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RESEARCH ARTICLE

Detecting clinical activity in systemic lupus erythematosus with an archaeal poly(ADP-ribose) polymerase-like thermozyme: a pivotal study

Maria Rosaria Faraone-Mennella[1,2], Raffaele Scarpa[3], Anna Petrella[1], Francesco Manguso[3], Rosario Peluso[3], and Benedetta Farina[1]

[1]*Department of Structural and Functional Biology, University 'Federico II,' Naples, Italy,* [2]*National Institute of Biostructures and Biosystems (INBB), Via delle medaglie d'oro, 00136 Rome, Italy, and* [3]*Department of Clinical and Experimental Medicine, University 'Federico II,' Naples, Italy*

Abstract

The clinical usefulness of an immunotest was evaluated by using purified poly(adenosine diphosphate (ADP)-ribose) polymerase from *Sulfolobus solfataricus* (PARP*Sso*) as an antigen to detect the presence of abnormal anti-PARP antibodies in the sera of patients with systemic lupus erythematosus (SLE) at different clinical stages. Sera from 44 patients with SLE, subgrouped on the basis of disease activity (16 with inactive disease, 28 with active disease) were analysed with a new immunotest to detect anti-PARP antibodies, and with an immunofluorescent (IIF) assay for antinuclear antibodies (ANA) detection. ANA detection by IIF revealed that sera of healthy subjects were negative, whereas sera from patients with SLE were positive in all cases (13 positive at 1:80, 15 at 1:160, 15 at 1:320, 1 at 1:640, v/v). Anti-PARP activity was higher in ANA-positive patients than in controls ($p = 0.005$). Within the group of SLE sera, disease and anti-PARP activity was increased more significantly in patients with active than in those with inactive disease ($p < 0.001$ and $p = 0.001$, respectively). Correlation between anti-PARP and disease activity in SLE patients was statistically significant ($p < 0.001$). PARP*Sso* seems to be suitable for detecting anti-PARP antibodies and could play a role as a serological marker of disease activity in patients with SLE.

Keywords: *Autoantibodies; autoimmunity; poly(adenosine diphosphate ribose) polymerase; serological marker;* Sulfolobus solfataricus

Introduction

Defining serological markers for disease monitoring in patients with previously assessed systemic lupus erythematosus (SLE) is of significant clinical value, as SLE does not always follow a linear course and therapeutic strategy may need to be tailored over time. A strict relationship between the poly(adenosine diphosphate (ADP)-ribosyl)ation system in SLE and the cell response to DNA damage has been suggested (Okolie & Shall 1979, Clayton et al. 1984, Jeoung et al. 2004, Muller et al. 1994). Indeed the synthesis of poly(ADP-ribose) (poly-ADPR) is an immediate response to DNA damage and is the first step in a cascade of events leading to either DNA repair or apoptosis (Caricchio & Cohen 1999, Bijl et al. 2001).

The original observation by Okolie and Shall (1979) that high levels of specific antibodies against poly-ADPR persisted, even when those against double-stranded (ds) DNA drastically decreased, enhances the diagnostic value of anti-poly-ADPR antibodies, which could flank other markers of SLE and could help in the early diagnosis and/or in the monitoring of the clinical stages of the disease.

Okolie and Shall (1979) gave particular emphasis to the diagnostic value of antibodies anti-poly-ADPR, which they already found related to SLE, and were therefore discriminating in its diagnosis. This evidence is

Address for Correspondence: Prof. Raffaele Scarpa, Department of Clinical and Experimental Medicine, University 'Federico II,' via Pansini 5, 80135 Naples, Italy. Tel.: +39 081 746 21 26. Fax: +39 081546 34 45. E-mail: rscarpa@unina.it

