Clin Exp Immunol 1993; 93:237–241

Clustering of neutrophil leucocytes in serum: possible role of C1q-containing immune complexes

G. STURFELT, H. JONSSON, G. HELLMER & A.G. SJÖHOLM* *Departments of Rheumatology and* **Medical Microbiology, University Hospital of Lund, Lund, Sweden*

(Accepted for publication 27 April 1993)

SUMMARY

Clustering activity for neutrophil granulocytes was generated in pooled normal human serum (NHS) by incubation of the serum with preformed IgG aggregates, but not in heat-treated NHS (56°C, 30 min), indicating that the function was complement-dependent. Judging from results of experiments with complement-deficient sera, and serum depleted of C1q, factor D and properdin, recruitment of the complement system beyond C1 was not required for induction of the activity. Zymosan treatment of NHS resulted in some neutrophil clustering activity, but recombinant C5a had a limited effect. C1q added to heat-treated NHS in conjunction with preformed IgG aggregates supported neutrophil clustering in a dose-dependent manner. The serum C1q inhibitor, a chondroitin 4-sulphate proteoglycan known to interact with the collagenous part of C1q, clearly reduced neutrophil clustering in heat-treated NHS supplemented with C1q and IgG aggregates. The C1q inhibitor also reduced the inherent neutrophil clustering activity of some sera from patients with systemic lupus erythematosus (SLE). Neutrophil clustering activity in SLE serum was earlier shown to be inversely related to the number of circulating neutrophils *in vivo*. Although the precise mechanisms remain unclear, we propose that C1q-containing immunoglobulin complexes mediate neutrophil clustering through C1q receptors, and that this might contribute to pathogenesis of immune complex diseases such as SLE.

Keywords neutrophil granulocytes neutrophil aggregation complement component C1q immune complexes systemic lupus erythematosus

INTRODUCTION

Intravascular formation of neutrophil granulocyte aggregates is thought to be of pathogenetic significance in systemic lupus erythematosus (SLE), particularly with regard to development of central nervous system and pulmonary disease manifestations [1–3].

We have described a simple assay for *in vitro* assessment of neutrophil clustering or aggregation in serum [4]. Sera from patients with severe SLE gave increased clustering of neutrophils from healthy donors. The clustering activity varied with the clinical course of the disease and was inversely related to the number of circulating neutrophils in the patients. C1q-binding immune complexes and neutrophil clustering activity were correlated, and clustering activity could be generated in normal serum with preformed IgG aggregates, indicating that immune complexes in conjunction with complement could be responsible.

The purpose of the present study was to identify possible complement-related ligands for induction of neutrophil clustering in human serum with preformed IgG aggregates. C1 subcomponents together with C4 and C3 proteins are known to be fixed to complement-activating immunoglobulin complexes [5], and contribution of C5-derived peptides generated in the course of complement activation [6] was also considered. The results suggested that C1q-containing immunoglobulin complexes were critically involved, and that further recruitment of the complement system was not required in the reaction.

Correspondence: G. Sturfelt, MD, PhD, Department of Rheumatology, University Hospital, S-22185 Lund, Sweden.

MATERIALS AND METHODS

Human sera

Sera were frozen in aliquots at −70°C within 4 h after blood sampling. Pooled normal human serum (NHS) from 20 healthy hospital staff members was used as a reference in all experiments. Sera from persons with homozygous deficiency of C1q, C4, C2, C5 or C7 were available in the laboratory. For some experiments, normal serum from one donor was depleted of C1q, factor D and properdin as described by Sjöholm *et al.* [7]. Where indicated, NHS was inactivated by heat treatment (56°C, 30 min). Zymosan treatment of serum was performed as described previously [8]. Sera with high granulocyte clustering

activity from five patients with definite SLE [4] were also investigated. The patients fulfilled four or more of the ACR classification criteria for SLE [9]. Disease activity was estimated by SLEDAI scoring [10].

