C1q-mediated chemotaxis by human neutrophils: involvement of gClqR and G-protein signalling mechanisms

Leonora E. A. LEIGH*, Berhane GHEBREHIWET†, Tim P. S. PERERA‡, Ian N. BIRD‡, Peter STRONG*, Uday KISHORE*, Kenneth B. M. REID* and Paul EGGLETON*[1]

*MRC Immunochemistry Unit, Department of Biochemistry, University of Oxford, Oxford OX1 3QU, U.K., ‡Yamanouchi Research Institute, Littlemore Park, Oxford OX4 4SX, U.K., †Department of Medicine, State University of New York, Stony Brook, NY 11794, U.S.A., and †Department of Pathology, State University of New York, Stony Brook, NY 11794, U.S.A.

C1q, the first component of the classical pathway of the complement system, interacts with various cell types and triggers a variety of cell-specific cellular responses, such as oxidative burst, chemotaxis, phagocytosis, etc. Different biological responses are attributed to the interaction of C1q with more than one putative cell-surface C1q receptor/C1q-binding protein. Previously, it has been shown that C1q-mediated oxidative burst by neutrophils is not linked to G-protein-coupled fMet-Leu-Phe-mediated response. In the present study, we have investigated neutrophil migration brought about by C1q and tried to identify the signal-transduction pathways involved in the chemotactic response. We found that C1q stimulated neutrophil migration in a dose-dependent manner, primarily by enhancing chemotaxis (directed movement) rather than chemokinesis (random movement). This C1q-induced chemotaxis could be abolished by an inhibitor of G-proteins (pertussis toxin) and PtdIns(3,4,5)P_3 kinase (wortmannin and LY294002). The collagen tail of C1q appeared to mediate chemotaxis. gC1qR, a C1q-binding protein, has recently been reported to participate in C1q-mediated chemotaxis of murine mast cells and human eosinophils. We observed that gC1qR enhanced binding of free C1q to adherent neutrophils and promoted C1q-mediated chemotaxis of neutrophils by nearly seven-fold. Our results suggests C1q-mediated chemotaxis involves gC1qR as well as G-protein-coupled signal-transduction mechanisms operating downstream to neutrophil chemotaxis.

INTRODUCTION

C1q, together with the proenzymes $C1r_2s_2$, constitute the first component of complement present in the blood. C1q is a 460 kDa glycoprotein with a complex hexameric 'tulip-like' structure, consisting of six globular heads, each derived from the C-terminal globular regions of three polypeptide chains (A, B and C). Each head is attached to a central fibril-like region by collagen-like tails [1]. Upon complement activation, both the globular heads of C1q (gC1q) and collagen-like tails of C1q (cC1q) interact with various plasma proteins. In the plasma, at least two of the six globular heads of C1q are required to form a strong interaction with antibody Fc region in the antibody–antigen complexes. This leads to the activation of C1r and C1s, which then can react with C1q inhibitor, resulting in dissociation of activated C1r and C1s from the C1q–immunoglobulin complex. Regions of gC1q and cC1q are then available for association with other plasma proteins within the peripheral blood circulation. The cC1q region of C1q provides binding sites for acute-phase plasma proteins, for example, C-reactive protein and serum amyloid protein [2,3].

Activated C1q also has the potential to interact with a number of C1q-binding proteins (C1q-bps) associated with the plasma membrane of leucocytes. Three C1q-bps, which interact with the collagen tail of C1q, of molecular masses 126, 57 and 68 kDa, have been described on the surface of neutrophils [4–6]. Another C1q-bp, called gC1qR, is known to interact with globular head regions of C1q [7,8]. gC1qR is a highly acidic protein and a tetramer of 33 kDa monomers. It can inhibit C1q-dependent haemolysis by preventing C1q–immune-complex interaction on the surface of sheep erythrocytes [9]. It is uncertain how the binding of C1q to these various proteins accounts for the diverse functions that appear to be enhanced upon association of C1q with the neutrophil cell surface. The interaction of C1q with leucocytes enhances several important immune functions, including up-regulation of complement and Fc-mediated phagocytosis [10], adhesion and superoxide burst [6]. Recent evidence suggests that low concentrations of C1q induce chemotaxis and chemokinesis in murine mast cells [11] and human eosinophils [12]. Normally, free C1q does not bind to leucocytes. However, free C1q is capable of associating with soluble C1q-bps (e.g. immune complexes, calreticulin and gC1qR), that are present in extracellular forms during inflammation. Possibly in complex with such proteins, C1q binding to cell surfaces is enhanced in one of several ways. (i) The globular head region of C1q binds specifically to complexes of immunoglobulins, which in turn bind to Fc receptors present on the cell surface. C1q has been shown to enhance immune-complex binding to the neutrophil cell surface [13], suggesting the C1q may bind to the cell through C1q-specific receptors for the collagen-like region. (ii) Free C1q can bind to cells undergoing apoptosis via calreticulin contained in endoplasmic reticulum vesicles that 'bleb' off from the cell surface [14]. (iii) C1q may bind to cC1qR (a 60 kDa C1q-collagen tail-binding protein; calreticulin homologue) after binding to a secreted form of gC1qR, as these two molecules have recently been shown to form a complex [15].

