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Nuclear-Targeting Autoantibodies Induced Nuclear PARP Cleavage Accompanied by More Pronounced Decrease of Peripheral White Blood Cells Than Ro/SSA and La/SSB Antigen-Targeting Autoantibodies

INGRID BÖHM[1,2]

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Autoantibody production and leukocytopenia may be linked in patients with lupus erythematosus (LE). Unclear is the ability of different autoantibody species to induce apoptosis and cell loss. Laboratory routine analyses (white blood cell counts, autoantibody detection), and flow cytometry (annexin V, CD3, CD4, CD8) have been performed in 126 consecutive LE-patients. Nuclei of PBMC were investigated flow cytometrically for the presence of the 85 kDa poly-(ADP-ribose)-polymerase (PARP) fragment. Peripheral total white blood cells (WBC), lymphocytes, T-cells, CD3+ CD4+, and CD3+ CD8+ cells were significantly decreased in patients with LE (P from 1.2×10^{-14} to $P < .0008$). In the presence of either antinuclear (P from 1.2×10^{-14} to $P < .0008$) or anti-dsDNA antibodies (P from 2.9×10^{-12} to $P < .007$) were significantly diminished. Differences in cell numbers in LE patients with versus without anti-Ro/SSA were less pronounced: significant differences could be only obtained in lymphocytes and T-cells ($P < .02$). Anti-La/SSB antibodies were accompanied by significant increased leukocytes ($P < .02$). PARP cleavage (85 kDa) in nuclei was preferentially observed in cases with nuclear targeting autoantibodies. These results indicate that nuclear targeting autoantibodies are associated to lower peripheral blood cells counts than Ro/SSA, and La/SSB cytoplasmic targeting autoantibodies. This provides an explanation for the pathogenesis of cytopenias associated with SLE.

KEY WORDS: Anti-dsDNA autoantibodies; Ro/SSA and La/SSB; leuko-/lymphocytopenia; PARP 85 kDa; nuclear apoptosis.

INTRODUCTION

Lupus erythematosus (LE), a prototype of autoimmune diseases, is characterized by autoantibody production of intra- and inter-individually varying degree and spectra. There is a body of evidence that autoantibodies recognize and may be derived from circulating non-phagocytosed apoptotic material (e.g., nucleosomes) (1–4). On the other hand, autoantibodies have the propensity to induce apoptosis (5), and possibly LE-associated cytopenias (6–9). Since autoantibody-penetration into living cells seems to disturb the cells and may kill them by apoptosis induction (10, 11), the present investigation has been undertaken to compare different autoantibody species directed either to nuclear (e.g., anti-dsDNA antibodies) or cytoplastic epitopes (e.g., anti-Ro/SSA and anti-La/SSB antibodies) with respect to circulating cell counts, and apoptosis induction.

MATERIALS AND METHODS

Patients

Blood was collected from 126 patients (men $n = 41$) with lupus erythematosus (LE) (mean age 43.6 years ±0.8 SD) with either biopsy-proven cutaneous LE (discoid LE [DLE] 48.4% or subacute cutaneous LE [SCLE] 31.5%) or SLE (20.1%). SLE was diagnosed by using the ACR criteria for the diagnosis of systemic lupus erythematosus (12). Patients with cutaneous LE did not fulfil the ACR criteria. Fifty-seven healthy persons (men $n = 24$) with a mean age of 40.8 years (11–84 years) served as controls. Peripheral mononuclear blood cells (PBMC) were separated by Ficoll-Hypaque density-gradient (1.077) centrifugation (Biochrom, Berlin, Germany). Routine laboratory parameters (peripheral blood counts of leukocytes, and lymphocytes) have been measured in parallel.

[1]Department of Radiology, University of Bonn, Sigmund-Freud Strasse 25, 53105 Bonn, Germany; e-mail: i.boehm@uni-bonn.de.
[2]Formerly with Department of Dermatology.