Complement components

C1q was purified according to Tenner *et al.* [11]. To remove possible aggregates, C1q preparations were consistently subjected to gel filtration on a TSK-4000 column (LKB, Bromma, Sweden) in an FPLC system (Pharmacia, Uppsala, Sweden). After gel filtration, C1q preparations were used within 2 days. Factor D and properdin were purified according to published methods [12,13]. Concentrations of the purified complement proteins were determined by electroimmunoassay, assuming a reference normal serum pool to contain C1q at 70 mg/*l*, properdin at 25 mg/*l* and factor D at 1 mg/*l* [6]. C1q inhibitor, a chondroitin-4 sulphate proteoglycan, was purified from 1·5 ml of outdated ACD plasma essentially as described by Silvestri *et al.* [14]. In brief, C1q inhibitor in complex with C1q was isolated from plasma by repeated euglobulin precipitation, and was then separated from C1q by chromatography on a 1·6 × 26 cm column of concanavalin A (Con A) Sepharose (Pharmacia) to which C1q was quantitatively bound [14]. The C1q inhibitor recovered in the effluent was dialysed overnight against 20 volumes of Tris 0·02 mol/*l*, NaCl 0·2 mol/*l*, pH 7·5, and was applied to a column of DEAE-Sepharose (Pharmacia) equilibrated in the same buffer. The inhibitor was eluted with a sharp NaCl gradient to 1 mol/*l*. C1q inhibitory activity was detected by haemolytic assay [15] and by single diffusion precipitation of purified C1q in agarose [14]. The sulphated glycosaminoglycan content of the C1q inhibitor preparation (0·92 mg/ml, total yield 2·8 mg) was kindly determined by Dr S. Björnsson (Lund, Sweden) according to a recently described procedure [16].

Other reagents

Commercial polyclonal IgG (Kabi, Stockholm, Sweden) at 10 g/*l* was aggregated by heat (63°C, 30 min). After treatment, the heat-aggregated IgG (HAIG) was centrifuged at 2000 *g* for 30 min, the supernatant being used for addition to serum. Recombinant human C5a was purchased from Sigma Chemical Co. (St Louis, MO).

Assay for measurement of neutrophil clustering

The assay for *in vitro* measurement of neutrophil clustering has been described in detail [4]. In short, neutrophils (0·01 ml, 10^7 neutrophils/ml) from healthy donors were added to 0·04 ml of test serum, incubated at room temperature for 1 min, whereafter single and clustered cells were counted within a standardized area. In each of duplicate preparations 200 single cells were counted. Clustering activity was expressed as the number of clustered cells in per cent of the total number of counted cells.

To generate neutrophil clustering activity, the sera were incubated for 60 min at 37°C with HAIG at a final concentration of 1 g/*l*. In reconstitution experiments, purified complement proteins were first added to serum depleted of C1q, factor D and properdin, or to heat-treated NHS at physiological or specified concentrations. Sera from patients with SLE were used without pretreatment with HAIG. C1q inhibitor at specified concentrations was added to HAIG-treated sera or to SLE sera, followed by incubation at 37°C for 10 min before the addition of cells.

Table 1. Neutrophil clustering (%) in normal and complement-deficient sera with and without treatment with heat-aggregated IgG (HAIG)

	Untreated serum	Serum treated with HAIG
Normal serum	5	26
C2-deficient serum		
I	7	36
II	10	35
III	12	54
C4-deficient serum	ND	51
Hereditary angio-oedema serum	12	26
Properdin-deficient serum	ND	32
C3-deficient serum*	10	32
C5-deficient serum	3	31
C7-deficient serum		
I	13	42
II	5	38
III	9	28
C8-deficient serum	25	43

* Acquired C3 deficiency in a patient with C3 nephritic factor.

