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# Impaired degradation and aberrant phagocytosis of necrotic cell debris in the peripheral blood of patients with primary Sjögren's syndrome

George E. Fragoulis [a, 1], Aigli G. Vakrakou [a, 1], Anna Papadopoulou [b], Anastasios Germenis [c], Emmanouel Kanavakis [b], Haralampos M. Moutsopoulos [a], Menelaos N. Manoussakis [a, d, *]

[a] Department of Pathophysiology, School of Medicine, National and Kapodestrian University of Athens, Greece
[b] Department of Medical Genetics, National and Kapodestrian University of Athens, Choremio Research Laboratory, "Aghia Sophia" Children's Hospital, Athens, Greece
[c] Department of Immunology and Histocompatibility, Medical School, University of Thessaly, Greece
[d] Hellenic Pasteur Institute, Athens, Greece

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ABSTRACT

Aberrant removal of necrotic debris is considered a feature with inflammatory consequences in SLE. Herein, primary Sjögren's syndrome (SS) patients were investigated for the first time for the capacity of their sera to degrade secondary necrotic cell remnants (SNEC) and DNA (endonuclease DNase1 activity), as well as for uptake of SNEC by blood-borne phagocytes. For comparison, specimens from unselected SLE and RA patients and from healthy blood donors (HBD) were also studied. Compared to HBD, the sera from SS and SLE patients studied (but not RA) were found to exhibit significantly impaired capacity for degradation of SNEC (both for $p = 0.007$) and deficient DNase1 activity (both for $p < 0.0001$). The deficient DNase1 activity in SS and SLE sera did not owe to decreased DNase1 protein levels. It correlated inversely with increased serum levels of circulating nucleosomes and cell-free DNA ($p < 0.0001$), as well as with the disease activity indices of SS ($r = -0.445$, $p = 0.0001$) and SLE ($r = -0.500$, $p = 0.013$). In ex-vivo whole blood analyses, SS and SLE patients (but not RA) also manifested significantly increased SNEC-phagocytosis by monocytes and granulocytes (all for $p < 0.0001$) that also correlated with disease severity indices of SS ($p = 0.001$) and SLE ($p = 0.01$). In various cross-admixture experiments, such aberration was found to reside in the hyperfunctional activity of phagocytes, the impaired degrading activity of serum DNase1 and the SNEC-binding capacity of serum IgG of SS and SLE patients. The sera of SS and SLE patients (but not of RA) induced significant SNEC-phagocytosis by healthy monocytes that correlated inversely with the DNase1 activity ($r = -0.634$, $p < 0.0001$) of these sera. In line with this, the inhibition of DNase1 in HBD sera by G-actin was found to lead to significantly diminished SNEC degradation and increased SNEC uptake by healthy phagocytes ($p = 0.0009$), supporting the important physiologic role of serum DNase1 in the prevention of SNEC-phagocytosis. Purified serum IgG preparations from SS and SLE patients manifested increased binding to SNEC and were able to enhance significantly the engulfment of SNEC by healthy phagocytes both directly (under serum-free conditions, $p \leq 0.009$) and via the prevention of physiologic degradation of SNEC by serum, most likely due to their "shielding" against endonuclease digestion ($p = 0.0005$). These data indicate that upon cell necrosis, the immune system of SS and SLE patients may be overly exposed to the necrotic debris, a fact that probably holds a key role in the pathogenesis of inflammatory and autoimmune reactions observed in these disorders.



*Abbreviations:* SNEC, secondary necrotic material (necrotic cell remnants); SRED, single radial enzyme diffusion assay; PI, propidium iodide; SS, primary Sjögren's syndrome; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; HBD, healthy blood donors.

* Corresponding author. Department of Pathophysiology, School of Medicine, National and Kapodestrian University of Athens, 75 Mikras Asias Str., 115 27 Athens, Greece. Tel.: +30 210 746 2677; fax: +30 210 746 2664.
*E-mail address:* menman@med.uoa.gr (M.N. Manoussakis).