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supported by a serological analysis of the cDNA expression library leading to identification of SLE-associated autoantibodies against the enzyme poly-ADPR polymerase (PARP) that synthesizes ADPR polymers (Lim et al. 2002, Decker et al. 2000). The presence of IgG antibodies in the serum of SLE patients reacting with both recombinant human PARP and a synthetic peptide which corresponds to the PARP domain directly involved in the specific recognition of double strand breaks in DNA was also reported (Jeoung et al. 2004). Moreover, it has been demonstrated that in SLE lymphocytes PARP is normally cleaved, indicating that the final biochemical pathway of apoptosis is not altered and SLE patients do not suffer from a major apoptotic abnormality (Caricchio & Cohen 1999). Human autoantibodies reacting with the zinc fingers of PARP involved in the recognition of damaged DNA totally prevent the cleavage of PARP by caspase-3 (Decker et al. 2000).

Poly(ADP-ribosyl)ation is a reversible post-translational modification of proteins catalysed by PARPs, a family of enzymes whose main member is PARP 1, a chromosomal enzyme that uses NAD^+ as a substrate to synthesize polymers of ADPR (Kim et al. 2006, Amé et al. 2004, Faraone-Mennella et al. 2003). Poly(ADP-ribosyl)ation is involved in the regulation of several cellular functions related to the maintenance of genomic integrity (DNA repair, gene amplification, apoptosis) and to the expression and propagation of the genetic information (DNA transcription and replication, differentiation, neoplastic transformation) (Faraone-Mennella et al. 2003, Amé et al. 2004).

Here, we propose antibodies directed against the poly(ADP-ribosyl)ation system as SLE biomarkers, possibly useful to set up a rapid, sensitive and cheap test contributing to the detection of disease activity. In particular, we have studied the cross-reactivity of an archaeal thermoprotein toward anti-PARP antibodies in autoimmune sera.

Over the past decade, in our group, particular interest has been addressed to the study of a PARP-like thermozyme (PARP*Sso*) from the extreme thermophilic archaeabacterium *Sulfolobus solfataricus*. The enzyme, thermophilic and thermostable, purified to homogeneity, showed properties similar to the eukaryotic PARP, such as elongation of the ADP-ribose chain to synthesize short oligomers, DNA-binding ability and cross-reactivity toward polyclonal antibodies against the mesophilic enzyme (Faraone-Mennella et al. 1996, 1998).

As the relationship between serological and clinical disease activity abnormalities in patients with SLE still remains a controversial issue, the aim of the present research was to evaluate the clinical usefulness of an immunotest using purified PARP*Sso* to detect the presence of abnormal anti-PARP antibodies in the sera of a cohort of patients with SLE at different clinical stages.

Patients and methods

Patients

In this study sera from 44 patients with SLE (one male and 43 female, median age 32 years with an interquartile range (IQR) of 12, all giving their oral consent to anonymous use of data) classified according to the 1982 American Rheumatism Association (ARA) criteria (Tan et al. 1982) and divided on the basis of disease activity according to the SLE Disease Activity Index (SLEDAI)-2K (Bombadier et al. 1992) (16 with inactive disease, median SLEDAI-2K score 4 (IQR 2), and 28 with active disease SLEDAI-2K score 10 (IQR 6), and from 23 healthy donors (one male and 22 female, median age 33 years with an IQR of 10) consecutively observed at the transfusional centre, matched for age ($p = 0.74$) and sex ($p = 1.00$) were selected. All sera were stored in two aliquots at –80°C until used.

Methods

Anti-PARP immunochemical procedure

Bovine serum albumin (BSA) and DNase I were from Sigma (Milan, Italy) (Tris was purchased from AppliChem (Del Chimica, Milan, Italy)). PARP-like thermozyme from *S. solfataricus*, was purified according to Faraone-Mennella et al. (1996) and the protein concentration was checked by the procedure described in a commercial kit (Pierce (Perbioscience, Lavsanne, Switzerland); SDS-PAGE and ultraviolet (UV) spectra determination were performed according to Faraone-Mennella et al. (1996, 1998). The purity of PARP*Sso* antigen was checked by SDS-PAGE followed by staining gels with either Coomassie blue or silver dye.