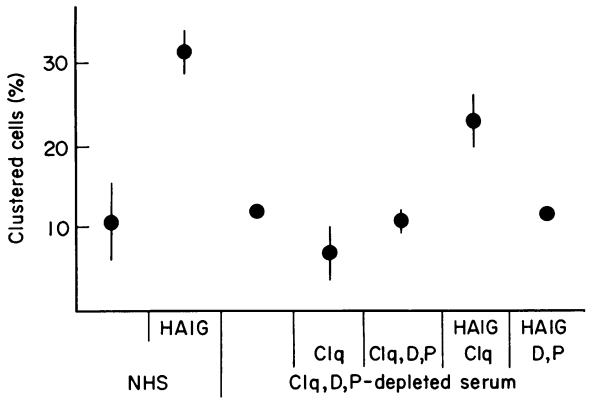

Fig. 1. Induction of neutrophil clustering with heat-aggregated IgG (HAIG) at 1 g/*l* in pooled normal human serum (NHS) and in normal serum depleted of C1q, factor D and properdin (C1q,D,P-depleted serum). Purified complement proteins were added to the depleted serum at physiological concentrations as indicated below the figure. Results of two separate experiments are shown. Neutrophil clustering activity is given as mean value and range.

RESULTS

Neutrophil clustering in complement-deficient and complement-depleted sera

In NHS the proportion of clustered cells varied between 5% and 25% of the total number of counted cells. In NHS treated with HAIG the number of clustered cells was consistently increased, and varied between 21% and 48%.

Addition of HAIG clearly promoted neutrophil clustering in sera from persons with homozygous deficiencies of C2, C5, C7 or C8 (Table 1). Similar findings were made in serum from a patient with hereditary angio-oedema and in serum from a

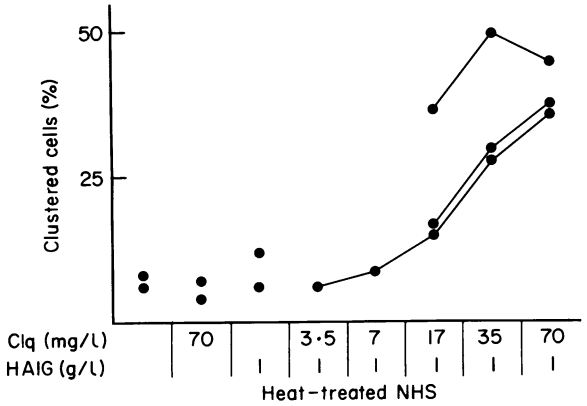

Fig. 2. Clq-dependent induction of neutrophil clustering activity in heat-treated (56°C, 30 min) pooled normal human serum (NHS). Clq and heat-aggregated IgG (HAIG) were added at the concentrations indicated below the figure. Results of three separate experiments are shown.

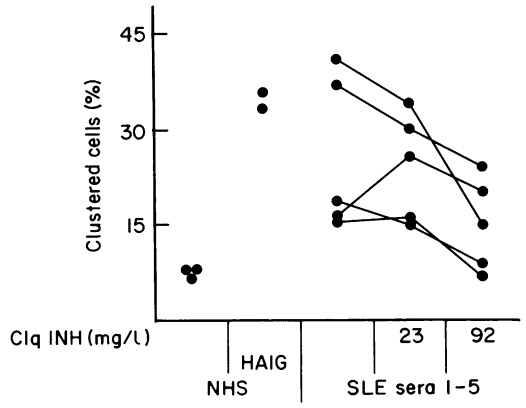

Fig 4. Influence of Clq inhibitor (Clq INH) on neutrophil clustering in sera from five patients with active systemic lupus erythematosus (SLE) (SLE sera 1–5). Clq INH was added at the concentrations indicated below the figure. Results of included control experiments with pooled normal human serum (NHS) and heat-aggregated IgG (HAIG, 1 g/*l*) are shown to the left.

patient with acquired C3 deficiency. In addition, high neutrophil clustering activity was found in C4-deficient serum and properdin-deficient serum treated with HAIG.

Further experiments were carried out with normal serum artificially depleted of Clq, factor D and properdin (Fig. 1). The depleted serum did not support enhanced neutrophil clustering activity in the presence of HAIG. However, reconstitution with Clq, but not with factor D and properdin before addition of HAIG, produced a marked response with regard to neutrophil-clustering activity.