Abbreviations used: cC1q, collagen-like tails of C1q; gC1q, globular heads of C1q; C1q-bp, C1q-binding protein; FMLP, *N*-formylmethioninyl-leucyl-phenylalanine; PLC, phospholipase C; PT, pertussis toxin.

[1] To whom correspondence should be addressed.

Chemotaxis and the respiratory burst are known to share some of the same signal transduction pathways [16]. Both responses frequently require the participation of one or more pertussis-toxin-sensitive G-proteins. However, Goodman and Tenner have shown the C1q-mediated respiratory burst in neutrophils is, at least, G-protein-insensitive [17]. This suggests that the signals operating downstream to C1q-mediated superoxide burst and chemotaxis are different. Direct evidence of divergence of regulatory events required for neutrophil chemotaxis and the respiratory burst has come from studies using oxidized and nonoxidized FMLP (N-formylmethioninyl-leucyl-phenylalanine). Oxidized FMLP triggers an oxidative burst but not chemotaxis, whereas the nonoxidized FMLP activates both functions [18]; binding affinities of the ligand are believed to influence the regulation of both functions. As there may be several C1q-bps associated with the cell surface at any one time, determining the number of C1q-bps and possible physiological roles of each protein is difficult. This is reflected in the reports for C1q-bps present on the surface of granulocytes, which range from 10^5 to 1.1×10^7 [19,20]. Further evidence that degranulating neutrophils export to their surface C1q-bps comes from a study in which biotinylated C1q was shown to bind five-fold more to FMLP-stimulated cells [21].

In this study, we sought to investigate the chemotactic properties of C1q with respect to the migration of human blood neutrophils. We also employed a number of signal-transduction inhibitors to examine the signalling pathways involved in C1q-mediated chemotaxis of human neutrophils. We also observed that the interaction of C1q with gC1qR, present on the cell surface of adherent human neutrophils, led to the enhancement of C1q-dependent human neutrophil locomotion, inhibitable by various signal-transduction inhibitors.

MATERIALS AND METHODS

Neutrophil isolation

Human neutrophils of 95% purity were isolated from the whole blood of 20 consenting normal donors by a one-step isolation procedure using Polymorphprep (Nycomed (UK) Ltd., Birmingham, U.K.) as previously described [22], according to the manufacturer's instructions.

Purification of C1q, cC1q and gC1q

Haemolytically active C1q was isolated from human serum, according to the method of Reid [23], and its purity assessed by SDS/PAGE on a 5–20% (w/v) polyacrylamide gel under reducing conditions. As a precaution, immediately prior to use, C1q was centrifuged at 4 °C at 14000 ***g*** for 15 min to eliminate aggregates that may have formed during storage. gC1q were prepared by digestion of C1q with collagenase purified from *Achromobacter iophagus* [24] and cC1q were purified by pepsin digestion of the purified C1q molecule [25].

Preparation of gC1qR and C1q antibodies

Polyclonal and monoclonal antibodies to human gC1qR were prepared as described by Ghebrehiwet et al., [8,26]. Rabbit polyclonal antibodies to C1q were raised against purified human C1q. The IgG fraction was further purified by passing the serum over a concanavalin A column. The specificity of the antibody was checked by ELISA and Western blotting.