Rabbit polyclonal antibodies against human PARP-1 catalytic domain (H-250; 200 μg ml^{-1}; 1:2000, v/v) and horseradish peroxidase (HRP)-conjugated secondary antirabbit antibodies (1:4000, v/v) were from Santa-Cruz, DBA, Milan, Italy and Pierce, respectively, both used to test the cross-reactivity of each purified PARP*Sso* preparation. *Polyvinylidene fluoride* (PVDF) membrane was from Amersham-Pharmacia. (now GE Healthcare, Zurich, Switzerland).

Immuno-dot-blot

An immuno-dot-blot was performed with a dot-blot apparatus (Bio-Rad, Milan, Italy) connected to a vacuum pump, following the instructions of the dot-blot booklet. Briefly the PVDF membrane was permeabilized by a rapid immersion in 100% methanol and equilibrated in Tris-saline buffer (TBS). Protein was adsorbed in duplicate on the PVDF membrane after blocking of aspecific sites with 1% BSA in TBS. Sera (10 μl) or commercial anti-PARP catalytic domain antibodies (1:2000, v/v) were added. After 1.5 h incubation the membrane was

washed three times with TBS/0.05% Tween and incubated with secondary HRP-labelled IgG. Following several rapid washings, cross-reactivity was revealed with a kit for chemiluminescence (Pierce; Super Signal West Dura) in a Chemi-Doc apparatus (Bio-Rad; Quantity One program). Fluorescence was measured as optical density per spot (arbitrary units) and acquired at 1.0 s.

Linear duplicate dilutions (1 to 0.2 μg) of PARP-like thermozyme (1 mg ml^{-1}) were preliminarily analysed with commercial rabbit antihuman PARP catalytic domain antibodies (1:2000, v/v) in order to determine the lowest protein sensitivity. Routine checks with commercial human anti-PARP antibodies were performed at 0.8 μg PARP*Sso*. PARP specificity of immunosignals was checked by SDS-PAGE of rat testis PARP crude preparation and, in parallel, of both *Sulfolobus* crude extract and purified PARP*Sso*, followed by anti-PARP immunoblotting. BSA and DNase I (negative) and rat testis PARP (positive) controls were used under the same conditions. Antirabbit secondary IgG were used at 1:4000 (v/v) dilution. Undiluted and diluted sera were tested at 0.4 μg protein and 1:10 000 (v/v) dilution of secondary antibodies as optimal conditions. The optical densities measured with the negative control were subtracted from values obtained with sera in the presence of PARP*Sso* and rat testis.

Indirect immunofluorescence

Antinuclear antibodies (ANA) were determined in one of the two aliquots of the available sera using a classic IIF procedure, with HEp-2 epithelial cells as substrate (Molden et al. 1984).

The two aliquots of each stored serum were analysed separately and blindly with IIF in the Department of Clinical and Experimental Medicine, and with anti-PARP immunochemical procedure at the Department of Structural and Functional Biology, respectively. Once collected, experimental data with IIF and immuno-dot-blot techniques were compared with clinical diagnosis and patients' symptoms, provided by the Department of Clinical and Experimental Medicine.

Statistical analysis

For categorical variables, the χ^2 test was performed by using the Fisher's exact test or exact method as appropriate. Continuous variables are expressed as median and IQR. The unpaired Mann–Whitney *U* test was used to compare patient groups. A receiver operating characteristics (ROC) curve was constructed taking into account the anti-PARP activity. Subjects were divided into a 'condition' present group, including all patients with SLE and a 'condition' absent group which included the healthy subjects. For this variable, the optimized cut-off for equally important sensitivity and specificity was calculated. Bivariate correlations computing Spearman's coefficient with their significance levels were calculated. All analyses were done with SPSS software, version 15.0.0. The ROC analysis was obtained with StatsDirect statistical software (version 2.6.2)