The results of experiments with complement-deficient and complement-depleted sera suggested that neutrophil clustering induced with HAIG required Clq or the C1 complex, and that further recruitment of the complement system was not necessary. In particular, the findings with C5-deficient serum argued against an important role of C5-derived peptides (C5a and $C5a_{des\ arg}$) in the assay. Zymosan-treated serum, known to be a reliable source of C5-derived peptides [8], produced slightly less neutrophil clustering than normal serum activated with HAIG. Addition of recombinant C5a at a final concentration of 1–10 mg/*l* to normal serum resulted in very little neutrophil-clustering activity.

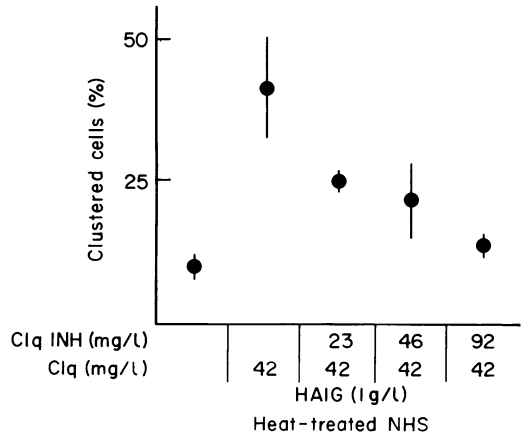

Fig 3. Influence of Clq inhibitor (Clq INH) on neutrophil clustering in heat-treated (56°C, 30 min) pooled normal human serum (NHS) after incubation of the serum with Clq and heat-aggregated IgG (HAIG). Final concentrations of Clq INH, Clq and HAIG are given below the figure. Results of two separate experiments are shown. Neutrophil clustering activity is given as mean value and range.

Neutrophil-clustering activity in heat-treated serum: effect of purified Clq

Background clustering in heat-treated NHS was similar to or less than that observed in NHS. Addition of HAIG to heat-treated NHS did not increase neutrophil clustering, nor did addition of Clq alone. When heat-treated NHS was reconstituted with Clq before addition of HAIG, Clq produced a dose-dependent increase of neutrophil-clustering activity in the serum (Fig. 2).

Inhibition experiments

In heat-treated NHS with Clq and aggregated IgG added, purified Clq inhibitor reduced neutrophil clustering in a dose-dependent manner (Fig. 3). Inhibitory doses were in the range 23–92 mg/*l*. The normal concentration of Clq inhibitor in serum is not known, but could be in the order of 20 mg/*l* assuming a 10% yield during the preparation procedure. This implies that the inhibitory doses were well beyond the normal concentration of Clq inhibitor.

Serum from five patients with active disease (SLEDAI scores 10–24) caused clustering from 17 to 43% of the total cell count. The morphology of cell clusters induced by SLE serum and by HAIG-activated serum was similar. In four of the five SLE sera, Clq inhibitor produced dose-dependent reduction of neutrophil clustering (Fig. 4).

DISCUSSION

Intravascular aggregation of neutrophil granulocytes is considered an important event in the inflammatory response, by promoting neutrophil contact for endothelial adhesion before transendothelial migration and accumulation of neutrophils at

extravascular sites [17]. Neutrophil expression of complement receptor type 3 (CR3), a member of the CD18 complex [18], appears to be critical for neutrophil aggregation and for adhesion to endothelium [19,20]. CR3-dependent neutrophil aggregation involves a cell surface counter-structure [21] that may be distinct from ICAM-1 and ICAM-2 [18]. Aggregation of neutrophils also requires cell activation as triggered by chemoattractants such as C5-derived peptides or other agents [19,21]. *In vivo*, intravascular chemoattractants and systemic complement activation induce neutropenia and neutrophil sequestration due to adherence of neutrophils to endothelium in the microvasculature [22,23].

Complement activation has been implied in neutrophil-mediated pathogenetic events associated with haemodialysis [24], the adult respiratory distress syndrome [25], and the 'post-perfusion syndrome' [26]. These studies have been focused on effects of C5-derived peptides. In the adult respiratory distress syndrome, neutrophil aggregating activity in plasma as ascribed to C5a was correlated with development of symptoms [25].