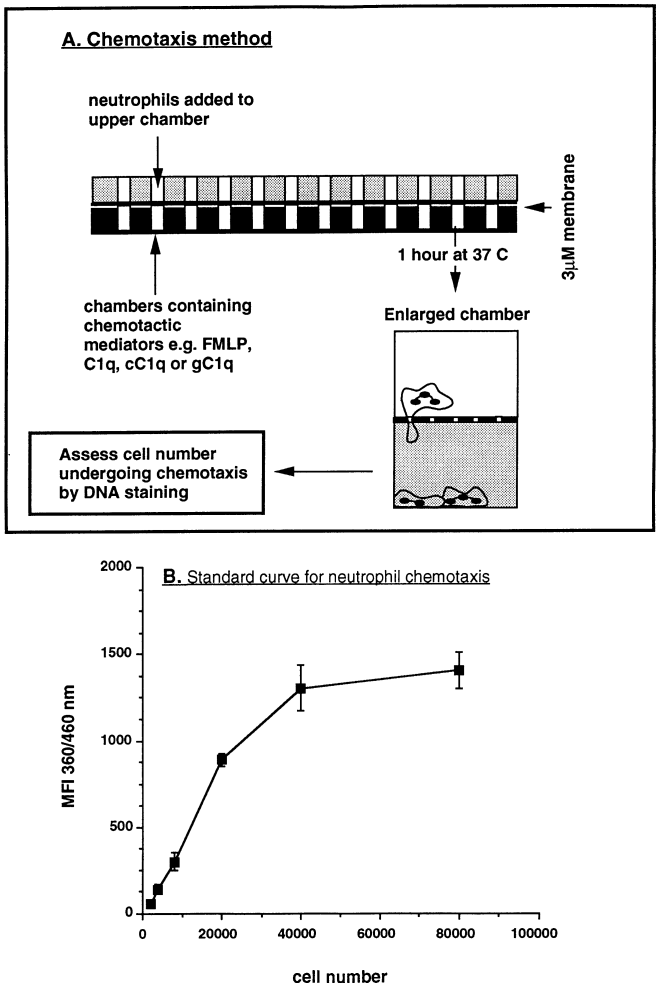

Figure 1 (A) Schematic diagram of chemotaxis method and (B) standard curve for neutrophil number as determined by Hoechst staining

Known numbers of neutrophils (2000–8000 cells in 100 μl aliquots) were placed in the lower chamber of the chemotaxis plate during each experiment and allowed to adhere for 1 h at 37 °C in a 5% CO_2 environment. The results show the mean ± S.D. fluorescence intensity (MFI) in arbitrary units with increasing cell number for duplicate determinations of between five and eight experiments.

Chemotaxis assay

Neutrophil-cell migration was determined using a Neuroprobe 96-well micro-chemotaxis chamber (Figure 1A). To accommodate the large biological variation observed in chemotactic responses for different individuals, neutrophil suspensions were initially incubated with a range of concentrations of native C1q, cC1q, gC1q and gC1qR suspended in the assay buffer (Gey's buffer, Dulbecco's modified PBS prepared in endotoxin-free water, pH 7.4, supplemented with 2% (w/v) BSA and 0.1% (w/v) glucose). The assay buffer solutions, with or without proteins, were placed in the lower compartments. All chemotaxis buffers and proteins were prepared in endotoxin-free water supplemented with 1000 units/ml of Polymyxin B to neutralize the activity of any endotoxin contamination derived from the gC1qR expression system. In parallel studies, FMLP was used as a positive control for chemotaxis; the lower chambers were filled with aliquots of FMLP over a concentration range of 0.1–1000 nM. All chambers were covered with 3 μm cellulose

nitrate filters and then 50 μl of the cell suspension (2×10^5 cells), treated with and without inhibitors of G-proteins (pertussis toxin, PT) and PtdIns(3,4,5)P_3 kinase (wortmannin and LY294002), were placed in the upper compartment. The chemotaxis chamber was then incubated for 1 h in a humidified incubator with 5% (v/v) CO_2 and 95% (v/v) air. Following incubation, non-chemotactic cells were removed from the upper chamber. The plate was then spun at 2000 rev./min for 10 min in a bench centrifuge to detach any migrating cells from the underside of the membrane, and the upper polycarbonate filter was then discarded. The test proteins and buffer were removed and the adherent cells allowed to air dry in a 60 °C oven for 15 min to permeabilize the cells. The cells that had traversed the filter were quantified by staining the cells with 200 μl of Hoechst dye (0.5 μg/ml in PBS containing 0.005% [w/v] SDS). Fluorescence intensity was determined 15 min later, employing a Cytofluor 2350 fluorimeter (Millipore) against the fluorescence reading for a known number of cells (Figure 1). The autofluorescence was subtracted from the fluorescence obtained from migrated cells to obtain specific fluorescence. The specific fluorescence from known numbers of cells placed in microtitre wells was used to construct a standard curve (Figure 1B). Fluorescence from test wells were then interpolated from the standard curve to obtain the cell number.

