Combination of Autoantibodies Against Different Histone Proteins Influences Complement-dependent Phagocytosis of Necrotic Cell Material by Polymorphonuclear Leukocytes in Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** Polymorphonuclear leukocytes (PMN) with autoantibody-coated engulfed necrotic cell material (NC) are frequently seen in systemic lupus erythematosus (SLE). We evaluated the roles of complement, different antihistone antibodies (anti-H ab), and oxidative burst in the phagocytosis of NC by PMN, as well as association to disease activity and clinical phenotype in SLE.

Methods. ELISA and immunoblot were used to measure antibodies to different histone proteins in sera from patients with SLE and complement-deficient individuals. Phagocytosis of NC by PMN and oxidative burst activity was assessed by flow cytometry.

Results. A clearly increased phagocytosis of NC was seen in patients with active SLE, which was associated with high levels of anti-H ab concentrations and oxidative burst activity. The complement system contributed to efficient phagocytosis of NC by PMN through activation of the classical pathway, and the phagocytosis was mediated by FcγRIIA, FcγRIIIB, and CR1 in combination. A pattern of high phagocytosis, consumption of classical pathway components, and a broad anti-H ab repertoire was seen particularly in patients with nephritis and serositis. The combination of antibodies to several different histone proteins, often with anti-DNA antibodies, promoted an efficient uptake of NC, whereas antibodies against only histone H1 or a few histones seemed to be of less importance.

Conclusion. The distributions of specificities among anti-H ab are of great importance in the complement-dependent phagocytosis of debris from NC in SLE. Measurement of anti-H ab could be useful in monitoring of this disease and contribute to improved understanding of the autoimmune process. (J Rheumatol First Release July 1 2012; doi:10.3899/jrheum.111511)

Key Indexing Terms:
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To a large extent, systemic lupus erythematosus (SLE) is an immune complex-mediated disease characterized by inflammation in several organ systems, and the severity of the disease can vary from mild to severe. The presence of immune complexes composed of autoantibodies directed against nuclear antigens such as nucleic acids and nucleic acid-associated proteins is a hallmark of SLE. These circulating immune complexes may deposit in tissue and cause inflammation that forms the pathogenetic basis of several SLE manifestations, including nephritis[1].

Apoptotic cells are thought to be the major source of autoantigen in SLE, partly because of impaired apoptotic cell clearance. If not eliminated, apoptotic cells will progress to secondary necrotic cells and modified autoantigens will chal-

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lenge immune tolerance, which may lead to autoantibody production[2,3,4,5].

Polymorphonuclear leukocytes (PMN) containing engulfed antibody-coated dead cell material were first described in bone marrow cell preparations from patients with SLE in 1948 by Hargraves, *et al*[6]. These PMN were named lupus erythematosus cells (LE cells). Later, the formation of LE cells was shown to require the presence of cell nuclei, antibodies against histone proteins (anti-H ab), and a functional complement system[7,8,9,10,11,12]. The original diagnostic LE cell test was based on microscopy. In a more recent method, described by Böhm, flow cytometry was used to measure phagocytosis of necrotic cell material by purified PMN in the presence of serum[13].

Antibodies against histone proteins are frequently found in SLE and are directed against individual histone proteins or histone complexes[14,15,16,17,18]. Histone H1 has been suggested as the main target protein for antibodies involved in the formation of LE cells, but reactivity toward other histone proteins, chromatin components, and histone-DNA complexes are all seen in LE cell-positive patients[12,17]. When PMN interact with immune complexes, then size, epitope density, IgG subclass, and complement incorporation are properties that determine PMN phagocytosis and the subsequent respiratory burst and degranulation[19,20]. The uptake of immune complexes in general is mediated by Fcγ receptors (FcγR) and complement receptors (CR) separately or in cooperation[20,21,22,23], and most likely similar uptake mechanisms are operating in the formation of LE cells.

The aims of our study were to analyze the levels and specificities of autoantibodies directed against different histone proteins involved in phagocytosis of necrotic cell material by PMN. Further, we also investigated the importance of the complement system for PMN phagocytosis of necrotic cell material by using serum samples from complement-deficient individuals. The roles of CR and FcγR in the uptake of necrotic cell material by PMN were assessed by the use of blocking antibodies. Activation of PMN can lead to tissue damage and inflammation; therefore oxidative burst activity was also measured and the relationship between phagocytosis of necrotic cell material by PMN and disease activity was investigated.

MATERIALS AND METHODS

Patients and serum samples. Serum samples from 100 patients with SLE taking part in a prospective control program at the Department of Rheumatology, Skåne University Hospital, were included in the study as well as samples from 100 healthy blood donors. Serum samples from patients with SLE were collected at 2 timepoints from each patient, 1 representing a disease flare with higher disease activity and 1 representing inactive disease with lower disease activity (n = 200), although there were a few patients in the lower disease activity group with serological or clinically detectable disease activity. Demographics and clinical characteristics are shown in Tables 1 and 2. An overview of American College of Rheumatology classification criteria for the 100 patients with SLE is presented in Table 2[24], and all patients fulfilled at least 4 of the criteria. In addition, serum samples and PMN from 15 healthy controls and 47 patients with SLE taking part in the same prospective program were collected and used in the oxidative burst assay. Disease activity was determined according to the SLE Disease Activity Index 2000 (Table 1)[25].

