IgG autoantibodies bound to surfaces of necrotic cells and complement C4 comprise the phagocytosis promoting activity for necrotic cells of systemic lupus erythaematosus sera

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ABSTRACT

Objective: Accumulation of dying and dead cells is thought to be involved in the etiopathogenesis of systemic lupus erythaematosus (SLE). Clearance has been described mainly for apoptotic cells; however, the knowledge of serum factors participating in the phagocytosis of necrotic cells is limited.

Patients and methods: Sera from 18 patients with SLE and 10 normal healthy donors (NHD), and macrophages from 3 NHD were included. Autoantibodies and complement were measured by ELISA and phagocytosis by flow cytometry. Binding of serum IgG to necrotic cells was assessed by flow cytometry and confocal microscopy.

Results: Sera from patients with SLE and NHD generally promoted the phagocytosis of necrotic cells by macrophages isolated from NHD. Five independent experiments with macrophages from three different NHD led to similar results. The sera from healthy controls displayed a homogeneous activity, whereas sera from patients with SLE showed a dichotomic behaviour. Only sera containing autoantibodies binding to the surfaces of necrotic cells and sufficient complement showed increased phagocytosis promoting activities. In SLE sera, C4 turned out to be the critical complement component in this process. Sera de-complemented by heat treatment strongly reduced phagocytosis of necrotic cells.

Conclusions: Serum components influence the uptake of necrotic cells by phagocytosis competent macrophages from NHD. Complement is required for this process and autoantibodies binding to the surfaces of necrotic cells additionally promote their phagocytosis.

Systemic lupus erythaematosus (SLE) is a chronic inflammatory autoimmune disease with a broad spectrum of symptoms and clinical manifestations. The presence of autoantibodies targeting double stranded DNA (dsDNA) and other nuclear constituents is a hallmark of SLE. The precise development of SLE is still unclear; however, the etiopathogenesis has been shown to be a multifactorial event.[1–3] Investigations on cellular and molecular level are providing growing evidence for the existence of a clearance deficiency that might lead to chronic autoimmunity.[4–7]

A well-organised phagocytic system has to ensure a correct discrimination between viable, apoptotic and necrotic cells (see review by Lauber *et al*).[8] Receptors on phagocytes and humoral factors are capable of modulating the phagocytic process. Complement factors, and particularly the component C1q, are supposed to play a weighty role herein[9 10] and are known to act as strong promoters of phagocytosis.[11 12] The pentraxins, C-reactive protein and serum amyloid P bind apoptotic cells, probably functioning as opsonins.[13 14] Immune complexes were also shown to bind to dying cells and immune complex-opsonised target cells positively influenced their uptake by macrophages.[15] Decreased uptake of dying cells was observed to be triggered by prostaglandins.[16] The existence of factors enhancing and repressing phagocytosis and the plethora of serum components involved indicate that clearance is controlled by a multitude of mechanisms reflecting complexity and importance of this process.

Most studies used apoptotic cells in phagocytosis experiments but less is known about the uptake of necrotic cells. Blood cells from patients with SLE were shown to have increased rates of apoptosis.[17–19] If these cells are not cleared in time they could accumulate and enter the secondary necrosis stage. Therefore, the occurrence of necrotic cells is of relevance for the pathogenesis of SLE (see review by Grossmayer *et al*).[20] In the present study we focused on the influence of serum on the phagocytosis by healthy macrophages of necrotic cells. Patient sera showed a much broader distribution with decreased or increased phagocytosis promoting activities when compared with sera of healthy controls. Phagocytosis was strongly dependent on heat labile serum components and the presence of autoantibodies.