Flow Cytometry

EDTA blood specimens were stained with CD8-fluorescein isothiocyanate (FITC), HLA-DR (FITC), CD4-phycoerythrin (PE), and CD3- peridin-chlorophyll protein (PerCP) antibodies (from Becton Dickinson Biosciences, San Jose, CA) following the manufacturer's instructions. Red cell lysis was done by using FACSLysing solution according to the manufacturer's instructions. Multicolor analysis of cell surface CD3, CD4, CD8, and/or HLA-DR molecules was performed by a FACScan flow cytometer and data were analyzed with CellQuest™ research software (BD). For every sample 0.5–1.0 $\times$ 10^4 cells were acquired.

Phosphatidylserine (PS) Externalization and Measurement of the PARP 85 kDa Cleavage Product

Cells suspended at a concentration of 1 $\times$ 10^6 were incubated with annexin V conjugated to FITC (ALEXIS, Germany) in a calcium-containing binding buffer (10 mM Hepes and 145 mM NaCl, pH 7.4 containing 2 mM $CaCl_2$) to detect PS exposure on PBMC. Propidium iodide (PI) was added at a final concentration of 50 ng/ml. After 15 min of incubation, data were acquired flow cytometrically.

The FITC-labeled anti-poly (ADP-ribose) polymerase 85 kDa cleavage product (PARP 85-kDa fragment) polyclonal rabbit anti-human antibodies (Biosource, Belgium) were used to stain isolated PBMC nuclei (13) that have been analyzed by flow cytometry.

Serum Autoantibody Determination

Antinuclear antibodies were detected on HEp2 cells by indirect immunofluorescence (IIF). In positive cases sera were analyzed for the presence of anti-dsDNA, anti-Ro/SSA, and anti-La/SSB antibodies by using ELISA (enzyme linked immuno-sorbent assay) (Pharmacia Upjohn, Freiburg, Germany) and/or Western blot technique according to the manufacturers' instructions (Innogenetics, Heidenheim, Germany).

Statistical Analysis

The normal distribution of the absolute cell counts measured was proved by the Kolmogorov-Smirnov test. Significance of differences between means was calculated using the Student's *t*-test or the Mann–Whitney nonparametric tests for comparison of the variables with skewed distribution. Correlations have been calculated by using the Spearman rank correlation. Differences between values were considered statistically significant at *P* less than 0.05.

RESULTS

LE-Patients had Decreased Numbers of Peripheral White Blood Cells and their Subsets

The numbers of circulating blood cells were significantly lower in LE-patients than in healthy controls: leukocytes/μL (5500.0 $\pm$ 165.0 versus 7308.0 $\pm$ 289.0; $P = 4.1 \times 10^{-8}$) (Fig. 1a), lymphocytes/μL (1255.7 $\pm$