Table 1. Clinical characteristics and treatment at the time of blood sampling for the 100 patients with systemic lupus erythematosus (SLE) and for the 47 patients with SLE included for analysis of autologous polymorphonuclear leukocytes (PMN) by the oxidative burst assay. Items included in the SLE Disease Activity Index 2000 (SLEDAI) are shown.

Characteristics	SLE[a] Lower, n = 100	SLE[b] Higher, n = 100	SLE[c], n = 47
Age, yrs, median (range)	43 (19–81)	41 (14–75)	47 (21–77)
Women, %	90	90	87
Disease duration, yrs, median (range)	7 (0–43)	4 (0–40)	7 (0–43)
SLEDAI 2K score, median (range)	2 (0–12)	9 (2–28)	2.5 (0–13)
Seizure	0	2	0
Psychosis	0	0	0
Organic brain syndrome	0	0	0
Visual disturbance	0	7	0
Cranial nerve disorder	0	0	0
Lupus headache	0	2	0
Cerebrovascular accident	0	2	0
Vasculitis	0	12	1
Arthritis	1	30	4
Myositis	0	2	0
Kidney involvement (urinary cast, hematuria, proteinuria, or pyuria)	1	35	3
Rash	4	31	1
Alopecia	1	7	1
Oral ulcers	0	7	0
Pleurisy	0	13	0
Pericarditis	0	7	0
Low complement (C3 or C4)	41	54	18
Anti-DNA antibodies	24	49	16
Fever	0	6	0
Thrombocytopenia	2	3	1
Leukopenia	6	10	2
Hydroxychloroquine	33	31	35
Chloroquine phosphate	4	6	0
Azathioprine	21	18	13
Mycophenolate mofetil	16	9	5
Rituximab (within 12 months)	1	0	3
Methotrexate	0	2	5
Cyclophosphamide	1	5	0
Cyclosporine A	9	5	1

[a] Patients from timepoint with lower disease activity. [b] Patients from timepoint with higher disease activity. [c] Patients included in the oxidative burst assay using autologous PMN.

Standard routine laboratory tests were used to measure complement components (C1q, C3, and C4) and autoantibodies against dsDNA (*Crithidia luciliae* immunofluorescence test). Serum samples from 100 blood donors were used as controls in the assay for phagocytosis of necrotic cell material (PNC assay) and in the different anti-H ab ELISA. Sera from individuals with a genetically determined deficiency of C1q, C2, C4, or properdin were used to evaluate the role of complement in the PNC assay. The characteristics of these sera have been described[26]. Informed consent was obtained from all participants and the study was approved by the research ethics board of Lund University (LU378-02).

Complement proteins and serum reagents. Complement proteins used to restore complement function in the deficient sera were purified as reported[26]. Serum samples from 3 patients with SLE who were positive in the PNC assay

Table 2. Clinical characteristics of the patients with systemic lupus erythematosus (SLE) according to American College of Rheumatology criteria, and associations with levels from the phagocytosis of necrotic cell (PNC) assay and antibodies against histone proteins.

Characteristics (%)	SLE Cohort, n = 100	PNC Assay-negative[a], n = 39	PNC Assay-positive[b], n = 61	p	No anti-H Ab, n = 48	Anti-H1 Ab Only, n = 11	Other Anti-H Ab, n = 41	p[c]	p[d]	p[e]
Malar rash	66	66.7	65.6	0.91	68.8	54.5	65.9	0.48	0.50	0.82
Discoid rash	30	38.5	24.6	0.14	35.4	54.5	17.1	0.31	**0.02**	0.06
Photosensitivity	62	71.8	55.7	0.11	77.1	45.5	48.8	0.06	1.00	**0.008**
Oral ulcers	29	35.9	24.6	0.22	33.3	18.2	26.8	0.48	0.71	0.64
Arthritis	84	89.7	80.3	0.21	87.5	100	75.6	0.58	0.10	0.17
Serositis	56	41	65.6	**0.016**	41.7	63.6	70.7	0.32	0.72	**0.01**
Renal disease	46	23.1	60.7	**< 0.0001**	33.3	45.5	61.0	0.50	0.50	**0.01**
Neurological disorder	8	2.6	11.5	0.11	4.2	0	14.6	1.00	0.32	0.14
Hematological manifestations	53	43.6	59	0.13	47.9	45.5	61.0	1.00	0.50	0.29
Leukopenia	43	35.9	47.5	0.25	39.6	27.3	51.2	0.51	0.19	0.29
Lymphopenia	23	12.8	29.5	0.06	18.8	18.2	29.3	1.00	0.71	0.32
Thrombocytopenia	22	23.1	21.3	0.84	22.9	9.1	24.4	0.43	0.42	1.00
Immunology	75	48.7	91.8	**< 0.0001**	56.3	81.8	95.1	0.17	0.19	**< 0.0001**
ANA	98	97.4	98.4	0.75	95.8	100	100	1.00	1.00	0.50
Anti-DNA antibodies	61	25.6	83.6	**< 0.0001**	37.5	54.5	90.2	0.33	**0.01**	**< 0.0001**
Anti-C1q antibodies	66	46.2	78.7	**0.001**	54.2	45.5	85.4	0.74	**0.01**	**0.003**

Significant values ($p < 0.05$) are shown in bold type. [a] PNC assay < 4.6. [b] PNC assay > 4.6. [c] Absence of antibodies against histones (anti-H ab) compared with presence of antibodies against histone H1 alone. [d] Presence of antibodies against only histone H1 compared with presence of antibodies against other histones or combinations of histones. [e] Absence of antibodies against histones compared with presence of antibodies against other histones than histone H1 alone. ANA: antinuclear antibody.

