls. On the other hand, in patients with SLE taking various doses of GCS, decreased calcineurin activity can be measured. The higher the concentration of GCS, the greater the decrease in calcineurin activity. The in vitro stimulation of PBMC by phorbol ester and Ca²⁺ ionophore (A23187) results in a pronounced decrease in the calcineurin activity of cells derived from patients with SLE or from healthy controls. GCS can reduce the calcineurin activity also in the mononuclear cells of healthy subjects in vitro.

Patients and methods

PATIENTS, CLINICAL DATA, AND CONTROL GROUP
The SLE study group comprised 32 patients, 29 women and three men, with a median age of 36 years (range 18–68). The mean SLE disease activity index (SLEDAI) was 4.1 (range 2–20). The average dose of GCS (methylprednisolone or equivalent of prednisolone) taken by the patients was 9.8 mg/day (range 0–32). These patients were free from any cytostatic drugs. The average duration of the disease was 8.5 years (range 0.5–35). Three subgroups of these patients were studied: (a) seven patients (six women, one man) not taking any GCS, median age 41 years (range 28–59), mean SLEDAI 2.43; (b) six patients (five women, one man) taking 8 mg of GCS/day, median age 39 years (range 18–55), mean SLEDAI 4.4; (c) six patients (five women, one man) taking 32 mg of GCS/day, median age 43 years (range 19–52), mean SLEDAI 11.5. Thirty five healthy white subjects (32 women, three men), median age 36 years (range 20–52), served as controls. Approval was given through the institutional review board, and informed consent was obtained from all participants. All patients with SLE fulfilled the diagnostic criteria of the American College of Rheumatology with at least four of the revised criteria for the diagnosis of SLE.¹⁷ Patients with a SLEDAI score $\leq$ 3 were considered to have inactive disease.¹⁸

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Figure 1 Negative correlation between the basal activities of calcineurin in the peripheral blood mononuclear cells (PBMC) of patients with systemic lupus erythematosus (SLE) and the doses of glucocorticosteroids (GCS) used for treatment. The coefficient of correlation between the basal calcineurin activities in the PBMC and the daily doses of GCS (0–32 mg/day) taken by patients with SLE was calculated. The calcineurin activity of healthy controls were taken as 100%. The number of patients is given in parentheses.

PREPARATION OF HUMAN MONONUCLEAR CELLS AND CHARACTERISATION BY FLOW CYTOMETRY
Human PBMC with an average of 88–95% lymphocytes and 5–12% monocytes were prepared from heparinised blood of patients with SLE and controls according to Boyum's method.¹⁹ The averages of the various subsets were detected by flow cytometry: CD3+ 69.4%, CD19+ 10.5%, CD56+ 1.8%, and CD14+ 8.3%. The suspensions of mononuclear cells (10^6 cells per sample) were labelled by saturating concentrations of anti-CD3-FITC (T3, Coulter, Hialeah, USA), anti-CD19-RD1 (B4, Coulter), anti-CD56-PE (Leu-19, Becton-Dickinson, Mountain View, USA), and anti-CD14-RD1 (MY4, Coulter). After staining and fixing, the cells were analysed by a Coulter EPICS XL flow cytometer (Coulter, USA).

STIMULATION OF PBMC BY PHORBOL ESTER AND CALCIUM IONOPHORE
Phorbol ester (PMA, phorbol-12-myristate-13-acetate, Sigma, St Louis, USA) 50 ng/ml and 5 μ mol/l calcium ionophore (A23187, Sigma, USA) were added to the cells at 37°C for four hours.

MEASUREMENT OF CALCINEURIN ACTIVITY
Cell suspensions were washed with phosphate buffered saline and collected by centrifugation. Cells were sonicated for 2×30 seconds with a Branson sonifier in two volumes of homogenisation buffer containing 50 mM Tris-HCl (pH 7.0), 0.5 M dithiothreitol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml trypsin inhibitor, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM benzamidine, and 0.3% Triton X-100. The homogenate was centrifuged for 10 minutes at 10 000 g, and supernatants were collected and stored in aliquots at -70°C before the assays.