Recombinant production and purification of gC1qR

The full-length gC1qR cDNA was used as a template for PCR. The forward primer, 5′ GCCATGGCTCTGCACACCGACGGAGAC 3′, was used to engineer an *Nco*I site at the 5′ end of the gC1qR open reading frame and a pBluescript reverse primer was used to generate an *Eco*RI restriction site at the 3′ end. The PCR product was digested with *Nco*I and *Eco*RI and the cleaved fragment was subcloned into Studier expression vector pET-21d between *Nco*I and *Eco*RI sites. Thus, the gC1qR construct was placed under the transcriptional control of bacteriophage T7ϕ10 promoter. The recombinant plasmid, called pBG-1, was introduced into an appropriate host cell, *Escherichia coli* BL21(DE3), that had an integrated copy of the T7 RNA polymerase gene under *lac*UV promoter control. Addition of isopropyl thiogalactoside induced the expression of T7 RNA polymerase and hence gC1qR protein. The protein was purified to near-homogeneity using Superose-12 gel-permeation and Mono-Q ion-exchange chromatography.

Binding of C1q on adherent neutrophils in the presence and absence of gC1qR

The binding of C1q to the neutrophil cell surface was evaluated by incubating 25 nM C1q with adherent cells (1×10^5 neutrophils) at 37 °C for 30 min. A fixed-cell ELISA method was used to detect C1q binding to adherent neutrophils. Monolayers of adherent neutrophils were prepared by resuspending the cells in Roswell Park Memorial Institute medium at a concentration of 1×10^6/ml and dispensing 100 μl aliquots into tissue culture microwell 96 FSI flat-bottomed microtitre plates for 1 h at 37 °C and 5% (v/v) CO_2 (Nunc, Roskilde, Denmark). Uniformity of cell adhesion in individual wells was determined by staining duplicate plates of cells with crystal violet as previously described [6]. A standard curve was obtained by plating known concentrations of cells and staining with crystal violet. The cells were washed in Gey's buffer and fixed to the plates by adding 100 μl of 0.1% (v/v) glutaraldehyde/well and incubating for 5 min at 25 °C. Non-specific binding sites were blocked with Gey's solution for a further 30 min. Then, an increasing concentration of gC1qR was mixed with C1q and added to the neutrophils for 30 min at 37 °C. The cells were washed three times in Gey's buffer, and bound C1q was detected by incubation with a 2.66 μg/ml dilution of rabbit anti-human C1q antiserum or 0.40 μg/ml rabbit anti-human gC1qR. Following three additional washes, binding of C1q to adherent neutrophils was examined by probing the cells with anti-rabbit IgG-peroxidase conjugate for 30 min at 37 °C, followed by TMB-peroxidase substrate (TMB, 3,3′,5,5′-tetramethylbenzidine in aqueous dimethyl formamide). Control preparations included cells with no primary and/or secondary antibody added, and antibodies but no C1q added. C1q binding to fluid-phase neutrophils was also assayed by flow cytometry employing a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA, U.S.A.), using FITC-labelled C1q directly, or non-labelled C1q probed with 1:50 dilution of 5.3 mg/ml rabbit anti-human C1q antiserum, followed by 1:50 dilution of anti-rabbit antisera conjugated to FITC. The binding to fluid-phase neutrophils was analyzed employing Lysis II software version 1.1 (Becton Dickinson).