PATIENTS AND METHODS

Patients

Routine serum diagnostics were carried out on 18 patients with SLE (2 male, 16 female) visiting our outpatient department. Simultaneously, serum was obtained from each patient for phagocytosis assays, for further determinations of autoantibody levels (Varelisa ReCombi ANA Profile, Pharmacia Diagnostics, Freiburg, Germany; Aeskulisa Phospholipid-8Pro-GM, Aesku Diagnostics, Wendelsheim, Germany) by ELISA, and for antibody binding assays. All patients fulfilled the American College of Rheumatology (ACR) 1997 revised criteria for the classification of SLE.[21] At the time of blood withdrawal 17 of 18 patients were receiving drug treatment including corticosteroids (n = 13; 2.5–30 mg/day prednisolone-equivalent),

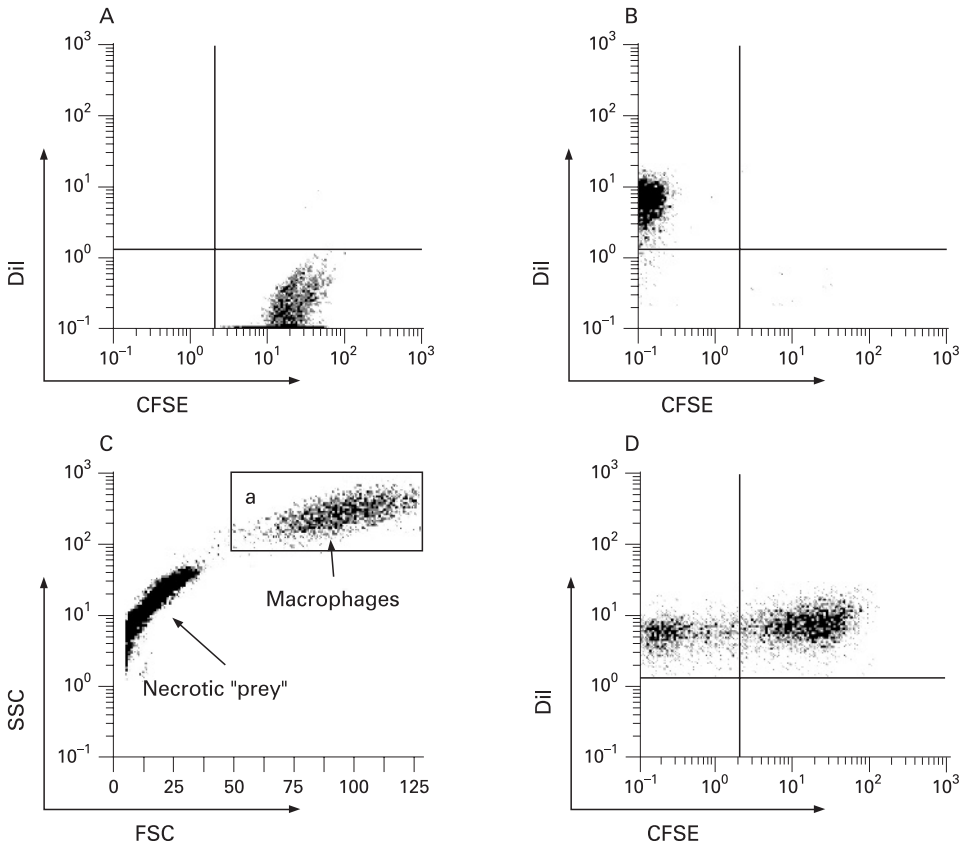

Figure 1 Necrotic Raji cell uptake by human monocyte-derived macrophages (HMDM) analysed by flow cytometry. Necrotic cells (A) were stained with carboxyfluorescein succinimidyl ester (CFSE)–diacetate and healthy HMDM (B) were labelled with the long-chain dialkylcarbocyanine membrane dye DiI. HMDM selected by size (gate a in C) and displaying positive staining for DiI were gated for analysis of phagocytosis. HMDM that had taken up necrotic cells became double positive for DiI and CFSE (D). FSC, forward scatter; SSC, side scatter.

hydroxychloroquine (n = 9), azathioprine (n = 7) and methotrexate (n = 5).

Serum samples

For the assays and measurements sera and heat-inactivated sera (IS; 56°C for 30 min) from 18 patients with SLE and from 10 normal healthy donors (NHD) were used. Heat treatment leads to inactivation of complement components concerning the classical and the alternative pathways of activation.[22] Serum aliquots were frozen on the day of blood withdrawal, stored at −80°C and thawed only once, immediately before each phagocytosis assay.