and normal human serum (NHS) were selected for IgG absorption on protein G sepharose (Amersham Pharmacia Biotech, Uppsala, Sweden). The concentration of IgG was measured before and after absorption (≥ 10 g/l and < 1 g/l, respectively). The same sera were also treated with the endoglycosidase EndoS (a kind gift from Mattias Collin, Lund University) at a concentration of 0.3 mg/ml at 37°C for 16 h. Removal of Fc glycans on IgG by EndoS affects the function of IgG by reducing the binding to FcγR and inhibiting the ability to activate the classical pathway of the complement system[27,28]. A pool of sera from 5 patients with high levels of anti-H ab was used as positive control in the measurement of oxidative burst, in the FcγR and CR blocking experiments, and in the different antihistone ELISA. All serum samples were stored at –80°C until analysis.

Preparation of phagocytic cells and cell nuclei. To obtain PMN and mononuclear cells (PBMC), freshly heparinized blood was used. The blood cells were isolated by density gradient centrifugation on Polymorphprep (Axis-Shield Poc AS, Oslo, Norway) according to the manufacturer's protocol. To obtain necrotic cell material (NC), PBMC were incubated 10 min at 70°C and then stained with propidium iodide (PI; BD Biosciences Pharmingen, San Diego, CA, USA). The PMN were stained with anti-CD45-FITC (Dako A/S, Glostrup, Denmark).

Assay for phagocytosis of NC material (PNC assay) and oxidative burst activity. Phagocytosis of NC by PMN was analyzed by flow cytometry, using an assay similar to the one described by Böhm[13]. Propidium iodide-labeled NC (4.5×10^5 cells) were incubated with 30 μl undiluted serum at room temperature for 20 min, allowing antibodies to bind to the NC. Anti-CD45-FITC-labeled PMN purified from healthy individuals were added, at a concentration of 1.0×10^6 cells/ml in a total volume of 300 μl, followed by incubation at 37°C for 15 min. NHS from a healthy donor was used as negative control as well as NHS supplemented with normal mouse IgG1 and IgG2a (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). As a positive control, NHS supplemented with anti-H ab (H5300-10; US Biological, Swampscott, MA, USA) was used. Cells were washed with phosphate-buffered saline pH 7.2 (PBS) containing 0.1% human serum albumin (Sigma-Aldrich, St. Louis, MO, USA) before analysis by flow cytometry. PMN were identified based on forward and side scatter properties and by computerized gating. Phagocytosis was calculated from the percentage of cells positive for both CD45 and propidium iodide.

To measure oxidative burst activity, dihydrorhodamine (DHR) 123 (Orpegen Pharma, Heidelberg, Germany) was added after 5 min incubation of serum, NC, and PMN at 37°C and followed by additional incubation at 37°C for 10 min before analysis by flow cytometry. Mean values of DHR-derived fluorescence intensity were used when quantifying oxidative burst activity.

FcγR and CR assay. PI-labeled NC (1.0×10^5 cells) were incubated with anti-H ab pool (6 μl) at room temperature for 20 min. Antibodies against CD11b, CD11c, CD16, CD18, CD32, CD35, and CD64 (Santa Cruz Biotechnologies) at 5–15 μg/ml were added to purified PMN from healthy individuals together with anti-CD45-FITC and incubated for 15 min at room temperature. The PMN were washed in PBS-HSA, spun down at 366 × g for 5 min and then added to the mix of NC and anti-H ab pool at a final concentration of 3.3×10^5 cells/ml in a total volume of 300 μl. Following incubations, washes and analyses were performed as in the PNC assay. Normal mouse IgG was used as negative control and 2000 events were counted per sample.

Antihistone ELISA. Microtiter plates (Maxisorp F96, Nunc, Roskilde, Denmark) were coated with 0.5 μg/well of histone H1 (H1), histone H3 (H3), or a mixture of the different histone proteins H1, H2A, H2B, H3, and H4 (H-mix) from calf thymus (Roche Diagnostics, Indianapolis, IN, USA) in PBS, then blocked with 1% bovine serum albumin (BSA; ICN Biomedicals Inc., Aurora, OH, USA) in PBS. Serum diluted in PBS-T containing 0.1% BSA was added and incubated for 2 h at room temperature, then washed before alkaline phosphatase-conjugated anti-human IgG, $F(ab')_2$ fragment (AP anti-IgG; Sigma-Aldrich), diluted in PBS-T, was added. After incubation, bound antibodies were visualized by adding disodium-*p*-nitrophenyl phosphate (Sigma-Aldrich) 1 mg/ml, dissolved in 10% (w/v) diethanolamine pH 9.8 containing 50 mM $MgCl_2$, and the absorbance at 405 nm was measured. The anti-H ab pool was included for normalization of the runs, and values reported are means of duplicates with the background absorbance subtracted. Concentrations were calculated as units/ml from the titration curves of the anti-H ab pool. The upper limit of the reference interval was set for antibodies to H1 (4.0 units/ml) and to the H-mix ELISA (1.7 units/ml) as the mean value +2 SD of the results from the 100 controls. Because of unspecific bind-

ing in the H3-ELISA, no upper limit was set, but the samples were also analyzed by immunoblot. Positivity for anti-H3 antibodies was defined as a positive reaction in the immunoblot together with antibodies detected in both the H3 ELISA and in the H-mix ELISA.