RESULTS

Human C1q induces neutrophil chemotaxis

As shown in Figure 2, freshly prepared human neutrophils were found to migrate towards a C1q concentration gradient in a dose-dependent manner. Basal migration, which was measured as the number of cells migrating in the presence of Gey's buffer alone, averaged 1113 ± 416 (mean $\pm$ S.E.M.; $n = 10$ subjects). Baseline control values were subtracted from all data shown. Neutrophil migration peaked at a C1q concentration of 25 nM, which enhanced neutrophil migration approximately 12-fold over that which occurred in the presence of buffer alone. Concentrations of C1q as low as 0.4 nM, which is approximately 400-fold lower than the levels of C1q found in plasma, stimulated migration approximately 2-fold. Although the physiological level of C1q is approximately 152 nM, C1q is normally present in the circulation in the form of a C1 complex and, therefore, much lower concentrations of free C1q would be expected to be present

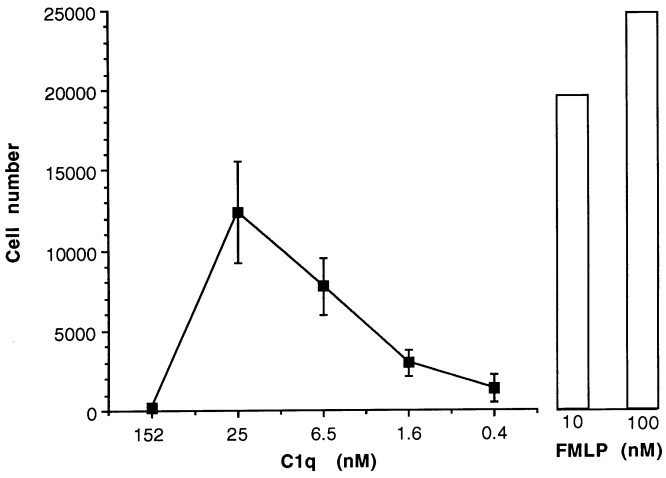

Figure 2 Neutrophil migration to C1q

Cells (2×10^5/50 μl aliquot) were allowed to migrate toward various concentrations of highly purified C1q for 1 h. Numbers represent mean $\pm$ S.E.M. of the total number of cells traversing a 3 μm cellulose membrane towards C1q above that observed with buffer alone for 10 subjects. The chemotactic response is compared with the mean response obtained for two concentrations of FMLP for eight subjects (bars on right).

Table 1 Checkerboard analysis of C1q-stimulated migration of human neutrophils

Values are means for two subjects performed in triplicate for C1q-mediated migration expressed as number of cells migrating into the lower chamber in 1 h at 37 °C and 5% CO_2. Cells were applied to the upper chamber. C1q in varying concentrations was applied to the upper, the lower, or both chambers.

C1q in lower compartment (nM)	C1q in upper compartment (nM) ...	Number of cells migrating into lower chamber			
		0	1.6	6.5	25
1.6		1415	369	1445	922
6.5		6801	1783	1446	1184
25		5502	3937	1967	984

during C1 activation, much of which would associate with immune complexes. The addition of 152 nM of free C1q in this study did not result in the migration of neutrophils. When compared with FMLP, a potent leucocyte chemoattractant, the migration response induced by 25 nM C1q was half as active as the optimal concentration of FMLP (100 nM). The mean dose-dependent response curve for 10 subjects is shown. For some individuals, concentrations lower than 25 nM were found to be optimal. To determine whether C1q stimulated directed migration (chemotaxis) or random migration (chemokinesis), a checkerboard analysis was performed in which C1q concentrations were varied above and below the filter (Table 1). When the concentration of C1q was higher in the lower compartment compared with the upper chamber, neutrophil migration occurred along the C1q concentration gradient, confirming that directional movement was taking place.

C1q-induced chemotaxis involves the collagen-like tail of C1q

To identify the region of C1q responsible for chemotaxis, C1q was proteolytically cleaved to generate gC1q and cC1q. As shown in Figure 3(A), cC1q, over a concentration range of 300–0.1 nM, stimulated migration. In contrast, gC1q gave a statistically insignificant increase in migration, observed for all concentrations tested (Figure 3B). The vehicle buffer for the preparation of gC1q and cC1q (10 mM Tris) was itself non-chemotactic.