Results

Anti-PARP immunoassay

Figure 1a shows the ability of PARP*Sso* to cross-react with commercial human anti-PARP catalytic site antibodies. No signal was detected after direct treatment of antigen with secondary antibodies (data not shown). Under the same experimental conditions, the densities measured at 0.4 μg antigen were comparable for both *Sulfolobus* and rat testis PARP. The signal was very weak with DNase I, and not significant with BSA; 0.4 μg protein gave the best antigen amount, suggesting a high sensitivity of anti-PARP autoantibodies towards the

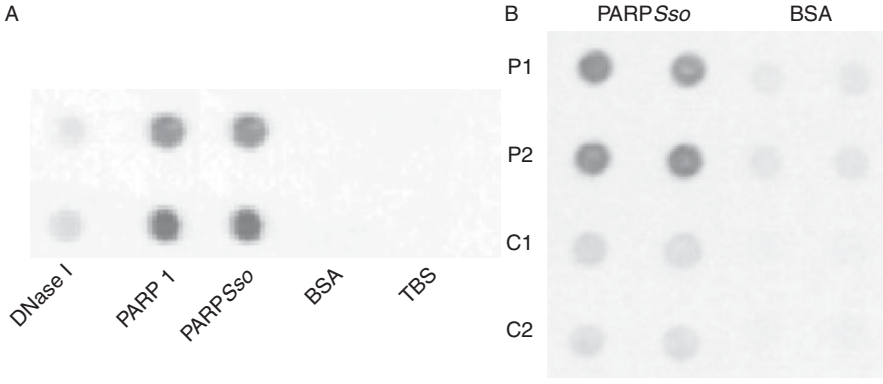

Figure 1. Antigenic ability of PARP*Sso*. (A) Dot-blot with commercial anti-PARP catalytic site antibodies (1:2,000, v/v). The thermozyme (PARP*Sso*) was analysed in comparison with eukaryotic PARP 1 and two negative controls, bovine serum albumin (BSA) and DNase I, at 0.4 μg. TBS, Tris buffer saline. (B) Dot-blot of PARP*Sso* (0.4 μg) with two control (C1, C2) and two patient (P1, P2) sera, in duplicate. BSA (0.4 μg) was used as negative control.

thermozyme. The following analyses were carried on at 0.4 μg antigen.

Analyses of sera, each in two aliquots, were performed by both IIF and the immunotest with PARP*Sso*. Cross-reactivity of PARP*Sso* (0.4 μg) with anti-PARP autoantibodies in human sera was tentatively tested by sampling randomly two sera from the control group and two from the patient groups. Linear dilutions of sera (0 to 1000) in TBS were used to test autoantibodies which were revealed with secondary antihuman IgG (1:4000 and 1:10 000, v/v in TBST/1% BSA).

Sera diluted 1:500 (v/v) gave the most relevant results, with optical densities for the control group at background levels and patient samples showing densities significantly higher than those of control group (Figure 1b). Dilutions of sera 1:500 (v/v) with a secondary antibody diluted 1:10 000 (v/v) seemed to give the most significant results and were taken as optimal conditions.