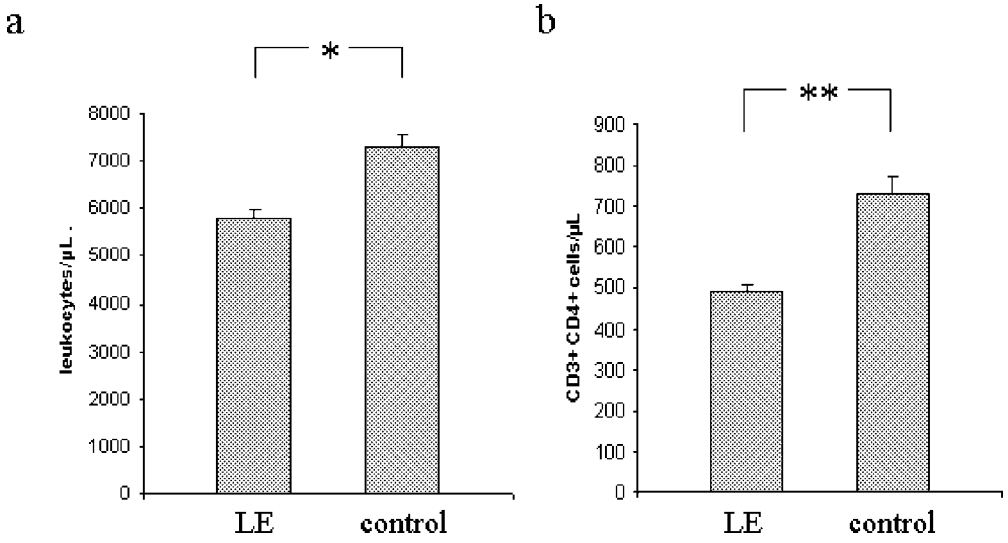

Fig. 1. Comparison of mean ±SEM absolute numbers of peripheral white blood cells in patients with LE versus healthy controls. a) Leukocytes and b) T-helper cells (CD3+ CD4+) were significantly lower in LE-patients than in healthy controls (* $P = 4.1 \times 10^{-8}$; ** $P = 1.2 \times 10^{-9}$.

55.6 versus 1838.3 ± 73.8; $P = 1.5 \times 10^{-8}$), CD3+ cells/μL (791.1 ± 33.5 versus 1263.0 ± 52.9; $P = 1.2 \times 10^{-12}$), CD3+ CD4+ cells/μL (455.6 ± 23.4 versus 734.2 ± 37.5; $P = 1.2 \times 10^{-9}$) (Fig. 1b), and CD3+ CD8+ cells/μL (297.0 ± 15.1 versus 487.8 ± 24.4, $P = 1.6 \times 10^{-4}$). LE-patients had non-significantly elevated absolute counts of CD3+ HLA-DR+ cells.

Patients with Autoantibodies Targeting the Nucleus have Lower Peripheral WBC Counts than those Without

Although autoantibodies targeting different subcellular compartments may induce different degree in peripheral WBC loss there are currently no data available that present an association between leuko-/lympho-cytopenia and autoantibodies targeting different subcellular epitopes.

Therefore, the above mentioned peripheral blood cell populations have been analyzed in dependency on detectable autoantibodies (i.e. antinuclear antibodies [ANA], anti-double stranded DNA [dsDNA] antibodies, anti-Ro/SSA, and anti-La/SSB antibodies. Patients with both ANA and anti-dsDNA antibodies had significantly decreased leukocytes (Fig. 2a), lymphocytes (Fig. 2b), CD3+ cells (Fig. 2c), T-helper cells (Fig. 2d), T-cytotoxic cells (data not shown), and activated T-cells expressing the HLA-DR molecule on their surface (data not shown). In patients with anti-Ro/SSA antibodies only lymphocytes (Fig. 2b), and T-cells (Fig. 2c) were significantly lower than in those without anti-Ro/SSA antibodies. The differences obtained for leukocytes (Fig. 2a), CD3+ CD4+ cells (Fig. 2d), CD3+ CD8+ cells, and CD3+ HLA-DR+ cells were statistically not significant when comparing patients with and without anti-Ro/SSA antibodies. LE-patients with anti-La/SSB antibodies showed significantly increased leukocytes (Fig. 2a) as compared to those without these autoantibodies. In association to anti-La/SSB antibodies the other investigated cell subsets did not differ significantly (Figs. 2b–d).

PARP is Preferentially Cleaved in Nuclei of PBMC in Association with Nuclear Targeting Autoantibodies

Annexin V binding capacity has been analyzed to order to show as to whether cell surface apoptosis marker correlate to apoptotic events observed within the nucleus. Percentage of annexin V binding cells in patients without (ANA−) and with anti-nuclear antibodies (ANA+), without anti-dsDNA (dsDNA−) and with anti-dsDNA antibodies (dsDNA+), without anti-Ro/SSA (SSA−) and with anti-Ro/SSA antibodies (SSA+), without anti-La/SSB (SSB−) and with anti-La/SSB antibodies (SSB+) did not differ significantly (data not shown).