Necrotic Raji cells

To induce necrosis, viable Raji cells were incubated at 56°C for 30 min. Necrosis was assessed by propidium iodide staining in the absence of detergents. More than 90% of the cells were positive for propidium iodide.

Assessment of serum antibody binding to necrotic cells by flow cytometry

Serum from each patient and NHD was added to a suspension (RPMI 1640 medium, Gibco Invitrogen, Paisley, UK; supplemented with 10% fetal calf serum (FCS)) of necrotic Raji cells (final human serum concentration 5%) and incubated at 4°C for 1 h allowing serum antibodies to interact with the cells. The cells were washed after incubation. To detect bound serum antibodies we used a γ-chain specific anti-human IgG R-phycoerythrin conjugated antibody (Sigma, St Louis, Missouri, USA) and incubated it (1:100) with the cells at 4°C for 0.5 h. After washing the cells antibody binding was analysed by flow cytometry (Epics XL-MCL; Beckman Coulter, Fullerton, California, USA).

Assessment of serum antibody binding to necrotic cells by confocal microscopy

In addition to the staining of bound antibodies to necrotic Raji cells described above the following stains were employed. The cell membrane was visualised by the lipophilic tracer DiO (Molecular Probes, Eugene, Oregon, USA) and double stranded nucleic acids were detected by staining with To-Pro-3 iodide (Invitrogen). Confocal microscopy was performed employing a Leica DMR confocal microscope (Leica, Wetzlar, Germany), using software V.2.00.

In vitro differentiation of human monocyte-derived macrophages (HMDM)

Three healthy volunteers with the blood group O served as donors for HMDM. Peripheral blood mononuclear cells (PBMC) were isolated from venous heparinised blood by Ficoll density-gradient centrifugation (Lymphoflot; Biotest, Dreieich, Germany). Depletion of platelets was achieved by density-gradient centrifugation through a cushion of FCS. Monocytes were isolated from PBMC by magnetic cell sorting (human CD14 MicroBeads and autoMACS; Miltenyi Biotec, Aubum, Georgia, USA) and stained with the long-chain dialkylcarbocyanine membrane dye DiI (Molecular Probes). To obtain HMDM cells were cultured for 7 days at 37°C in a humidified atmosphere containing 5.5% CO_2 in RPMI 1640 medium supplemented with 10% autologous serum, 1% L-glutamine (Gibco Invitrogen), 1% *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Merck, Darmstadt, Germany), 1% penicillin-streptomycin (Gibco Invitrogen) and 10 ng/ml of

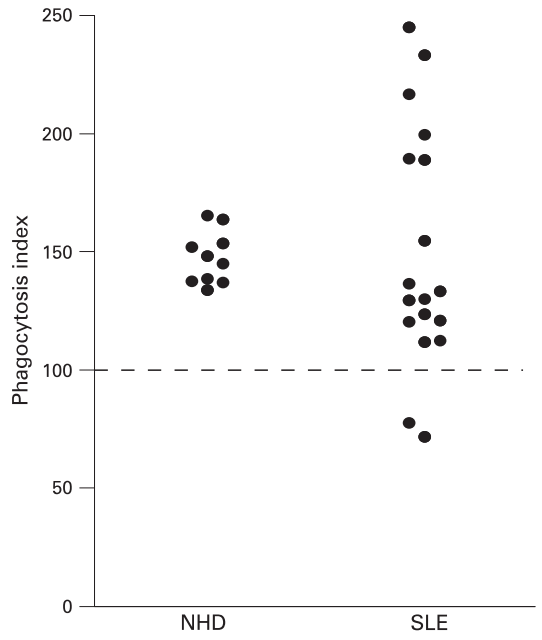

Figure 2 The phagocytosis promoting activity of sera from patients with systemic lupus erythaematosus (SLE) shows a dichotomic behaviour. The general phagocytosis index (PIdx) as average value of five independent experiments each carried out in duplicate is shown for each serum. Necrotic Raji cells and individual sera were added together to allogeneic human monocyte-derived macrophages (HMDM) from healthy individuals. Phagocytosis was analysed by flow cytometry after coculture at 37°C for 1 h. All normal healthy donor (NHD) sera and 16 of 18 sera of patients with SLE increased phagocytosis related to controls containing 10% fetal calf serum (PIdx = 100) only. Of the SLE sera, 6 enhanced phagocytosis even better than NHD sera, and most of the remaining 12 patient sera led to a weaker increase of phagocytosis in comparison to NHD sera.