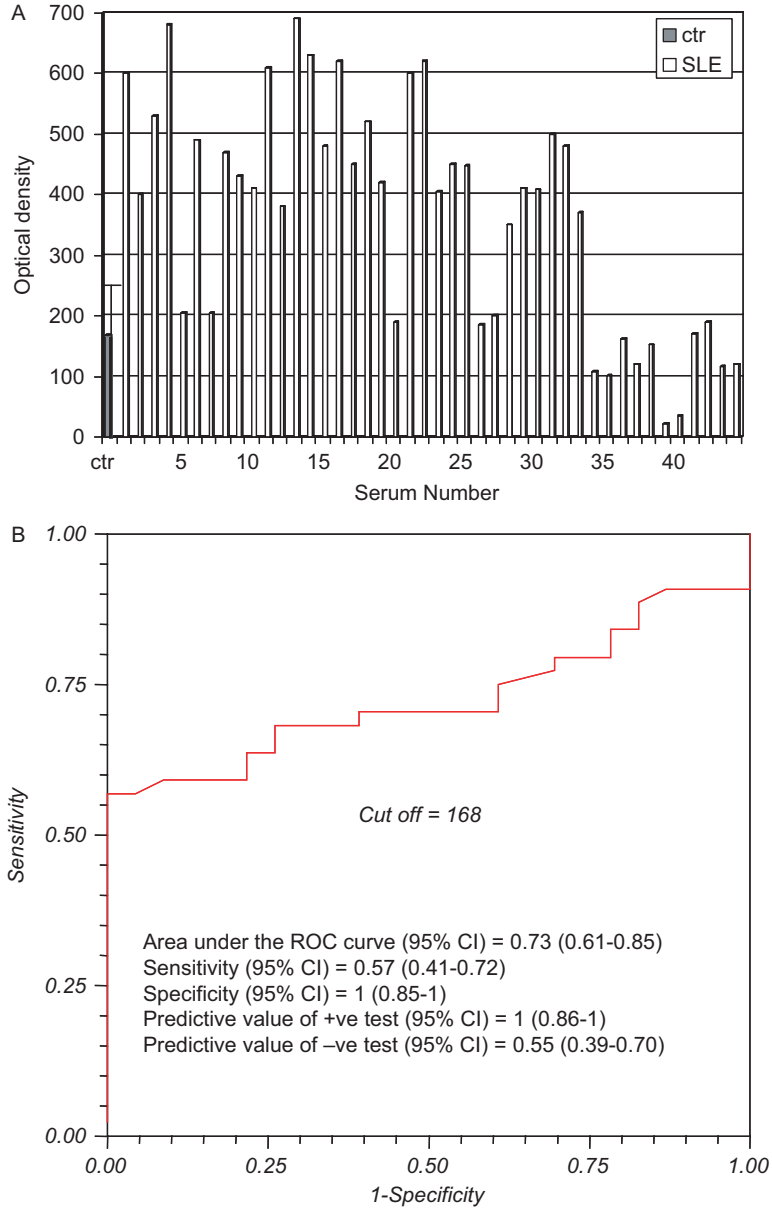

Figure 2. Detection of anti-PARP antibodies in sera of patients with systemic lupus erythematosus (SLE). (A) Optical densities of immunospots from dot-blot experiments with antinuclear antibody (ANA)-negative and -positive sera. The values of the 23 ANA-negative samples (controls) were reported as an average mean (grey column). White columns (from 1 to 44 numbers) refer to ANA-positive sera, all from SLE patients. (B) Plot of a receiver operating characteristic (ROC) curve. The curve defines the cut-off point to appraise the discrimination ability of the anti-PARP antibodies in sera and to predict patients with SLE. In the area under the curve, sensitivity, specificity, positive and negative predictive values are also shown.

Anti-PARP antibody detection in SLE sera

IIF tests on all 67 samples revealed that the ANA-negative sera were from the 23 healthy subjects (controls in Figure 2A), whereas the ANA-positive sera, as expected, were from the 44 patients with SLE (starting dilution 1:80, v/v; 13 cases positive at 1:80, 15 at 1:160, 15 at 1:320 and 1 at 1:640, v/v) (Table 1).

Figure 2A shows the optical densities measured after immunodetection of anti-PARP antibodies in all 67 serum samples. The levels of anti-PARP antibodies from the 23 ANA-negative subjects were reported as mean value and taken as background. Twenty-four (nearly 50%) of 44 sera positive for SLE were above the mean value of the 23 controls. The statistical significance of these results was confirmed by plotting them on a ROC curve, which allowed us to calculate the density cut-off value (168) discriminating among healthy (below the cut-off) and pathological (above the cut-off) subjects (Figure 2B and Table 1).

Among the 44 SLE patients, clinical parameters indicated that 16 were SLE inactive and 28 SLE active. It is worth noting that the inactive SLE patients had anti-PARP activity means below the cut-off level (147.5), whereas the value was higher in patients with active SLE (241.5) (Table 1). Eleven out of 16 inactive sera had anti-PARP activity below the cut-off level (normal). Abnormal anti-PARP activity (i.e. largely above the cut-off level) was observed for 19 sera from the active group (Table 1).