Neutrophil-cell migration to C1q is enhanced in the presence of gC1qR

As shown in Table 2, the addition of gC1qR (30 nM) together with C1q (25–1.6 nM) in the lower wells of the chemotaxis chamber causes a dose-dependent enhancement of cell migration into the lower chamber of the chemotaxis apparatus. In control experiments, gC1qR alone enhanced neutrophil migration to a similar level as 1.6 nM C1q. However, when both C1q and gC1qR were added to the lower chamber together, the neutrophil chemotaxis was enhanced approximately seven-fold. The increase in chemotaxis could not be explained by the sum of the cumulative chemotactic response of C1q and gC1qR. In control experiments, gC1qR was incubated with cC1q (0.1–100 nM) alone or together with gC1q (100 nM). The presence of gC1qR did not enhance cC1q-mediated chemotaxis to any significant degree. When cC1q and gC1q were added to the chemoattractant chambers without gC1qR, the chemotactic response induced by cC1q was much lower than that induced by cC1q alone. The addition of gC1qR appeared to marginally enhance the cC1q/gC1q-mediated chemotaxis. To test the specificity of the C1q-induced chemotaxis, an attempt was made to neutralize the chemotaxis by addition of Fab fragments of anti-C1q. However this had the unexpected effect of enhancing chemotaxis (results not shown). Interestingly, a previous study has demonstrated that $F(ab')_2$ fragments of anti-cC1qR induce superoxide release by neutrophils, suggesting both anti-C1q and anti-C1qR $F(ab')_2$ fragments of antibodies can activate neutrophil functional responses [27]. Therefore an alternative neutralization procedure was adopted, in which 25 nM

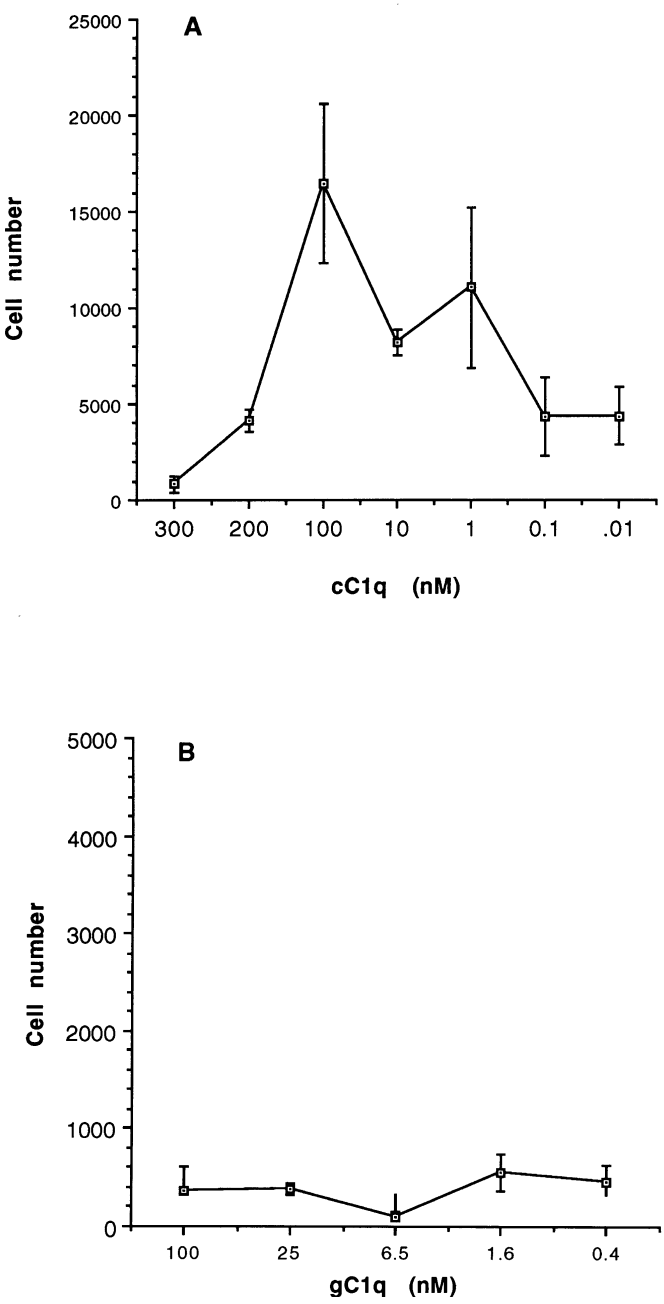

Figure 3 Neutrophil migration to cC1q and gC1q

Cells (2×10^5/50 μl aliquot) were allowed to migrate toward various concentrations of highly purified cC1q (**A**) or gC1q (**B**) for 1 h at 37 °C. Numbers represent mean ± S.E.M. for duplicate samples for five subjects of the total number of cells traversing a 3 μm cellulose membrane towards cC1q.