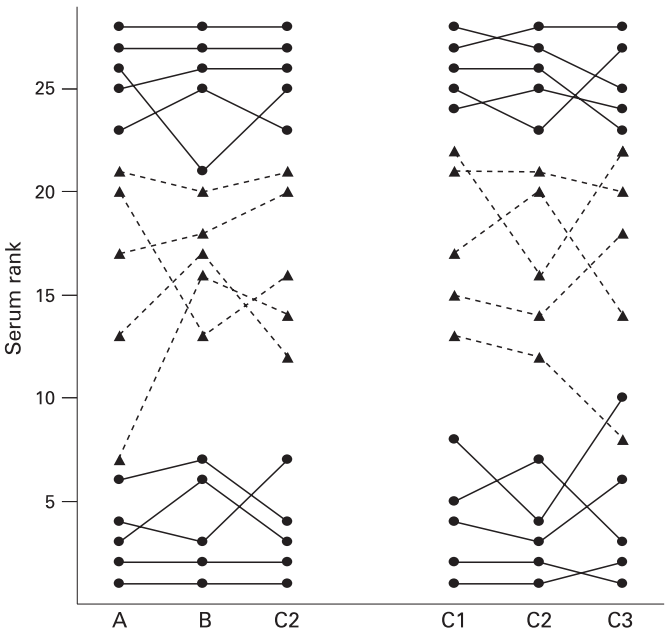

Figure 3 The rankings of sera within all phagocytosis assays remain stable. A serum ranking was created for each experiment on the basis of individual phagocytosis indices (PIdx; 28 = highest individual PIdx, 1 = lowest individual PIdx). Sera from patients with systemic lupus erythaematosus (SLE, solid lines) bearing the five highest and five lowest PIdx and five exemplarily chosen sera from normal healthy donors (NHD, dashed lines) are shown. Assays were performed with human monocyte-derived macrophages (HMDM) from three different healthy donors (A, B, C) and three independent experiments were carried out with HMDM from donor C (C1–C3). The sera generally maintained their ranking positions. Sera in the upper and lower extreme positions showed the least variability.

granulocyte-macrophage colony-stimulating factor (GM-CSF; Behringwerke, Marburg, Germany). The cell numbers per well amounted to 50 000. On day 3 and 5 of cultivation the same medium was added to the cultures.

Phagocytosis assay and flow cytometry

The phagocytosis assays were performed using HMDM stained with DiI and necrotic Raji cells labelled with carboxyfluorescein succinimidyl ester (CFSE)–diacetate, serving as "prey" (Molecular Probes, Leiden, The Netherlands). The latter were stained 1 day before the phagocytosis assay and cultured at 37°C in 5.5% CO_2 in medium (see above) without GM-CSF and with 10% FCS instead of human serum. Directly before the phagocytosis experiments cultured CFSE-stained Raji cells were treated with heat (see above) to induce necrosis. Each serum sample was added, together with 100 000 necrotic Raji cells, to 7-day-old HMDM. This was determined to be the optimal necrotic cell number per well in pilot experiments. After 1 h of incubation at 37°C in 5.5% CO_2 in medium (see above) supplemented with 10% FCS only or additionally with 10% SLE or NHD serum the phagocytosis was stopped by placing the culture plates on ice. Adherent HMDM were detached by rinsing with cold phosphate buffered saline (PBS; Gibco Invitrogen, Karlsruhe, Germany) containing 2 mM ethylenediamine tetraacetic acid (EDTA; Merck) and analysed by flow cytometry. HMDM from 1 NHD were used to perform 120 tests in parallel. To monitor the uptake of necrotic cells by HMDM, we used two-colour flow cytometry. HMDM were identified by size using forward scatter/side scatter analysis (fig 1C) and by positive staining for DiI. HMDM that took up necrotic cells were double positive for DiI and CFSE. The internalisation of the necrotic material was confirmed by confocal laser microscopy.