Calcineurin activity was measured by the release of $^{32}\text{P}_i$ from ^{32}P labelled protein phosphatase inhibitor 1²⁰ as described by Yang

*et al*²¹ with slight modifications. The assay mixture (30 μ l) containing 50 mM Tris-HCl (pH 7.0), 0.3 mM dithiothreitol, 0.2 mM CaCl_2 , 1 mM MnCl_2 , 0.04 mg/ml calmodulin or 2 mM EGTA, an appropriate amount of extract (0.5–1.5 mg/ml protein), and ^{32}P labelled protein phosphatase inhibitor 1 (12–25 000 cpm/reaction mixture) was incubated at 30°C for 10 minutes. All mixtures contained 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml trypsin inhibitor, 1 mM PMSF, 5 mM benzamidine as protease inhibitors, and 20 nM okadaic acid as a protein phosphatase 2A inhibitor. The activity of protein phosphatase 1 was not assayed under the conditions given above, because 200 nM of okadaic acid did not result in any inhibition. The reaction was terminated by the addition of 100 μ l of 10% trichloroacetic acid. $^{32}\text{P}_i$ of the supernatant fraction was determined after centrifugation by Cerenkov counting in a liquid scintillation counter. The activity of calcineurin was calculated as the difference between $^{32}\text{P}_i$ counts in the absence and presence of $\text{Mn}^{2+}/\text{Ca}^{2+}$ /calmodulin.

CULTURING OF PBMC IN THE PRESENCE OF PREDNISOLONE SODIUM SUCCINATE
PBMC of five healthy controls were cultured for 72 hours in the absence and presence of 10^{-4} M prednisolone sodium succinate (Diadreson-F-Aquosum, Organon, The Netherlands) in RPMI medium with fetal calf serum (10%).

STATISTICAL ANALYSIS
Statistical means and SD values were calculated to compare the activity of calcineurin in the patients with SLE and in the healthy controls. The statistical significance of the differences was evaluated by Student's unpaired *t* test. During measurements of the in vitro effects of GCS on calcineurin activity, each value was calculated as the mean of the data from five healthy control subjects. In these experiments the statistical significance was calculated by Student's paired *t* test. The correlation coefficient between the calcineurin activities and the doses of GCS was determined in the patients with SLE. In these studies the calcineurin activities of the individual patients were expressed as a percentage of the activities measured in the healthy controls, which were considered to be 100%.

Results

During the first phase of the experiment we compared the activity of calcineurin in the PBMC of patients with SLE and in healthy controls. As the calcineurin activities obtained from the patients with SLE were diverse, the daily doses of GCS taken by the subjects were considered when the data were analysed.

NEGATIVE CORRELATION BETWEEN THE BASAL ACTIVITIES OF CALCINEURIN IN PBMC OF PATIENTS WITH SLE AND THE DOSES OF GCS USED FOR THE TREATMENT

The correlation between the basal calcineurin activities in the PBMC and the daily doses of GCS (0–32 mg/day) taken by the 32 patients

Figure 2 Calcineurin activities in the peripheral blood mononuclear cells (PBMC) of patients with systemic lupus erythematosus (SLE) with or without glucocorticosteroid (GCS) treatment and in healthy controls. Calcineurin activities were assayed in the supernatants of non-stimulated and stimulated PBMC as described in "Patients and methods". The statistical significance of the differences was calculated by Student's unpaired t test. Asterisk denotes significant difference compared with the controls.

with SLE was calculated. It was found to be highly significant: $r_s = -0.52$ ($p < 0.001$). The inhibition of calcineurin activity in the PBMC of patients with SLE may be a new factor in the immunosuppression caused by GCS. There was no significant difference in the calcineurin activities of seven GCS-free patients with SLE and those of 35 healthy controls (fig 1).