To determine whether caspases become activated in relation to different autoantibody findings in the LE-patients' sera, PARP, a known substrate of caspase-3 and some caspase-3-like caspases was studied. As shown in Fig. 3a, when isolated PBMC nuclei were analyzed by flow cytometry with anti-PARP 85 kDa polyclonal antibodies, percentage of PARP 85 kDa cleavage product was more abundant in patients with ANA and anti-dsDNA antibodies (the latter was statistically significant at $P < 0.03$). Patients with and without SSA/SSB autoantibodies did not differ with respect to PARP cleavage (Fig. 3).

Circulating anti-dsDNA antibodies inducing both leukocytopenia and PARP cleavage are shown in Fig. 4a. In situ (PBMC nuclei) IgG can be detected by direct immunofluorescence (Fig. 4b).

DISCUSSION

These data provide evidence that nuclear targeting autoantibody species seemed to be preferentially involved in loss of peripheral WBC and their subsets.

Although both increased anti-dsDNA autoantibodies and leuko-/lymphocytopenia are ACR criteria for the diagnosis SLE (12), the latter has been rarely investigated in patients with LE (6, 7). In comparison to healthy controls LE-patients have diminished peripheral WBC and subsets (Fig. 1). Autoantibody-related apoptosis induction could be discussed as an attractive underlying mechanism.

In addition to anti-nuclear (ANA) and anti-dsDNA antibodies LE-patients are known to have a broad spectrum of autoantibodies targeting different epitopes (14–16). Of these anti-Ro/SSA and anti-La/SSB antibodies characteristic features for patients with Sjögren's syndrome may arise in patients with different LE manifestations (17–20). Anti-SSA/Ro antibodies are detected in the sera of 30% of patients with SLE, even during preclinical setting; anti-Ro/SSA are strongly associated (90%) with some subtypes of SLE such as old-onset (>50 years) SLE, subacute lupus erythematosus, drug-induced subacute lupus erythematosus and in patients with hereditary C2 or C4 or C1q deficiency with lupus or lupus-like disease; and are also associated with primary Sjögren's syndrome and with undifferentiated connective tissue disease (UCTD) (21). Both anti-dsDNA and anti-La/SSB antibodies have been related to neutropenia in patients with LE (22, 23). Since these autoantibodies may be related to different clinical features, it could be likely that these autoantibodies may induce different degrees of leuko-/lympho-cytopenia. Therefore, in relation to the autoantibody-specificity the absolute number of WBC, lymphocytes, and some of their