Table 2 Effect of gC1qR on neutrophil migration towards C1q, cC1q and gC1q

Cells (2×10^5/50 μl aliquot) were allowed to migrate toward various concentrations of highly purified C1q, cC1q and gC1q in the presence and absence of 30 nM (1 μg/ml) gC1qR for 1 h at 37 °C. Numbers represent the mean total number of cells traversing a 3 μm cellulose membrane towards C1q for two subjects, performed in triplicate.

	Number of neutrophils migrating (per 100000 cells added)	
Chemotaxis conditions	Without gC1qR	With 1 μg/ml (30 μM) gC1qR
C1q		
25 nM	9600	70400
6.5 nM	4300	52300
1.6 nM	3100	31250
0.0 nM	1471	4100
cC1q		
100 nM	23893	13783
10 nM	9011	10480
1 nM	10917	7948
0.1 nM	8572	12573
cC1q + 100 nM gC1q		
100 nM	4579	7845
10 nM	5593	6650
1 nM	2198	1849
0.1 nM	2850	1735

C1q with 1 μg/ml gC1qR were incubated in the presence of 100 and 500 nM gC1q. This markedly reduced the chemotactic response of the neutrophils towards C1q as shown in Table 3. We next sought to determine if gC1qR binds directly to neutrophils using a fixed-cell ELISA. Additional gC1qR at concentrations ranging between 1 and 40 μg/ml (Figure 4A) was shown to bind to the cells. Using monoclonal and polyclonal anti-human gC1qR antiserum in this and a previous study [26], gC1qR was also detected on the surface of adherent neutrophils.

gC1qR enhances C1q binding to human neutrophils

A prerequisite for C1q-mediated chemotaxis is the binding of C1q to the cell surface. The aim of these experiments was to determine if any of the C1q-bps enhanced association of C1q with the neutrophil-cell surface. Monomeric C1q conjugated directly to FITC, or detected by FITC-labelled anti-C1q antisera, bound very weakly or not at all to the surface of human neutrophils in fluid-phase suspension as detected by FACS analysis (results not shown). In contrast, C1q bound to adherent neutrophils, but binding of C1q was increased in the presence of gC1qR in a dose-dependent manner (Figure 4B). However, saturation binding of C1q binding to adherent neutrophils occurred in the presence of 10 μg/ml gC1qR, but as little as