Immunoblot analysis. Serum samples from the 100 controls and from the 100 patients with SLE were also analyzed for anti-H ab proteins by immunoblot. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis under nonreducing conditions (NuPAGE 12% Bis-Tris Gel, NuPAGE MOPS SDS running buffer; Invitrogen, Carlsbad, CA, USA) was used. H-mix was loaded onto the gel and electrophoresed histone proteins were transferred to nitrocellulose membranes (Invitrogen). After blocking with 1% BSA, the membranes were incubated with serum, then washed before AP anti-IgG was added. The membranes were stained with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich).

Statistical analysis. Nonparametric statistical analyses were used: the Wilcoxon matched-pairs rank test, Mann-Whitney U test, and Spearman rank correlation test. A p value < 0.05 was considered statistically significant.

RESULTS

Phagocytosis of NC by PMN correlates with disease activity and presence of autoantibodies. Flow cytometry was used to measure phagocytosis of NC by PMN in the serum from patients with SLE and from controls. Among the 100 paired SLE sera, selected for timepoints of higher and lower disease activity, sera from 61 patients with SLE facilitated increased phagocytosis of NC. Sera from timepoints of higher disease activity had significantly higher ability to promote phagocytosis of NC compared to sera obtained from timepoints of lower disease activity ($p < 0.0001$; Figure 1). At both higher and lower disease activity timepoints, a significantly increased phagocytic capacity of NC was seen compared to healthy controls ($p < 0.0001$, $p = 0.019$, respectively).

To verify the involvement of autoantibodies in the phagocytosis of NC by PMN, sera from 3 patients with SLE who were positive in the PNC assay and NHS were selected for IgG absorption and EndoS treatment. When using SLE sera, a clear reduction of the capacity for phagocytosis of NC by PMN was seen after depletion of IgG (82%, 78%, and 86% reduction, respectively) and after treatment with EndoS (75%, 87%, and 86% reduction). No increased ability to promote phagocytosis of NC was seen when NHS was added to the IgG-depleted serum (data not shown). Further, when monoclonal anti-H ab were added to NHS, the capacity for phagocytosis of NC increased from median 1.4% (range 0.75–3%) to median 15% (range 6.2–34%). Thus, we have demonstrated that IgG autoantibodies are necessary for an efficient phagocytosis of NC by PMN and such antibodies are frequently found in serum samples from patients with SLE, especially when obtained at timepoints of active disease.

IgG-mediated phagocytosis of NC by PMN is dependent on the classical pathway of complement activation. The relative importance of the complement system in the PNC assay was

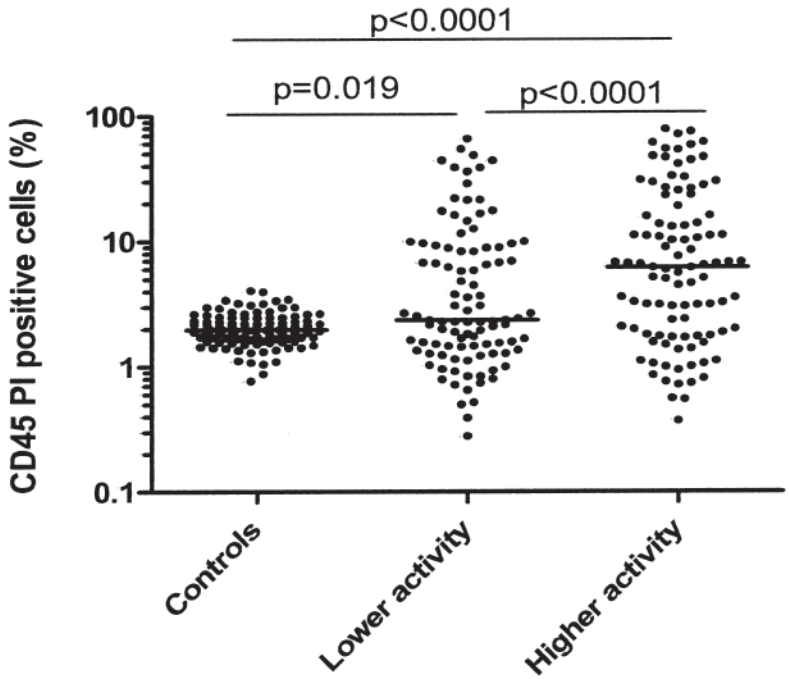

Figure 1. Results from the phagocytosis of necrotic material assay. Paired serum samples (n = 200) from 100 patients with SLE selected for timepoints of lower and higher disease activity and healthy control samples (n = 100) were used. A significantly increased capacity for phagocytosis of necrotic cell material (NC) by polymorphonuclear leukocytes (PMN) was seen when using sera sampled at timepoints of higher disease activity compared to timepoints of lower disease activity ($p < 0.0001$). At both timepoints, a significantly increased capacity for phagocytosis of NC by PMN compared to control was seen ($p < 0.0001$, $p = 0.019$, respectively). Lines represent median values. PI: propidium iodide.

significant because heat treatment (56°C, 30 min) of serum samples with known capacity to promote phagocytosis of NC