Within the group with SLE, the SLEDAI score and anti-PARP activity were significantly increased in the active compared with the inactive patients ($p<0.001$ and $p=0.001$, respectively) (Table 1). Interestingly, the correlation between anti-PARP activity and SLEDAI in SLE patients was statistically significant ($r=0.74$; $p<0.001$; Figure 3), whereas ANA titres did not correlate with the disease activity index ($r=-0.12$, $p=0.58$) (data not shown).

Once correlation of anti-PARP activity and the SLEDAI was determined, statistical analysis was performed with the aim of finding any interdependence between PARP antibody activity and each SLEDAI variable (Table 2).

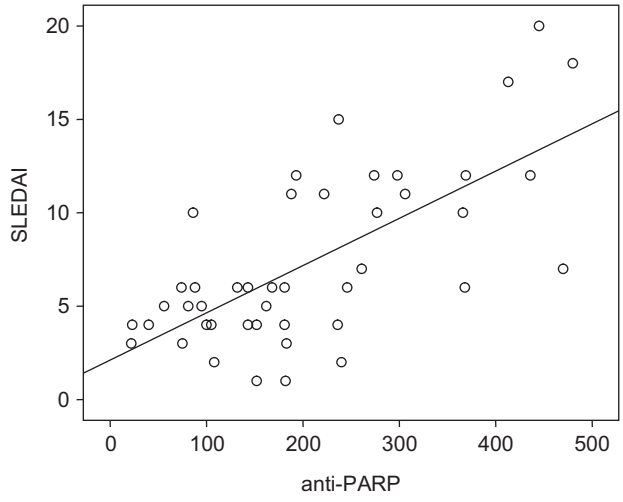

Figure 3. Spearman's correlation between anti-PARP activity and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in patients with SLE ($r=0.74$; $p<0.001$).

Table 1. Anti-PARP, antinuclear antibody (ANA) class and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) in active and inactive patients with SLE.

	SLE		
	Inactive ($n=16$)	Active ($n=28$)	p-Value
Anti-PARP, median (IQR)	147.5 (101)	241.5 (233)	0.005
Anti-PARP, n (%)			0.02
Normal	11 (68.8)	9 (32.1)	
Abnormal	5 (31.3)	19 (67.9)	
ANA class, n (%)			0.004
1:80	9 (50)	4 (14.3)	
1:160	1 (6.3)	14 (50)	
1:320	6 (37.5)	9 (32.1)	
1:640	0 (0)	1 (3.6)	
SLEDAI, median (IQR)	4 (2)	10 (6)	<0.001

IQR, interquartile range. For more details see under Statistical analysis in Materials and methods.

Table 2. Systemic lupus erythematosus clinical variables and PARP test.

	PARP		
Variables	Negative ($n=20$)	Positive ($n=24$)	p-Value
Seizure	0 (0)	0 (0)	–
Psychosis	0 (0)	0 (0)	–
Brain	0 (0)	0 (0)	–
Visual	0 (0)	0 (0)	–
Cranial	0 (0)	0 (0)	–
Headache	0 (0)	0 (0)	–
CVA	0 (0)	3 (12.5)	0.24
Vasculitis	0 (0)	6 (25)	0.03
Arthritis	0 (0)	0 (0)	–
Myositis	0 (0)	1 (4.2)	1.00
Casts	0 (0)	0 (0)	–
Haematuria	0 (0)	0 (0)	–
Proteinuria	7 (35)	12 (50)	0.32
Pyuria	0 (0)	0 (0)	–
Rash	1 (5)	1 (4.2)	1.00
Alopecia	3 (15)	1 (4.2)	0.32
Mucosal	0 (0)	3 (12.5)	0.24
Pleurisy	0 (0)	5 (20.8)	0.05
Pericarditis	2 (10)	7 (29.2)	0.15
Low complement	10 (50)	13 (54.2)	1.00
Anti-DNA	11 (55)	8 (33.3)	0.15
Fever	3 (15)	11 (45.8)	0.03
Thrombocytopenia	1 (5)	6 (25)	0.11
Leucopenia	7 (35)	11 (45.8)	0.47

Data are n (%).