Sera from patients with SLE or Felty's syndrome have been reported to produce aggregation adherence to endothelium and superoxide generation of neutrophils *in vitro* [2]. Attempts to identify the serum factor(s) involved have been inconclusive [2–4].

In the present investigation, neutrophil aggregating activity in serum was assessed with a simple neutrophil clustering assay [4], whereas turbidimetry measurements have been employed in other studies [2,3,24,25]. Thus, findings might not be strictly comparable. Changes in light scattering are influenced by cell polarization and modification of subcellular structures [27]. Furthermore, cytochalasin B, an agent that enhances neutrophil aggregation responses [28] by interaction with actin [29], was used in the study of Abramson *et al.* [3]. In our assay, preformed IgG aggregates readily generated neutrophil clustering activity in normal serum, while no such effect was obtained in heat-treated serum. This argued against a role of cell activation mediated through FcγRII and FcγRIII [30], and clearly suggested a requirement for complement. It is questionable if the clustering effect of Zymosan-treated serum was due to C5-derived peptides, since recombinant C5a showed very limited activity.

The principal finding was that recruitment of the complement system beyond C1 was not necessary for efficient induction of neutrophil clustering. However, preformed IgG aggregates in combination with C1q produced marked activity, even in heat-treated serum. We conclude that the reaction was triggered by C1q-containing IgG complexes through interaction with neutrophil surface receptors for C1q [31].

C1q receptors recognize the collagenous part of C1q [32,33], and collagen structures of C-type lectins that resemble C1q [34]. The receptor binding site of C1q is masked by C1r and C1s in the $C1q(C1r\text{-}C1s)_2$ complex [32], but disassembly of the molecule by C1 inhibitor in conjunction with activation [35] exposes the ligand structure of fixed C1q. Like C1q receptors, the C1q inhibitor in serum [14] binds to the collagenous part of C1q [15]. C1q inhibitor virtually abolished neutrophil clustering elicited with C1q-containing complexes and the inherent neutrophil clustering activity of some SLE sera, indicating that the collagenous part of C1q was a significant ligand in the reaction.

To our knowledge, neutrophil clustering by a C1q-dependent mechanism has not been previously described. The previous finding of inverse correlation between neutrophil clustering activity and the number of circulating neutrophils in SLE [4] suggests that the activity is not a biologically inconsequential phenomenon. C1q can activate neutrophils as expressed by an oxidative response [36] with recruitment of partly unique signal transduction pathways [37]. It remains to be established if C1q-triggered neutrophil clustering resembles neutrophil aggregation induced by other stimuli in the requirement for continuous cell activation and CD18 leucocyte adhesion molecules [21], or if other mechanisms are involved.

The ligand requirements for induction of neutrophil clustering activity in serum strongly suggested involvement of C1q receptors, even if the precise mechanisms remain unclear. As proposed for platelets [38] and endothelial cells [39], C1q receptors might contribute to localization of C1q-containing immune complexes to neutrophil surfaces. Assuming that this leads to neutrophil clustering and enhanced margination of the cells, the findings relate to previous observations in disease conditions such as SLE [1–4]. Interestingly, C1 dissociation with possible exposure of immune complex-bound C1q is pronounced in active SLE [40,41]. We propose that C1q bound to immune complexes could be an important ligand for interaction with neutrophils in SLE, and perhaps also in other immune complex diseases.

ACKNOWLEDGMENTS

The study was supported by the Swedish Medical Research Council (B93-27X-09528-03B and B93-16I-07921-07A), the National Board for Industrial and Technical Development, the Medical Faculty of the University of Lund, the Swedish National Association against Rheumatism, King Gustaf V's 80th Birthday Fund, Greta och Johan Kock's Foundation, Alfred Österlund's Foundation, Anna-Greta Crafoord's Foundation, Crafoord's Foundation, Nanna Svartz' Fund, Thelma Zoega's Fund, and the Foundation for Aid to the Disabled in Scania.

REFERENCES

1 Churg A, Franklin W, Chan KL, Kopp E, Carrington CB. Pulmonary hemorrhage and immune-complex deposition in the lung: complications in a patient with systemic lupus erythematosus. Arch Pathol Lab Med1980; **104**:388–91.