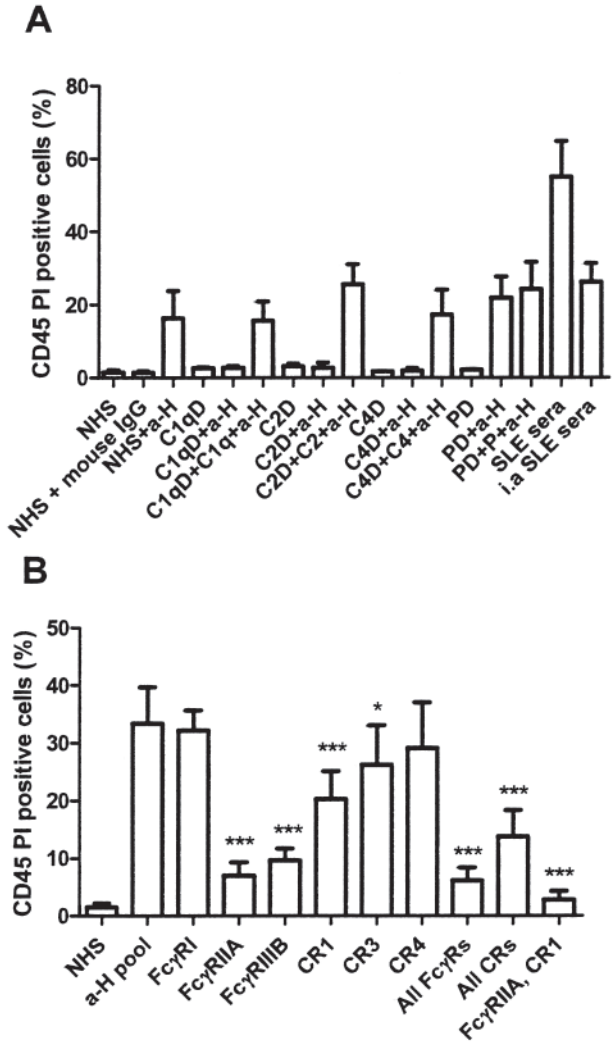

Figure 2. A. Serum samples from individuals deficient in the complement components C1q (C1qD), C2 (C2D), or C4 (C4D) showed decreased ability to phagocytose necrotic cell material (NC) in the presence of antihistone antibodies (a-H) when compared to normal human serum (NHS) with a-H added. Reconstitution of these complement-deficient sera by addition of the respectively missing complement protein restored the capacity for phagocytosis of NC by polymorphonuclear leukocytes (PMN), equaling that of NHS with a-H added. The serum deficient of properdin with a-H added showed equal phagocytosing capacity to NHS containing a-H. Addition of properdin did not increase the capability to phagocytose the NC. Untreated serum from 4 patients with SLE with known capacity to promote phagocytosis of NC, and the same samples with heat treatment (56°C, 30 min), showed at least a 50% reduction of phagocytosis of NC by PMN after the heat treatment. B. Phagocytosis of NC by PMN, incubated with antibodies against FcγR and CR prior to the addition of a-H pool containing NC. Phagocytosis of NC by PMN was significantly reduced (*$p \leq 0.05$, ***$p \leq 0.001$) when FcγRIIA, FcγRIIIB, CR1, or CR3 were blocked with monoclonal antibodies. Combinations of antibodies against FcγR, CR, or against both FcγRIIA and CR1 resulted in further reduction for phagocytosis of NC by PMN as compared to blocking of the different receptors separately. Normal mouse IgG was added to the PMN as a negative control and no effect was seen on the ability of the a-H pool to promote phagocytosis. Mean values and SD of 3 and 6 separate experiments, respectively, are shown. PI: propidium iodide.

by PMN resulted in at least a 50% reduction of NC phagocytosis (Figure 2A). To further clarify the importance of the complement system for an efficient phagocytosis of NC, we used serum samples from individuals with different complement deficiencies, and anti-H ab were added to promote phagocytosis of NC. Sera from individuals with deficiencies in the classical pathway (C1q, C2, or C4) all showed clearly decreased ability to support phagocytosis of NC by PMN even though anti-H ab were present (Figure 2A). After reconstitution of the sera with the missing complement protein, the phagocytosis of NC by PMN equaled that of NHS. PD-deficient serum showed an equal capacity to promote phagocytosis of NC as NHS, and no difference was seen after reconstitution (Figure 2A). To further evaluate the role of the alternative pathway, we used a C2-deficient serum at a concentration of 20% and 40%, allowing activation of the alternative pathway. No increased capacity for phagocytosis of NC by PMN could be seen with higher serum concentrations (data not shown). Thus, under these conditions, the antibody-mediated phagocytosis of NC by PMN is clearly dependent on the classical complement activation pathway but not on the alternative pathway.

Phagocytosis of NC by PMN is dependent on FcγRIIA, FcγRIIIB, and CR1 in combination. To investigate the involvement of different FcγR and CR in phagocytosis of NC by PMN, the ligand-binding sites of the receptors were blocked with antibodies. Preincubation of PMN from healthy individuals with antibodies against CD16 (FcγRIIIB), CD32 (FcγRIIA), CD35 (CR1), or CD11b/CD18 (CR3) all significantly reduced the capability for phagocytosis of NC (Figure 2B). A further reduction was seen when blocking all FcγR or all CR or when blocking FcγRIIA in combination with CR1. These results show the importance of FcγR and CR in autoantibody-mediated phagocytosis of NC by PMN and that there are synergistic effects when FcγR and CR are activated simultaneously.

Oxidative burst activity induced by PMN after phagocytosis of NC. We studied whether uptake of immune complexes composed of autoantibodies and NC in PMN induced oxidative burst activity. When using heterologous PMN from healthy individuals and serum from the 100 patients with SLE, a highly significant correlation was seen between phagocytosis of NC and oxidative burst activity ($r = 0.72$, $p < 0.0001$; Figure 3).