Phagocytosis was monitored in five independent experiments each performed in duplicate. The assays were performed with HMDM from three different NHD. HMDM from one NHD selected by chance were used for three separate and independent experiments. A product was calculated by multiplying the percentage of double-positive HMDM with the mean CFSE fluorescence intensity (amount of phagocytosed material) of double-positive HMDM. To ensure comparability of results in the assays this product was standardised (mean value of human serum free controls containing only 10% FCS was set to 100) and defined as phagocytosis index of one individual experiment (individual PIdx). The mean value of the individual PIdx of the five assays was defined as the general phagocytosis index (PIdx).

Statistical analysis

The heteroskedastic two-tailed Student t test for unpaired data was applied for comparisons of the patient and NHD cohort (Excel, Microsoft, Redmond, Washington, USA). For analyses of correlations we used the Pearson correlation test (SPSS V. 14.0, SPSS, Chicago, Illinois, USA). Levels of $p<0.05$ were considered as statistically significant.

RESULTS

Quantitative determination of phagocytosis of necrotic cells by HMDM

We established a phagocytosis assay based on flow cytometry allowing the quantification of necrotic cell uptake by HMDM. Raji cells were stained with CFSE and incubated at 56°C for 30 min to induce necrosis. The CFSE-labelled necrotic cells (fig 1A) could clearly be distinguished from HMDM stained with DiI (fig 1B). HMDM displayed higher forward scatter/side scatter properties (gate a in fig 1C) in comparison to the necrotic "prey". Only DiI-stained HMDM were included in the evaluation. HMDM that had taken up necrotic cells were positive for DiI and CFSE (fig 1D). This uptake was further confirmed by confocal microscopy (not shown).

Sera generally increase phagocytosis of necrotic cells

The impact of sera on the uptake of necrotic cells by HMDM from NHD was analysed in phagocytosis assays. The PIdx for each SLE and NHD serum is shown in fig 2. All NHD sera and 16 out of 18 sera from patients with SLE increased the phagocytosis of necrotic cells. Two sera decreased the uptake of necrotic cells to 72% and 78% of the FCS control (PIdx = 100), respectively.

The phagocytosis promoting activity of NHD sera is homogenous, that of SLE sera shows a dichotomic distribution

The PIdx of NHD sera are homogeneous without outliers (fig 2). By contrast, the PIdx of SLE sera are widely scattered over a much greater range than NHD sera. The majority of patients' sera augmented the uptake activity weaker than those of the NHD cohort. Surprisingly, some patients' sera greatly enhanced necrotic cell uptake—even better than all NHD sera samples.

The influence of serum on phagocytosis is independent of the monocyte donor

To answer the question if the donor of monocytes has an effect on phagocytic activity, HMDM from three different NHD were used in the assays. In total, five assays were performed and monocytes from one NHD were isolated on three separate days and used in independent assays. Phagocytosis took place in the presence of serum from patients or NHD. To compare the results of all experiments a serum ranking was created for every experiment based on the individual PIdx. Rankings (28 = highest individual PIdx, 1 = lowest individual PIdx) of SLE sera with the five highest and five lowest PIdx and five exemplarily chosen NHD sera are illustrated in fig 3 for each of the five experiments. Three independent assays performed with the same (C1–C3) and three different monocyte donors (A, B, C2) are shown on the right and left panel, respectively. Across all experiments, sera underwent only a slight change of position and generally retained their ranking. This was particularly true for sera in the upper and lower extreme positions. The midfield was mainly represented by sera from NHD. Since the differences of the individual PIdx are scarce in this range the sera showed some variability in their rankings. In the experiments performed three times with HMDM from the same donor rankings were only weakly altered. This underlines the stability of the assay. A major influence on our phagocytosis assay by the monocyte donors was not observed, since HMDM from diverse donors showed similar ranks for individual sera. Instead, the impact of serum factors has to be considered.