CALCINEURIN ACTIVITIES IN THE PBMC OF PATIENTS WITH SLE WITH OR WITHOUT GCS TREATMENT AND IN HEALTHY CONTROLS

In four groups of subjects the calcineurin activities of PBMC were assayed and compared. The cells were either non-stimulated or

stimulated by PMA and Ca^{2+} ionophore. In the non-stimulated cells of six patients with SLE taking 32 mg of GCS/day, there was a significant decrease in the calcineurin activity compared with the value of six healthy controls (9.7 mU/mg v 15.1 mU/mg, $p < 0.05$). In the seven GCS-free patients or in the six patients taking 8 mg of GCS/day, the changes were not significant compared with the values of the healthy controls (14.6 mU/mg and 12.8 mU/mg v 15.1 mU/mg). In the cells stimulated by PMA and Ca^{2+} ionophore for four hours there was a marked decrease in the calcineurin activities of all groups compared with their

Figure 3 In vitro effect of prednisolone sodium succinate (PRED) on the activity of calcineurin in peripheral blood mononuclear cells (PBMC) of healthy subjects. PBMC of five healthy controls were cultured for 72 hours in the absence and presence of 10^{-4} M PRED. Calcineurin activities were determined and the statistical analysis was carried out as described in "Patients and methods". Asterisks denote significant differences compared with the respective controls. For the calculation of statistical significance Student's paired t test was used.

non-stimulated counterparts: 5.2, 4.9, 4.5, and 3.8 mU/mg *v* 15.1, 14.6, 12.8, and 9.7 mU/mg ($p < 0.001$). The differences between the calcineurin activities of the stimulated cells in the four groups were not significant, but the changes showed a pattern similar to those found in the non-stimulated cells—the highest activity was measured in the healthy controls, the lowest in the patients receiving 32 mg of GCS/day (fig 2). In addition, we found no significant differences in the calcineurin activities of men or women with SLE taking or not taking GCS, but each group contained only one man (data not shown).

IN VITRO EFFECT OF PREDNISOLONE SODIUM SUCCINATE ON THE ACTIVITY OF CALCINEURIN IN PBMC OF HEALTHY SUBJECTS

To verify the inhibitory effect of GCS on calcineurin activity, the mononuclear cells of healthy controls were cultured with or without 10^{-4} M prednisolone sodium succinate (PRED) for 72 hours. This non-toxic dose of the drug had significantly inhibited calcineurin after 24 hours: 16.8 mU/mg *v* 6.4 mU/mg ($p < 0.001$), whereas a slight but not significant increase of calcineurin activity was seen in the control cells (15.1 mU/mg *v* 16.8 mU/mg). The activity of calcineurin increased even further in the GCS-free cultures over the next two days: 26.7 and 21.6 mU/mg at 48 and 72 hours, respectively ($p < 0.001$ and $p < 0.01$). A similar tendency was seen in the cells treated with PRED. The activity of calcineurin increased significantly from the value at 24 hours (6.4 mU/mg) to 14.2 mU/mg and 12.9 mU/mg at 48 and 72 hours, respectively. However, the calcineurin activities measured in the presence of PRED remained always significantly lower than those in the GCS-free cultures (fig 3). An assay of lactate dehydrogenase activities in the supernatants of cultured cells showed that neither the culturing nor the presence of PRED caused any cellular damage (data not shown). Thus the decreased calcineurin activity may be related to the effect of PRED.

Discussion

The goal of these experiments was to measure the activity of calcineurin in the PBMC of patients with SLE. The major results are as follows. Firstly, there is no difference in the calcineurin activities of PBMC in the GCS-free patients with SLE and healthy control subjects. Secondly, as far as we know, this is the first experimental evidence of decreased calcineurin activity of PBMC induced by GCS used for the treatment of patients with SLE.

Unfractionated PBMC—that is, suspensions of T and B lymphocytes, NK cells, and monocytes, were used in this study because the calcineurin activity assays required a large number of cells. As patients with SLE are lymphopenic it would have been difficult to obtain a sufficient volume of blood for the separation of various purified subsets of the cells. As the subsets of PBMC were characterised by flow cytometry, and as it was found that 69.4% of the cells were T cells, these preparations could be regarded as “T cell-rich suspensions”.