Oxidative burst activity in autologous PMN from patients with SLE. We also measured whether PMN from patients with SLE had equal capacity to engulf antibody-coated NC and induce oxidative burst activity as compared to PMN from healthy individuals. Oxidative burst activity could clearly be seen when anti-H ab pool and NC were added to the PMN, and no difference was seen in oxidative burst activity between PMN isolated from patients with SLE compared to controls (data not shown). When serum from patients with SLE was mixed with NC and incubated with autologous PMN, a correlation between oxidative burst activity and presence of anti-H ab was seen ($r = 0.47$, $p = 0.012$).

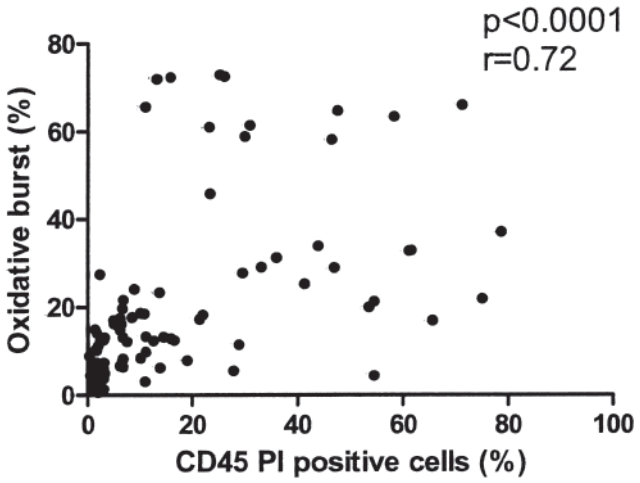

Figure 3. Correlation between phagocytosis of necrotic cell material by heterologous polymorphonuclear leukocytes and oxidative burst activity analyzed with serum samples from the 100 patients with SLE. PI: propidium iodide.

Antibodies against histone proteins display different specificity and correlate with phagocytosis of NC by PMN. We found antibodies against one or more histone proteins in 52 of the 100 patients. Antibodies against only histone H1 were found in 11 patients and the remaining 41 patients had antibodies to other histone proteins or histone proteins in combination (Table 3). Levels of autoantibodies against histone H1, H3, and of the mix of histone proteins were significantly higher when using sera sampled at timepoints of higher disease activity compared to lower disease activity ($p = 0.02$, $p = 0.009$, and $p = 0.0002$, respectively). Phagocytosis of NC by PMN correlated significantly to levels of anti-H ab and anti-H3 antibodies, and strongly with antibodies against the mix of different histone proteins ($r = 0.47$, $p < 0.0001$; $r = 0.41$, $p < 0.0001$; and $r = 0.70$, $p < 0.0001$, respectively). A highly significant correlation could also be seen between phagocytosis of NC by PMN and antibodies against DNA ($r = 0.76$, $p < 0.0001$). Thus, we found not only anti-H1 antibodies in sera from patients with SLE, but also antibodies against other histone proteins, and sera reactive against a broad spectrum of histones and DNA promote NC phagocytosis by PMN more efficiently. Further, anti-H ab proteins correlated to disease activity.

Table 3. Distribution of autoantibodies against different histone proteins found in sera from 100 patients with systemic lupus erythematosus (SLE). Antibody reactivity in the sera was determined by ELISA and immunoblot analysis.

Anti-histone Antibody Specificity	Patients with SLE, n = 100
H1, H3	12
H1	11
H3	5
H1, H2A, H3	5
H2B, H3	4
H1, H2B, H3	3
H1, H2B, H3, H4	3
H1, H3, H4	2
H4	1
H2A	1
H2A, H4	1
H2B, H3, H4	1
H2A, H2B, H3	1
H2A, H2B, H3, H4	1
H1, H2A, H2B, H3, H4	1
Negative	48

SLE serum containing antibodies against several different histone proteins promotes increased phagocytosis and complement consumption. As shown, the NC phagocytosis was clearly dependent on the complement classical pathway. Next, we wanted to know whether autoantibody reactivity against none, 1, or many histones was associated with a differential decrease in complement levels, and also with differences in NC phagocytosis and oxidative burst activity. The highest level in the PNC assay of each patient was used and grouped according to the specificity of the anti-H ab reactivity. Sera containing antibodies against more than one histone protein showed a significantly increased activity in the PNC assay compared to sera containing antibodies against only histone H1 or to serum with no detectable anti-H ab ($p = 0.0009$ and $p < 0.0001$, respectively; Figure 4A). Serum containing antibodies against only histone H1 also showed a significantly increased capacity for phagocytosis of NC, compared to serum with no detectable anti-H ab ($p = 0.001$; Figure 4A). A similar pattern could be seen when comparing the different combinations of anti-H ab and oxidative burst activity (Figure 4B). A significantly decreased concentration of the complement proteins C1q, C3, and C4 was seen in sera with a broad specificity of antibodies against different histone proteins compared to serum without detectable anti-H ab ($p = 0.005$, $p = 0.0003$, and $p = 0.002$, respectively; Figure 4C, 4D, and 4E). No differences between complement concentrations were seen when comparing sera containing only anti-H1 antibodies with serum without a detectable level of anti-H ab. Thus, autoantibodies against more than one histone protein are strongly associated with increased capacity to promote phagocytosis of NC and oxidative burst activity, and with decreased levels of classical pathway components, supporting the observation that this process involves complement activation resulting in complement consumption.