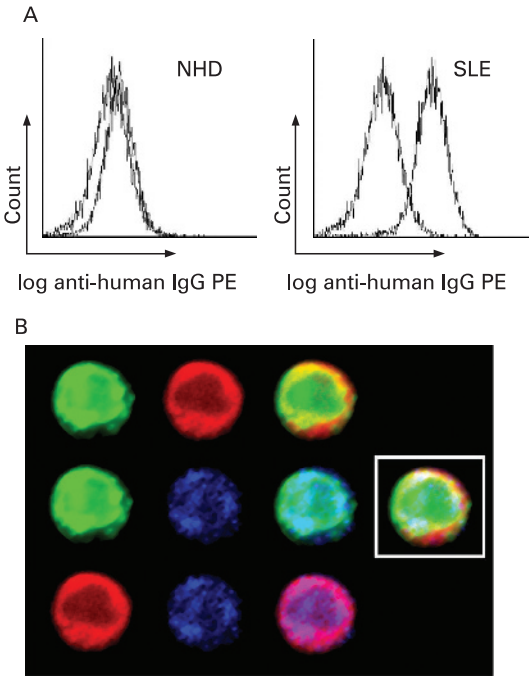

Figure 4 Serum IgG binds to the surfaces of necrotic cells. Necrotic Raji cells were incubated with serum and bound IgG were detected by indirect immunofluorescence. A. The phycoerythrin (PE) fluorescence of necrotic cells after incubation with sera of a representative healthy donor (NHD) and of a patient with systemic lupus erythaematosus (SLE) in comparison to the human serum free control is displayed. SLE sera showed a higher binding of serum IgG to necrotic cells than NHD sera. B. Bound serum IgG antibodies (red), cell membrane (green) and double stranded nucleic acids (blue) were visualised by confocal microscopy. Single stainings are shown in the first and second column, superimpositions of those stainings in the third column, and the superimposition of all stainings within the white frame. Binding of serum IgG was detected on the cell membrane and in cytoplasmatic near-membrane areas (first line). Double stranded nucleic acids were distributed over near-membrane and membrane areas and also in the central cytoplasm (second line). Binding of serum IgG was partially colocalised with double stranded nucleic acids in the membrane regions of necrotic cells (third line).

Patients' serum IgG bind to the surfaces of necrotic cells

Next, we searched for an explanation for the inhomogeneous distribution of the phagocytosis promoting activities of the SLE sera. We wondered if autoantibodies present in the sera of patients contributed to activity. First we examined if autoantibodies bind to necrotic cells. Necrotic cells were incubated with heat-inactivated serum and bound IgG was detected by indirect immunofluorescence. Quantitative analysis by flow cytometry revealed a twofold higher binding of IgG to necrotic cells for sera of patients with SLE (mean fluorescence 7.9) when compared to those of NHD (mean fluorescence 3.9; $p = 0.011$). Figure 4A shows data for one NHD and one SLE serum in comparison to the human serum free control. Next, we examined if autoantibodies bound to the surfaces of necrotic cells and were, therefore, potentially accessible for phagocytes. To clarify this we stained necrotic cells with autoantibodies by indirect immunofluorescence (red), DiO (membrane; green) and To-Pro (dsDNA; blue). The images taken via confocal microscopy are shown in fig 4B. There was a detectable binding of serum IgG on the cell membrane and to cytoplasmatic near-membrane areas (fig 4B, first row). dsDNA was also distributed in membrane areas (second row). Moreover, binding of serum