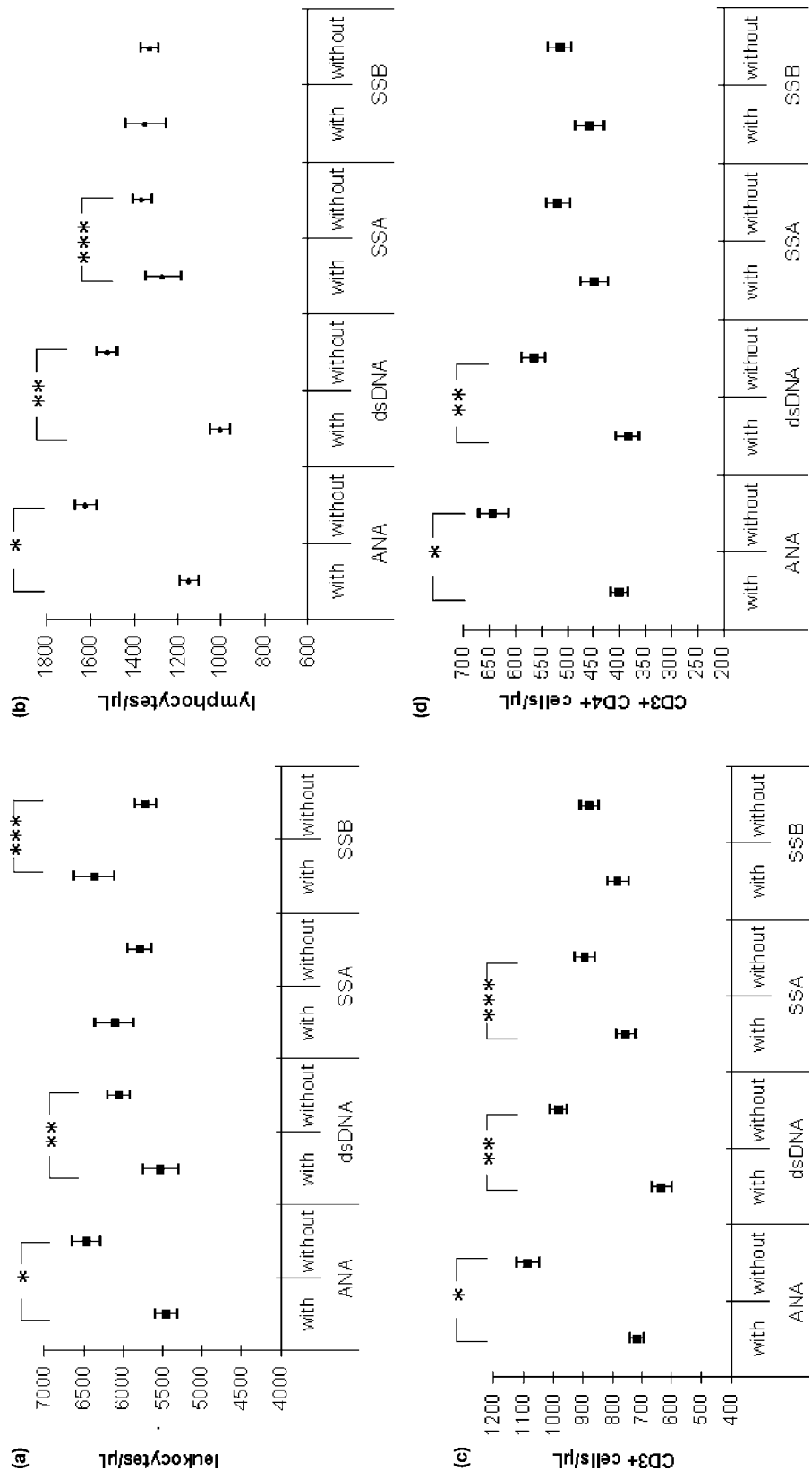

Fig. 2. LE-patients with a positive autoantibody finding (designated as 'with') were compared to those without detectable autoantibodies (designated as 'without'). (a) When comparing the means of the absolute counts of leukocytes, in presence of antinuclear antibodies (ANA) ($^{*}P = 1.71906 \times 10^{-6}$), anti-dsDNA antibodies (dsDNA) ($^{**}P = 0.0062$), anti-Ro/SSA (SSA), and anti-La/SSB antibodies (SSB) ($^{****}P = 0.0137$) were as indicated significantly higher than in those without such autoantibodies. (b) The means of the absolute counts of lymphocytes, in presence of antinuclear antibodies (ANA) ($^{*}P = 1.21014 \times 10^{-14}$), anti-dsDNA antibodies (dsDNA) ($^{**}P = 3.81428 \times 10^{-12}$), anti-Ro/SSA (SSA) ($^{***}P = 0.01339$), and anti-La/SSB antibodies (SSB) significantly higher values had been found in comparison to patients without such autoantibodies. (c) The means of the absolute counts of total T-cells (CD3+ cells), in presence of antinuclear antibodies (ANA) ($^{*}P = 4.04121 \times 10^{-14}$), anti-dsDNA antibodies (dsDNA) ($^{**}P = 2.91223 \times 10^{-12}$), anti-Ro/SSA (SSA) ($^{***}P = 0.01449$), and anti-La/SSB antibodies (SSB) significantly higher values had been found in comparison to patients without such autoantibodies. (d) The means of the absolute counts of T-helper cells (CD3+ CD4+ cells), in presence of antinuclear antibodies (ANA) ($^{*}P = 7.04992 \times 10^{-14}$), anti-dsDNA antibodies (dsDNA) ($^{**}P = 1.36133 \times 10^{-7}$), anti-Ro/SSA (SSA), and anti-La/SSB antibodies (SSB) significantly higher values had been found in comparison to patients without such autoantibodies.