Clinical findings related to the specificity of anti-H ab. We found that the different specificity of the anti-H ab could be linked to different patterns of disease manifestations. Sera from patients with a history of nephritis or serositis supported increased phagocytosis of NC ($p < 0.0001$ and $p = 0.016$, respectively) compared to sera from patients without these manifestations (Table 2). In analogy with these observations, sera from patients with SLE containing antibodies with specificity toward many different histone proteins were significantly associated with nephritis and serositis ($p = 0.01$ and $p = 0.01$, respectively). A history of skin involvement was

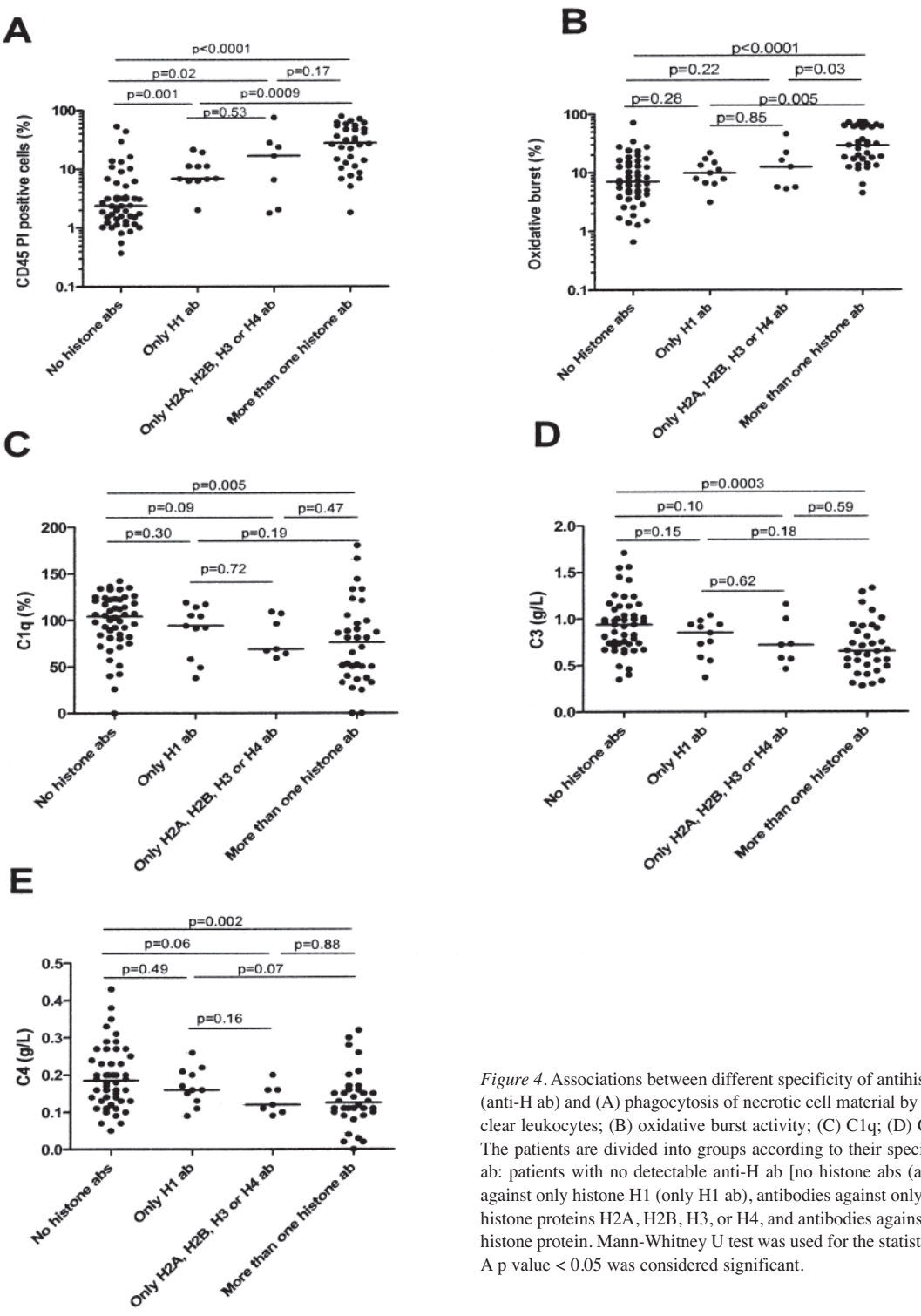

Figure 4. Associations between different specificity of antihistone antibodies (anti-H ab) and (A) phagocytosis of necrotic cell material by polymorphonuclear leukocytes; (B) oxidative burst activity; (C) C1q; (D) C3; and (E) C4. The patients are divided into groups according to their specificity of anti-H ab: patients with no detectable anti-H ab [no histone abs (antibodies)], abs against only histone H1 (only H1 ab), antibodies against only one of the core histone proteins H2A, H2B, H3, or H4, and antibodies against more than one histone protein. Mann-Whitney U test was used for the statistical calculation. A p value < 0.05 was considered significant.

inversely associated with a broad specificity of anti-H ab (Table 2). Altogether, autoantibody specificity against several different histone proteins was seen in patients with more severe SLE, especially nephritis, whereas patients with milder disease such as skin involvement had autoantibodies against fewer histone proteins.