[1] Contributed equally.

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## 1. Introduction

Dead cells and cellular debris may elicit inflammatory reactions, however, under healthy conditions this is effectively prevented by their rapid elimination from tissues [1]. On the other hand, experimental evidence from patients and animal models indicates that systemic lupus erythematosus (SLE) is characterized by defective clearance of apoptotic cells, whereby chronic autoimmune reactions and organ damage are thought to ensue [2,3]. Normally, dying cells are rapidly cleared at early stages of apoptosis via mechanisms that involve several types of membrane receptors and serum bridging molecules, including components of the complement system [4–6]. However, in the absence of efficient uptake, the apoptotic cells proceed to the state of "late apoptosis" or "secondary necrosis", where cells lose their cell membrane integrity and spill out their content, including modified autoantigens and "danger signals" such as alarmins [7–9]. Although such secondary necrotic material (termed SNEC) may be rendered accessible to the cells of the immune system, recent studies indicate that SNEC is not engulfed by phagocytes from healthy individuals, whereas it is readily ingested by monocytes and granulocytes from SLE patients, thus resulting in the secretion of several proinflammatory cytokines [10,11]. In fact, SNEC normally undergoes efficient degradation by serum DNase1 with the assistance of serum cofactors [12,13], a fail-safe mechanism that is reportedly defective in SLE [14,15]. In lupus patients, serum autoantibodies to nuclear antigens, such as anti-dsDNA, have been also reported to promote the uptake of SNEC by blood-borne phagocytes [10,11,16]. Taken together, the impaired phagocytosis of apoptotic cells in conjunction with increased uptake of necrotic material by peripheral blood phagocytes of SLE patients appears to contribute to the pathogenesis of the disease [17].

Primary Sjögren's syndrome (SS) is a chronic autoimmune disorder with a broad spectrum of clinical symptoms that extends from disease confined to the exocrine glands (organ-specific exocrinopathy) to various extraglandular manifestations (systemic disease) and the development of B-cell lymphoma [18]. SS patients present polyclonal B-cell hyperactivity, illustrated by profound hypergammaglobulinemia, multiple autoantibodies, circulating immune complexes and complement consumption [18,19]. SLE shares with SS the above features of B-cell hyperactivity, as well as the frequent occurrence of defective uptake of apoptotic cells by phagocytes (Manoussakis et al., submitted). In this context, we presently sought to comparatively investigate the handling and removal of necrotic cell debris by the peripheral blood components of SS, SLE and RA patients, including the degradation of SNEC and DNA by serum and the uptake of SNEC by phagocytes.

## 2. Patients and methods

### 2.1. Patients

Blood specimens were obtained after informed consent from 70 consecutive unselected patients with primary SS, 32 with SLE and 15 with rheumatoid arthritis (RA) using established criteria [20–22] (Table 1), as well as from 50 healthy blood donors (HBD) matched for age and sex to the SS (HBD-1; $n = 25$) and SLE groups (HBD-2; $n = 25$). The study was approved by the Medical Council of "Laikon" University Hospital. At the time of investigation, none of the patients or controls studied displayed evidence of infection, including past or current infection by hepatitis viruses or human immunodeficiency virus. Patients' medical records were retrospectively analyzed for demographic variables, clinical and laboratory features. At the time of the study, patients studied were assessed for disease activity by calculation of ESSDAI (for SS) [23], SLEDAI (for SLE), DAS28 (for RA) and for disease severity by SSDDI (for SS) [24], SLICC-ACR (for SLE) [25] and by global disease severity index (for RA). Patients with SS were also analyzed for the presence of extraglandular manifestations (arthritis, Raynaud's phenomenon, purpura, peripheral neuropathy, vasculitis, and hepatic, pulmonary, or renal involvement), as well as for the presence of manifestations of type-I disease, as previously [26]. In RA patients, the occurrence of extra-articular disease (subcutaneous nodules, secondary Sjogren's syndrome, episcleritis/scleritis, serositis, interstitial lung disease, and vasculitis) was recorded. The global severity of RA (assigning patient's global disease condition and not just the inflammatory status of the joints) was rated independently and blindly to the other results by two of the authors (MNM and HMM), on a graded scale ranging from 0 (mildest disease) to 10 (most severe disease) (Table 1). All blood specimens were processed immediately. Sera were stored in aliquots at −20 °C until tested.