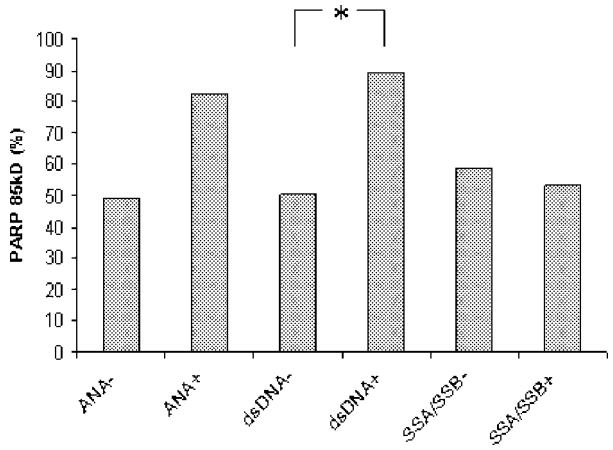

Fig. 3. a) Percentage of PARP 85 kDa positive PBMC nuclei in patients without (ANA−) and with anti-nuclear antibodies (ANA+), without anti-dsDNA (dsDNA−) and with anti-dsDNA antibodies (dsDNA+), without anti-Ro/SSA (SSA−) and with anti-Ro/SSA antibodies (SSA+), without anti-La/SSB (SSB−) and with anti-La/SSB antibodies (SSB+). Patients with increased percentage of PARP cleavage (78.72%) had increased corresponding DNA fragmentation (34.86%; sub-G1 peak), while a lower percentage of PARP 85 kDa positive PBMC nuclei correspond to lower DNA fragmentation (1.14%).

subsets have been analyzed. Thereby, it has been found that patients with ANA and anti-dsDNA antibodies had significantly lower counts of leukocytes, lymphocytes, T-cells (CD3+), T-helper cells (CD3+ CD4+), T-cytotoxic (CD3+ CD8+) cells, and activated T-cells (CD3+ HLA-DR+) (Figs. 2a–d). In contrast patients with anti-Ro/SSA antibodies only had significantly lower counts of lymphocytes, and T-cells (CD3+); the difference between patients with versus without in the latter group was less pronounced than in patients with versus without nuclear targeting autoantibodies (Fig. 2a–d). Leukocytes were significantly increased in patients with as compared to those without anti-La/SSB antibodies (Fig. 2a), but did not show any further hint of a significant difference (Fig. 2b–d).

The presence of anti-SSA/SSB antibodies is related to blood cytopenia, increased photosensitivity, congenital heart block, pulmonary disease, keratoconjunctivitis sicca suggesting that they have an immunopathologic role (22, 24–26). Both anti-dsDNA and anti-SSA/SSB antibodies have been found in association with peripheral blood cytopenias (22–24). The reason for this phenomenon is currently unclear, but apoptosis-induced cell loss could be likely (23, 27). The herein presented data show that patients with anti-dsDNA autoantibodies had the lowest peripheral blood cell counts (Figs. 2a–d). How can one explain this phenomenon? The anti-dsDNA antibody is thought to move across the cell membrane, and targets the nucleus that may result in apoptosis induction (11, 28, 29, 30). Anti-dsDNA antibodies can bind to numerous cellular receptors that exist on various cell types including (a) localization and prolonged residence on the membrane, (b) penetration and cytoplasmic localization, and (c) penetration and nuclear localization (31).