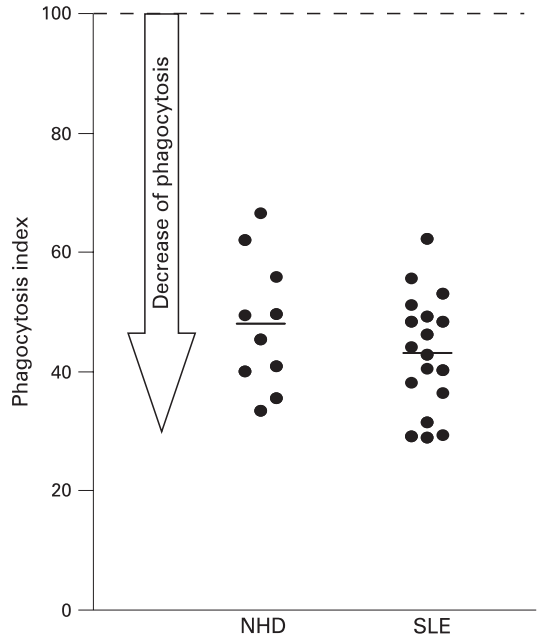

Figure 5 Heat-inactivated serum reduces phagocytosis of necrotic cells by human monocyte-derived macrophages (HMDM) from healthy donors. The phagocytosis index (PIdx) as average value of five independent experiments each carried out in duplicate is shown. Necrotic Raji cells and individual sera were added together to allogeneic HMDM. Phagocytosis was analysed by flow cytometry after coculture at 37°C for 1 h. The presence of heat-inactivated serum from normal healthy donors (NHD) or patients with systemic lupus erythaematosus (SLE) reduced the mean PIdx in comparison to controls containing 10% fetal calf serum only (PIdx set to 100; dashed line) to 48 and 43, respectively. Note that no significant difference in the PIdx of heat-inactivated NHD and SLE sera was observed ($p = 0.261$). Bars = mean.

IgG was partially colocalised with dsDNA in membrane regions of necrotic cells (third row). However, the binding of serum IgG to necrotic cells showed no significant correlation with the PIdx ($p = 0.684$). One may conclude that autoantibodies binding to the surfaces of necrotic cells are involved in phagocytosis but require cofactors to determine the PIdx of individual sera.

Heat inactivation of sera strongly reduces their phagocytosis promoting activities

To evaluate the influence of heat labile serum components on the phagocytosis of necrotic cells we compared the PIdx in the presence or absence of heat-inactivated human serum (IS; fig 5). All IS led to a notably reduction of necrotic cell uptake in comparison to 10% FCS only (PIdx set to 100). The mean (SD) PIdx of sera of NHD and patients with SLE was 48 (11) and 43 (10), respectively. The decline of phagocytosis was similar in the presence of sera from NHD or patients with SLE ($p = 0.261$).

Complement and autoantibodies synergise in promoting phagocytosis of necrotic cells

The observation that IS inhibits the phagocytosis of necrotic cells points to the involvement of complement in this process. Complement levels ($n = 16$) in the sera of our patient cohort were as follows: 8 patients had decreased levels of C3 and 13 patients decreased levels of C4 (normal range 16–47 mg/dl). The sera that led to a decreased uptake of necrotic cells had the lowest C4 concentrations (2.6 and 2.4 mg/dl, respectively). The

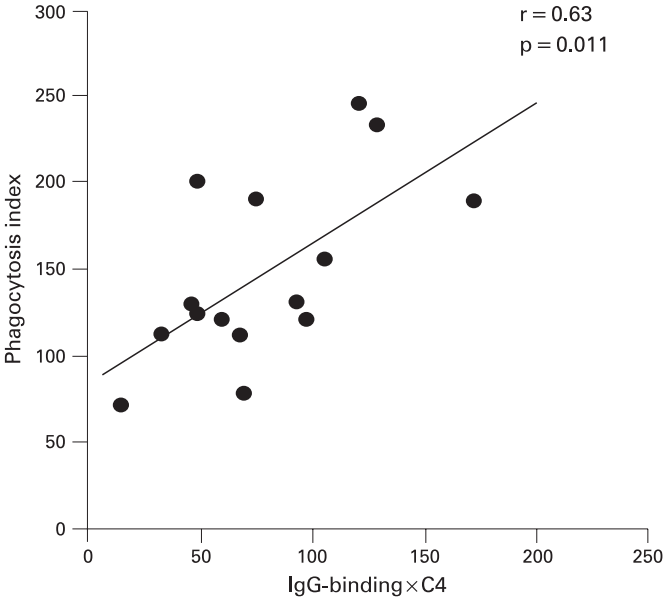

Figure 6 Correlation between phagocytosis of necrotic cells and the combination of C4 levels and serum IgG binding to necrotic cells ($n = 15$). The correlation was calculated by Pearson correlation test.

average C4 level of the other sera was 13.5 mg/dl. Moreover these two sera have been drawn from patients with the highest European Consensus Lupus Activity Measurement (ECLAM) scores (five) of the cohort. Nevertheless, overall, neither the ECLAM nor C4 complement levels significantly correlated with the PIdx.