**Table 1**
Anthropometric, clinical and serologic features of the SS, SLE and RA patients studied.

| Features | SS ($n = 70$) | SLE ($n = 32$) | RA ($n = 15$) |
|---|---|---|---|
| Age, years, median (range) | 57.5 (33–78) | 39.5 (19–42) | 62.0 (42–80) |
| Sex, women: men | 66:4 | 30:2 | 11:4 |
| Disease duration, years, median (range) | 10 (2–22) | 10 (1–20) | 9 (2–15) |
| Sicca manifestations, no. positive (%) | 70 (100) | 0 (0.0) | 1 (6.7) |
| Disease activity, median (range)[a] | 8.4 (0–30) | 4 (0–16) | 5.4 (3.9–7.1) |
| Disease severity, median (range)[a] | 7 (2–7) | 2 (0–7) | 2.7 (1.0–5.2) |
| Type-I SS disease, no. positive (%)[a] | 51 (72.8) | NA | NA |
| ANA, titer$^{-1}$, median (range) | 320 (0–2560) | 640 (0–2560) | 0 |
| Anti-Ro/SSA, no. positive (%) | 48 (68.6) | 13 (40.6) | 0 (0.0) |
| Anti-La/SSB, no. positive (%) | 22 (31.4) | 0 (0.0) | 0 (0.0) |
| Anti-dsDNA, no. positive (%) | 0 (0.0) | 14 (43.7) | 0 (0.0) |
| Anti-chromatin, no. positive (%) | 0/27 (0.0) | 5/18 (22.2) | 0 (0.0) |
| Anti-histone, no. positive (%) | 0/27 (0.0) | 7/18 (38.9) | 0 (0.0) |
| Anti-C1q, no. positive (%) | 5/27 (18.5) | 9/18 (50.0) | 0 (0.0) |
| Low serum C3 and/or C4, no. positive (%)[b] | 42 (60.0) | 21 (65.6) | 3 (20.0) |
| Low serum C1q, no. positive (%)[c] | 6/22 (27.3) | 5/14 (35.7) | ND |
| RF, no. positive (%) | 32 (45.7) | NA | 11 (73.3) |
| Cryoglobulinemia, no. positive (%) | 20 (28.6) | ND | ND |

NA: non-applicable, ND: not determined.
Median serum levels of C3 (range) were in SS: 104 mg/dL (60–168), in SLE: 91 mg/dL (39–154) and in RA: 107 mg/dL (82–163). Median serum levels of C4 (range) were in SS: 19 mg/dL (2–46), in SLE: 14 mg/dL (4–33) and in RA: 23 mg/dL (16–37). Median serum levels of C1q (range) were in SS: 17.6 mg/dL (3.5–43.6) and in SLE: 18.5 mg/dL (12.0–25.0).
[a] Defined as described in Patients and Methods.
[b] Low serum C3 < 90 mg/dL and C4 < 20 mg/dL.
[c] Low serum C1q < 15 mg/dL.

### 2.2. Serological evaluations

Sera studied were assessed for anti-Ro/SSA, anti-La/SSB by counter immunoelectrophoresis and for anti-C1q, anti-native histone and anti-chromatin antibodies by ELISA (Quanta Lite, Inova Diagnostics, USA). Serum levels of C3 and C4 complement components were measured by radial immunodiffusion, of C1q by nephelometry and of nucleosomes by ELISA (Roche Diagnostics). Serum DNase1 activity was assessed by single radial enzyme diffusion (SRED) assay, as previously described [27], as well as by a commercially available ELISA assay (Orgentec GmbH, Mainz, Germany), according to the instructions of the manufacturer. In SRED assays,

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