Anti-SSA/Ro and anti-SSB/La autoantibodies recognize different epitopes on polypeptides associated with small RNAs called scYRNA that are most commonly located in the cytoplasmic compartment (70%) and few in the nuclear compartment (30%) (21). The 52 kDa Ro antigen resides mainly cytoplasmic, Ro 60 kDa both nuclear and cytoplasmic, while La 48 kDa only resides in the nucleus under normal conditions (4, 32, 33). During early apoptosis, La 48 kDa is dramatically redistributed to the cytoplasm, while the localization of Ro 52 kDa and Ro 60 kDa is maintained (4, 32). Induction of apoptosis in cultured cardiocytes also results in surface translocation of Ro/La and recognition by Abs

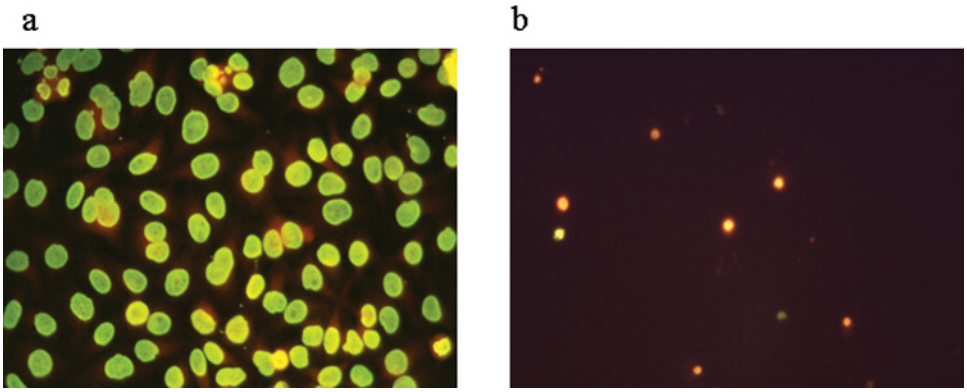

Fig. 4. (a) Indirect immunofluorescence analysis on HEp2-cells using anti-dsDNA autoantibody containing serum and FITC-labelled anti-human IgG showing homogenous fluorescence of the nuclei. (b) Immunostaining for human IgG (goat anti-human IgG Cy3) in PBMC nuclei.

(25). In addition, it could be shown that that Ro/SSA antigen expression on keratinocyte cell membrane can be induced by different factors such as heat shock, phorbol 12-myristate 13-acetate, estradiol and ultraviolet B irradiaton (34). The subcellular antigen localization and the interaction with antigen targeting autoantibody may serve some clues to get some insight into the mechanisms responsible for the different degrees of cell loss. Therefore, phosphatidylserine externalization as detected by annexin V binding, and the caspase 3-dependent cleavage product of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) within the nucleus has been analyzed in parallel to different autoantibody findings. PARP, a 116-kDa protein, is cleaved to yield an 85-kDa product during PCD (35, 36). The obtained findings suggest that caspase-3, and/or a caspase-like enzyme, may be preferentially activated in LE-patients with nuclear targeting autoantibodies (ANA, anti-dsDNA antibodies). This is further supported by the recent identification of autoantibody against PARP fragment as a serological marker in systemic lupus erythematosus (37). Moreover, the finding that neutrophils from SLE-patients demonstrate increased nuclear DNA damage (38) may also support the presented findings. In contrast anti-Ro/SSA and anti-La/SSB autoantibodies that target autoantigens located within the cytoplasm seem to induce less pronounced cell damage.

Beside pathogenetic aspects the potential use of a modified autoantibody as a delivery system to target the cell nucleus seems to function as new therapeutic tool (30). In contrast to damaging effects in association with LE-related anti-dsDNA antibodies, a murine monoclonal anti-dsDNA antibody was found to penetrate living cells and localize in the nucleus without pathologic effects (30).

Taken together, the herein presented results provide evidence that the presence of autoantibody species targeting to different subcellular compartments may be involved in different parts of the machinery of PCD. Especially anti-dsDNA antibodies targeting the nucleus appears to break down the DNA repair enzyme PARP within the nucleus that is accompanied by cell loss which can be finally detected as peripheral cytopenias. If so, this finding would provide further evidence that anti-dsDNA antibodies play a central role in the pathogenesis of LE. As to whether modified anti-dsDNA antibodies can be used as vectors for the delivery of desired molecules into the nucleus (30) could be a fascinating question for further investigations.

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