When we combined the autoantibody binding and the C4 levels the resulting value significantly correlated with the PIdx of our sera (fig 6; $r = 0.63$, $p = 0.011$). We conclude that two of the factors determining the phagocytosis promoting activity for necrotic cells are represented by autoantibodies binding to their surfaces and by complement C4.

DISCUSSION

The clearance failure of apoptotic cells leads to the accumulation of secondarily necrotic cells, a feature that is observed in a subgroup of patients with SLE. We wanted to study the mechanism of the clearance of these necrotic cells and focused on the involvement of serum factors in this process.

Phagocytosis is a multifactorial process controlled by a plethora of serum constituents including pathological factors. The central role of complement for the efficient uptake of apoptotic and necrotic cells has previously been described.[23–25] It is well known that complement levels are often low in patients with SLE and genetic deficiencies of complement components are highly correlated with the occurrence of this disease.[6 26–28] In concordance with these facts the majority of our patients had decreased levels of C4. We had previously shown[24] that C1q markedly contributes to the clearance of necrotic cells by professional phagocytes. We demonstrated in our previous work that adding physiological concentrations (25 μg/ml) of heat labile C1q to serum-free medium or heat-inactivated autologous serum led to a slight increase of phagocytosis of necrotic cells by macrophages. However, reconstitution of C1q depleted as well as C1q deficient serum with C1q significantly elevated the phagocytosis of necrotic cells, indicating that other heat labile serum components, such as other complement proteins, may

also be involved in the C1q-mediated uptake process of primary necrotic cells by macrophages. Complement components such as C1q, C3 and C4[10] and the prototypic long pentraxin PTX3[29] are important innate opsonins. Very interestingly, PTX3 decreases the C1q deposition and subsequent complement activation on dying cells.[30 31] However, natural IgM-mediated opsonisation of apoptotic cells with complement accelerates their clearance by phagocytes[32] highlighting that a plethora of serum components are directly or indirectly involved in the clearance of apoptotic as well as necrotic cells.

It was also shown that the presence of pathological factors like increased levels of autoantibodies affects phagocytosis activity. To date, over 100 autoantibodies have been described for SLE, each with a differing pathogenicity and prevalence.[33] In particular, autoantibodies directed against nuclear constituents and phospholipids were reported to be competent in enhancing the phagocytic process.[34–37] Of our patients, 78% were positive for at least three anti-nuclear antibodies (ANA) and 50% for at least three anti-phospholipid antibodies. We also performed experiments with sera of patients with rheumatoid arthritis (RA). These sera had normal levels of complement and were ANA negative or had only very low levels of ANA. The uptake by HMDM of primary necrotic cells in the presence of sera from patients with RA was comparable to that of NHD (data not shown), indicating that the wide distribution of phagocytosis promoting serum activities is specific for SLE. Analysing possible effects of immunosuppressive therapy, drug dosage, age at diagnosis, disease duration and disease activity revealed no significant correlation to the phagocytosis index and no significant differences between the sera increasing phagocytosis and the sera decreasing phagocytosis (not shown).

Here, we demonstrate that IgG antibodies of SLE serum bind to the surfaces of necrotic cells and are accessible for phagocytes. These opsonising autoantibodies are prone to enhance the phagocytic process. If these autoantibodies are the exclusive factor determining the phagocytosis promoting activity, both parameters would correlate—but they did not. Since heat-inactivated serum (56°C) did not support phagocytosis of necrotic cells, we tested whether complement is a necessary cofactor for this process. However, neither C3 nor C4 serum levels correlated significantly with the PIdx. Complement and autoantibodies mutually depend on each other to efficiently opsonise their targets. Therefore, we calculated the combined influence on the PIdx of autoantibodies and C4 and found a significant correlation. The presence of complement is required for an efficient uptake process and to enable autoantibodies to exert their phagocytosis promoting activity.

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IgG autoantibodies bound to surfaces of necrotic cells and complement C4 comprise the phagocytosis promoting activity for necrotic cells of systemic lupus erythaematosus sera

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