There was no significant difference in the proportions of the various subsets of PBMC in the patients with SLE and the controls.

PRED was chosen for the *in vitro* experiments because the derivatives of prednisolone were mostly used also for the treatment of the patients with SLE. PRED was judged not toxic in these studies, according to the lactate dehydrogenase measurements. It is known that all GCS have both genomic and non-genomic membrane effects.^{22–23} According to these reports, the 32 mg/day dose of GCS in our patients and 10^{-4} M PRED applied in our *in vitro* experiments might have had a significant non-genomic influence, playing a part in the reduction of calcineurin activity. We found no decrease in the amount of calcineurin detected by immunoblotting (data are not shown) either in the cells of patients or in the cells of *in vitro* experiments treated with high doses of GCS, suggesting that the GCS induced inhibition of calcineurin activity was independent of the synthesis of the enzyme. However, in these cells an increased rate of apoptosis was detected at 24 and 48 hours of culturing compared with the GCS-free cells (data are not shown). This observation is in accordance with an earlier observation that the rate of apoptosis is increased in the T cells of patients with SLE treated with GCS.²⁴ Therefore, we suppose that cabin 1, a newly discovered endogenous inhibitor of calcineurin, might possibly be one of the key molecules playing a part in this phenomenon, coupling the processes of steroid receptor regulation, apoptosis, and reduction of calcineurin activity in the PBMC.^{25–27} Furthermore, cabin 1 may also play a part in the reduced calcineurin activity seen in the cells stimulated by phorbol ester and Ca^{2+} ionophore in our experiments.²⁸

Our results confirmed the observation of Rider *et al.*¹³ who found no difference in the amounts of calcineurin mRNA in patients with SLE and healthy controls. According to our data this similarity was also reflected in the calcineurin activities of the PBMC of patients with SLE and healthy subjects. The significantly decreased calcineurin activity of PBMC measured in patients with SLE treated with 32 mg GCS/day, however, is a new observation. In addition, a significant negative correlation was found between the doses of GCS used in the treatment and the calcineurin activity (fig 1). This effect of GCS may also be an element of the immunosuppression related to the inhibition of the production of NF-AT dependent cytokines¹¹ (for example, IL2, IL4, IL8, and IL13) in patients with SLE, and this pathway may coexist with other, well known influences of GCS.²⁹ Calcineurin is a Ca^{2+} dependent enzyme,¹⁵ and, therefore, the inhibition of Ca^{2+} influx by GCS³⁰ may have an important role in the GCS dependent decrease of calcineurin activity. This fact may partly explain the earlier finding that GCS inhibit the calcineurin dependent activation of IL2³¹ and IL4³² genes. However, the inhibition of calcineurin by GCS is not a phenomenon specific to SLE because it can be induced also in a dose dependent manner in the PBMC of healthy subjects *in vitro*.³³

The mononuclear cells of healthy controls cultured in vitro showed rather different patterns of calcineurin activity in the untreated and PRED treated series (fig 3). The gradually increasing calcineurin activities of control cultures could be attributed to the production of several cytokines (for example, TNF or IL1) derived from the mononuclear cells attached to the surface of plastic dishes used for the culturing.³⁴ By increasing the intracellular levels of Ca²⁺, TNF and IL1 could significantly increase the calcineurin activity of cells cultured for longer than 24 hours.³⁵ The significant inhibition of calcineurin seen in the cells cultured in the presence of PRED for 24 hours was described recently.³³ However, the recovery of calcineurin activity in the PRED treated cells from its reduced level at 24 hours may be explained by the appearance of the cytokines (TNF and IL1) during the second day of culturing (48 hours). As PRED can also inhibit the cytokine production of cultured cells activated by the plastic surface, the calcineurin activity increased by the effect of cytokines could never reach the values seen in the GCS-free counterparts of these cells.

Our data show that the decreased calcineurin activity induced by high doses of GCS may represent a new element in the immunosuppressive treatment of patients with SLE.

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