Anti-PARP activity was strictly related to inflammatory indexes rather than other markers of organ damage (pleurisy $p = 0.05$, fever $p = 0.03$, vasculitis $p = 0.03$).

Discussion

Defining serological markers for disease monitoring and/or for outcome measure of patients with SLE is still a controversial issue (Esdaile et al. 1996, Kallenberg et al. 1997). In practice, to standardize outcome measures in SLE, several complex clinical indexes have been identified for the detection of disease activity, damage from disease and health status (Bombadier et al. 1992, Negri et al. 1990).

For instance, anti-dsDNA and serum complement levels are generally considered useful for detecting active phases or for predicting clinical flares of the disease (Esdaile et al. 1996, Zonana-Nacach et al. 1995, Mollnes et al. 1999).

The present study was performed with the aim of evaluating another laboratory test that could play a role as a serological marker of disease activity, also able to reveal clinical exacerbations. On the basis of our results, PARP*Sso* from *S. solfataricus* seems to be a useful candidate for recognizing, specifically and with suitable sensitivity, anti-PARP autoantibodies in the serum of patients with active SLE. It is worth noting that in the serum of patients with inactive SLE (under prolonged immunosuppressive treatment and/or in clinical remission stage after therapy) anti-PARP antibodies drastically drop, even if ANA levels are still high (Figure 3). The thermozyme exhibited both specificity and sensitivity among autoimmune sera, even at 1:500 (v/v) dilution, and was able to discriminate between sera from patients with active or inactive SLE.

There is a wide literature discussing the possible relationship between poly(ADP-ribosyl)ation and SLE. Most papers report immunoassays (filter binding or immunoadsorbent) detecting in autoimmune sera antibodies against the product of this reaction, poly-ADPR (Okolie & Shall 1979, Clayton et al. 1984, Muller et al. 1994). However, the sensitivity and specificity of these tests depend on the chain length of the antigen, as antibodies do not recognize short chains (4–5 residues) of poly-ADPR (Kanai et al. 1977).

In a few articles the enzyme PARP from mesophiles is used and the formation of complexes with antibodies is assayed by different methods (enzyme-linked immunosorbent assay, Western blotting, enzyme activity) (Jeoung et al. 2004, Muller et al. 1994, Caricchio & Cohen 1999, Lim et al. 2002, Decker et al. 2000). With either antigen, all the authors state that only in part do SLE sera cross-react and parallel ds-DNA assays in detecting clinical activity. None, except our group, has correlated the levels of antibodies with disease activity, supporting data with a detailed statistical analysis. Furthermore, the fact that ANA are still high in patients in remission, whereas anti-PARP antibodies decrease in parallel with the reduction of SLE activity, is reported and documented in our research for the first time. Our method of detecting anti-PARP antibodies not only provides evidence of a strict correlation between antibodies and SLE disease activity but, among SLEDAI criteria, identifies inflammation as mainly responsible for high anti-PARP levels.

It is well known that extensively damaged DNA is irreparable. Therefore PARP is cleaved by apoptotic caspases (Muller et al. 1994, Bijl et al. 2001, Scovassi & Poirer 1999) and lacks its role of 'genome guardian'. The variability of anti-PARP antibodies in sera of patients with inactive and active SLE might be related to the functional state of the cells: recovering cells regain the ability to repair DNA and do not undergo apoptosis.

In the light of these observations, we believe that the proposed immunoassay, which uses the thermozyme PARP*Sso*, might be a good complement to other SLE immunotests. The relevance and diagnostic potential of our research results impose their validation on a wider number of samples.

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