2 Hashimoto Y, Ziff M, Hued E. Increased endothelial cell adherence, aggregation and superoxide generation by neutrophils incubated in systemic lupus erythematosus and Felty's syndrome sera. Arthritis Rheum 1982; **25**:1409–18.

3 Abramson SB, Given WP, Edelsson HS, Weissman G. Neutrophil aggregation induced by sera from patients with active systemic lupus erythematosus. Arthritis Rheum 1983; **26**:630–6.

4 Jonsson H, Sturfelt G. A novel assay for neutrophil clustering activity of human sera: relation to disease activity and neutropenia in systemic lupus erythematosus. Ann Rheum Dis 1990; **49**:46–50.

5 Schifferli JA, Ng YC. The role of complement in the processing of immune complexes. Baillieres Clin Immunol Allergy 1988; **2**:319–34.

6 Cooper NR. Laboratory investigation of complement proteins and complement receptors. Baillieres Clin Immunol Allergy 1988; **2**:263–93.

7 Sjöholm AG, Selander B, Östenson S, Holmström E, Söderström C. Normal human serum depleted of C1q, factor D and properdin: its use in studies of complement activation. APMIS 1991; **99**:1120–8.

8 Sturfelt G, Linder C, Sjöholm AG, Svensson B. Impaired monocyte motility in sera from patients with systemic lupus erythematosus. Acta Path Microbiol Immunol Scand (C) 1983; **91**:151–8.

9 Tan EM, Cohen AS, Fries JF *et al.* The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982; **25**:1271–7.

10 Bombardier C, Gladman DD, Urowitz MB, Chang CH, Charron D. Development and validation of the SLEDAI: an index of disease activity in SLE. Arthritis Rheum 1992; **35**:630–40.

11 Tenner AJ, Lesavre P, Cooper NR. Purification and radiolabeling of human C1q. J Immunol 1981; **127**:648–53.

12 Truedsson L, Sturfelt G. Human factor D of the alternative pathway: purification and quantitation by enzyme amplified electroimmunoassay. J Immunol Methods 1983; **63**:207–14.

13 Medicus RG, Esser AF, Fernandez HN, Muller-Eberhard HJ. Native and activated properdin: interconvertibility and identity of amino- and carboxy-terminal sequences. J Immunol 1980; **124**:602–6.

14 Silvestri L, Baker JR, Rodén L, Stroud RM. The C1q inhibitor in serum is a chondroitin 4-sulphate proteoglycan. J Biol Chem 1981; **256**:7383–7.

15 Ghebrehiwet B. C1q inhibitor (C1qINH): functional properties and possible relationship to a lymphocyte membrane-associated C1q precipitin. J Immunol 1981; **126**:1837–42.

16 Björnsson S. Simultaneous preparation and quantitation of proteoglycans by precipitation with Alcian Blue. Anal Biochem 1993; **210**:282–91.

17 Harlan JM. Leukocyte–endothelial interactions. Blood 1985; **65**:513–25.

18 Arnaout MA. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood 1990; **75**:1037–50.

19 Schwartz BR, Ochs HD, Beatty PG, Harlan JM. A monoclonal antibody-defined membrane antigen complex is required for neutrophil-neutrophil aggregation. Blood 1985; **65**:1553–6.

20 Tonnesen MG, Anderson DC, Springer TA, Knedler A, Avdi N, Henson PM. Adherence of neutrophils to cultured human microvascular endothelial cells. Stimulation by chemotactic peptides and lipid mediators and dependence upon the Mac-1, LFA-1, p150, 95 glycoprotein family. J Clin Invest 1989; **83**:637–46.

21 Kuypers TW, Koenderman L, Weening RS, Verhoeven AJ, Roos D. Continuous cell activation is necessary for stable interaction of complement receptor type 3 with its counter-structure in the aggregation response of human neutrophils. Eur J Immunol 1990; **20**:501–8.