DISCUSSION

We found that broad autoantibody specificity against histone proteins in sera from patients with SLE was associated with phagocytosis of NC material by PMN and that activation of the classical complement pathway contributed, together with FcγR and CR, to more efficient phagocytosis by PMN. A

broad specificity of antibodies against different histone proteins was also associated with complement consumption and more severe clinical manifestations such as serositis and glomerulonephritis. In contrast, sera from patients with milder disease, a phenotype predominated by skin involvement, had antibodies against only histone H1.

Our results confirm findings from several previous investigations that anti-H ab promote phagocytosis of NC by PMN, often referred to as the LE cell phenomenon[11,12,17,29,30,31]. It has been shown that the LE cell phenomenon is dependent on antibodies against dsDNA and that patients positive for LE cells have a more active disease[17,29]. It has also been suggested that histone H1 and not the core histones is the major autoantigen in patients with SLE, generating a T cell response with a proinflammatory Th1 phenotype that is not seen in patients with antibodies against histone H3 or histone H4[18]. However, in our study anti-H3, especially in combination with anti-H1, + H2A, H2B, or H4 antibodies, correlates more strongly with phagocytosis of NC by PMN. Further, in our cohort, sera from some patients with SLE supported high phagocytosis of NC by PMN without any detectable antibodies against histone H1. Most patients with serum antibodies to more than one histone protein were also positive for antibodies against dsDNA. In all, this suggests that autoantibodies against only histone H1 are of less importance. Instead, a broad diversity of antibodies against histone proteins, other nuclear proteins, and DNA are more important in facilitating phagocytosis by PMN.

In sera from SLE patients with antibodies against several histone proteins, low concentrations of classical pathway complement components were seen, most likely because of activation of the complement system. Possibly, the activation of the complement system seen in the patients with a broad histone autoantibody profile, sometimes in combination with antibodies against DNA, reflects a higher density of bound antibodies to the autoantigen. This would facilitate C1 binding, resulting in complement activation, complement consumption, and a more efficient phagocytosis. Inherited deficiencies of components in the classical pathway are strong risk factors for development of SLE[32]. It is well known that complement enhances antibody-mediated phagocytosis of NC by PMN, which has previously been shown by heat treatment of serum and by the use of EDTA to stop complement activation[11,29]. With our investigation, we add to this knowledge about the role of complement in phagocytosis of NC by PMN by using sera from patients with deficiencies of distinct complement proteins. We could demonstrate that the classical pathway was essential for enhancement of NC phagocytosis, and the results also indicated that the alternative pathway had no role in the system used. Consequently, decreased concentrations of the complement proteins in the classical pathway, which is frequently seen in active SLE, could contribute to a reduced phagocytosis of dead cells by the PMN. This could lead to reduced clearance of dying cells and prolonged exposure of such autoantigens to the immune system and eventually to formation of circulating immune complexes.

We found that both FcγR and CR1 are needed for phagocytosis of NC by PMN. Thus FcγR-mediated phagocytosis of antibody-coated nuclear material is not sufficient, but cooperative binding of complement fragments to CR1 seems necessary for efficient phagocytosis. Under normal conditions, nuclei of dead cells are enzymatically cleaved, but it has been suggested that the presence of antibodies against dsDNA could prevent cleavage and support uptake by circulating PMN to form LE cells that could lead to an inflammatory response[33,34]. It has been suggested that neutrophil extracellular traps (NET) are an important autoantigen source in SLE[34]. NET are released following PMN activation and are composed to a large part of histones, but it should be noted that histone H1 is not present in NET[35,36]. The correlation we found between oxidative burst activity and presence of anti-H ab suggests that circulating autoantibody-coated necrotic material could enhance activation of PMN and thereby the release of NET. We found that PMN from patients with SLE have equal capacity to induce degranulation and oxidative burst as PMN from healthy controls. In our observations, oxidative burst activity from PMN was thus related to autoantibodies and not to cellular factors. Immune complex-induced NETosis and subsequent degradation of the NET in the presence of autoantibodies against specific histone proteins would be of great interest to examine further because impaired degradation of NET has been shown in the presence of antibodies against DNA[34]. Our findings are also compatible with the concept that immune complexes composed of autoantibodies and NC induce degranulation and oxidative burst activity in PMN-generating reactive oxygen species that contribute to inflammatory clinical manifestations and organ damage.

Our investigation is based on *in vitro* analyses, but we found clear associations with clinical manifestations. The broad spectrum of autoantibody specificities against histone proteins and high levels of phagocytosis of NC by PMN were associated with high disease activity, which indicates that these antibodies might influence the disease process. Both serositis and glomerulonephritis were found in high frequency in patients with antibodies against combinations of different histone proteins, whereas SLE patients without anti-H ab or with antibodies directed only to histone H1 more often had milder disease, predominantly with involvement of the skin. We suggest that determination of an anti-H ab profile of patients with SLE could be important in assessing the disease phenotype and monitoring disease activity.

Thus, the presence of antibodies specific for various histone proteins in combination have a great influence on complement consumption because of classical pathway activation in PMN uptake of histone autoantibody-coated NC. This broad range of autoantibody specificity toward histone proteins was associated with a more severe disease progression,

possibly because of the activation of PMN-generating reactive oxygen species that could contribute to more severe clinical manifestations. Our findings confirm previous reports and suggest that measurement of a broad panel of anti-H ab could be useful in SLE as a marker for ongoing dysregulated handling of apoptotic and necrotic cells contributing to the autoimmune process in the disease.

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