22 McCall CE, DeChatelet LR, Brown D, Lachmann P. New biological activity following intravascular activation of the complement cascade. Nature 1974; **249**:841–3.

23 Fehr J, Jacob HS. *In vitro* granulocyte adherence and *in vivo* margination. Two associated complement-dependent functions. J Exp Med 1977; **146**:641–52.

24 Craddock PR, Hammerschmidt D, White JG, Dalmasso AP, Jacob HS. Complement (C5a)-induced granulocyte aggregation *in vitro*: a possible mechanism of complement mediated leukostasis and leukopenia. J Clin Invest 1977; **60**:260–4.

25 Hammerschmidt D, Weaver L, Hudson L, Craddock PR, Jacob HS. Association of complement activation and elevated plasma C5a with adult respiratory distress syndrome. Lancet 1980; **i**:947–9.

26 Chenoweth DE, Cooper SW, Hughli TE, Stewart RW, Blackstone EH, Kirklin JW. Complement activation during pulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins. N Engl J Med 1981; **304**:497–502.

27 Yuli I, Snyderman R. Light scattering by polymorphonuclear leukocytes stimulated to aggregate under various pharmacologic conditions. Blood 1984; **64**:649–55.

28 Kaplan HB, Edelson HS, Friedman R, Weissman G. The roles of degranulation and superoxide anion generation in neutrophil aggregation. Biochim Biophys Acta 1982; **721**:55–63.

29 Ohmori H, Toyama S, Toyama S. Direct proof that the primary site of action of cytochalasin on cell motility processes is actin. J Cell Biol 1992; **116**:933–41.

30 Crockett-Torabi E, Fantone JC. Soluble and insoluble immune complexes activate human neutrophil NADPH oxidase by distinct Fcγ receptor-specific mechanisms. J Immunol 1990; **145**:3026–32.

31 Ghebrehiwet B. Functions associated with the C1q receptor. Behring Inst Mitt 1989; **84**:204–15.

32 Tenner AJ, Cooper NR. Analysis of receptor-mediated C1q binding to human peripheral blood mononuclear cells. J Immunol 1980; **125**:1658–63.

33 Arvieux J, Reboul A, Bensa JC, Colomb MG. Characterization of the C1q receptor on a human macrophage cell line, U937. Biochem J 1984; **218**:547–55.

34 Malhotra R, Thiel S, Reid KBM, Sim RB. Human leukocyte C1q receptor binds other soluble proteins with collagen domains. J Exp Med 1990; **172**:955–9.

35 Laurell A-B, Mårtensson U, Sjöholm AG. C1 dissociation. Spontaneous generation in human serum of a trimer complex containing C1 inactivator, activated C1r, and zymogen C1s. J Immunol 1987; **139**:4145–51.

36 Tenner AJ, Cooper NR. Stimulation of a human polymorphonuclear leukocyte oxidative response by the C1q subunit of the first complement component. J Immunol 1982; **128**:2547–52.

37 Goodman EB, Tenner AJ. Signal transduction mechanisms of C1q-mediated superoxide production. Evidence for the involvement of temporally distinct staurosporine-insensitive and -sensitive pathways. J Immunol 1992; **148**:3920–8.

38 Peerschke EIB, Ghebrehiwet B. Platelet interactions with C1q in whole blood and in the presence of immune complexes or aggregated IgG. Clin Immunol Immunopathol 1992; **63**:45–50.

39 Daha MR, Miltenburg AMM, Hiemstra PS, Klar-Mohamed N, Van Es LA, Van Hinsbergh VWM. The complement subcomponent C1q mediates binding of immune complexes and aggregates to endothelial cells *in vitro*. Eur J Immunol 1988; **18**:783–7.

40 Sturfelt G, Johnson U, Sjöholm AG. Sequential studies of complement activation in systemic lupus erythematosus. Scand J Rheumatol 1985; **14**:184–96.

41 Jonsson H, Sjöholm AG, Mårtensson U, Laurell A-B, Sturfelt G. C1 subcomponent complexes and C2 cleavage in active systemic lupus erythematosus. Complement Inflamm 1991; **8**:1–12.