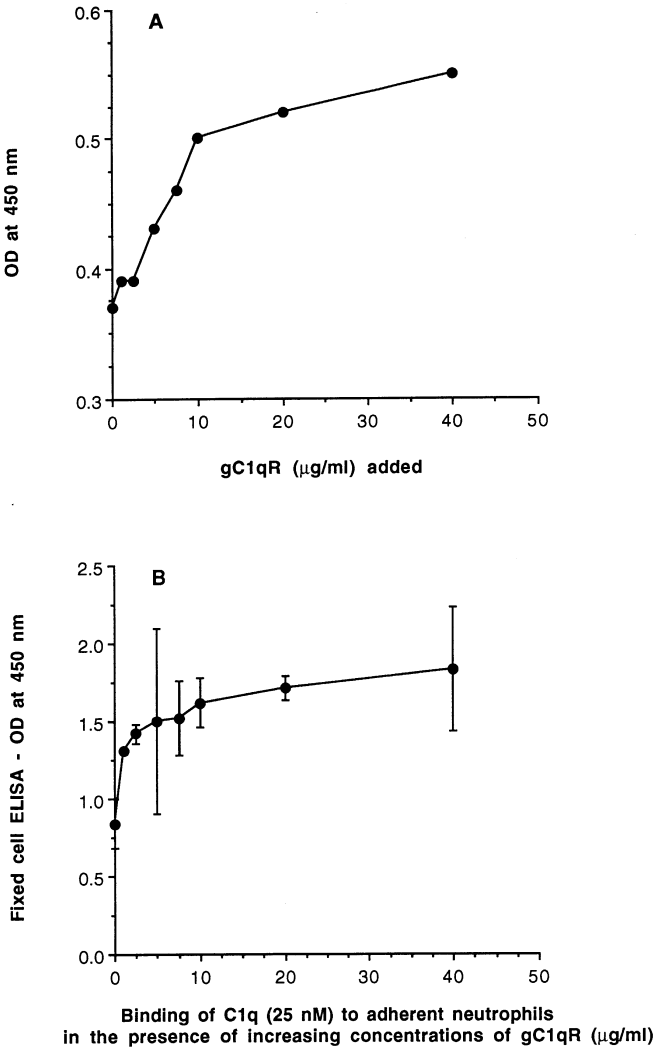

Figure 4 Effect of gC1qR on C1q binding to neutrophils

(**A**) Binding of free gC1qR to adherent neutrophils. Cells (2×10^5/100 μl of Roswell Park Memorial Institute medium) were allowed to adhere to microtitre plates for 1 h at 37 °C. The cells were washed in Gey's buffer and fixed to the plates with 0.1% (v/v) glutaraldehyde for 5 min at 25 °C. Neutrophils were incubated at 37 °C for 45 min with seven concentrations of purified human gC1qR (1–40 μg/ml). Antiserum to human gC1qR was used to reveal the binding of gC1qR to adherent neutrophils. The results show the mean of two subjects performed in duplicate. (**B**) Binding of C1q to adherent neutrophils in the presence of gC1qR. Cells were allowed to adhere to microtitre plates as described above. Neutrophils were incubated at 37 °C for 45 min with purified human C1q (25 nM) with a range of concentrations of gC1qR (1–40 μg/ml). Antiserum to human C1q was used to reveal the binding of C1q to adherent neutrophils. The results are the mean ± S.D. for three subjects each performed in triplicate. OD = absorbance.

Table 3 Effect of gC1q on gC1qR-enhanced C1q-mediated chemotaxis

Numbers represent the mean total number of cells traversing a 3 μm cellulose membrane towards C1q for two different subjects, performed in triplicate.

	Number of neutrophils migrating (per 100000 cells added)	
Chemotaxis conditions	Subject 1	Subject 2
25 nM C1q	20654	2524
25 nM C1q + 1 μg/ml gC1qR	30651	25040
25 nM C1q + 1 mg/ml gC1qR + 100 nM gC1q	20182	1316
25 nM C1q + 1 mg/ml gC1qR + 500 nM gC1q	7200	379

Table 4 Effect of various metabolic inhibitors on C1q-mediated chemotaxis

Chemotaxis of human blood neutrophils to 25–1.6 nM C1q was examined in the presence of inhibitors of PtdIns3 kinase (wortmannin and LY294002) and G-proteins (PT). $n = 2$ subjects, performed in triplicate. Values represent the actual number of neutrophils migrating across the membrane towards various concentrations of C1q in the presence and absence of chemotaxis inhibitors.

		Wortmannin			LY294002			PT (ng/ml)		
	PBS	10 nM	50 nM	100 nM	0.5 μM	1μM	10 μM	100 ng	500 ng	1000 ng
25 nM C1q	8461	0	0	0	1876	0	0	4071	363	876
6.5 nM C1q	7921	0	0	0	0	0	0	0	0	0
1.6 nM C1q	112	0	0	0	0	0	0	0	0	0

1 μg/ml gC1qR led to a significant increase on C1q binding to the cell surface.

Effect of signal-transduction-pathway inhibitors on C1q-mediated chemotaxis

The data in Table 4 demonstrate the efficacy of wortmannin, LY294002 and PT to inhibit C1q-mediated chemotaxis of human neutrophils. Cells incubated in the presence of between 100 ng to 1 μg PT, a G-protein inactivator, caused a dose-dependent inhibition of cell migration towards 25 nM C1q by 50–90 %. The PtdIns(3,4,5)P_3 kinase inhibitors, wortmannin and LY294002, appeared to be more potent inhibitors of C1q-mediated chemotaxis. This is of interest since PtdIns(3,4,5)P_3 kinase is rapidly produced upon exposure of neutrophils to some chemoattractants such as FMLP. Both wortmannin and LY294002 at the concentrations tested are not known to be toxic to neutrophils, as assessed by the ability of the cells to generate a respiratory burst [28,29]

DISCUSSION

Diverse host-defence functions have been described for human C1q. In particular, the interaction of C1q with various types of leucocytes results in enhancement of several important immune functions, including Fc-mediated phagocytosis [10], increase in adhesion [6] and respiratory burst [30]. Other inflammatory mediators such as FMLP and C5a, which can also induce multiple functional responses in cells of the immune system, are known to induce chemotaxis. Normal levels of C1q in the serum are 170 nM as part of the C1 complex. But only 5–10 % of the available C1q is thought ever to be found in a free state. The results presented here demonstrate that free C1q (concentrations ranging between 1.6 and 25 nM) can stimulate chemotaxis. The levels of C1q used in this study are similar to those found in various tissue sites during injury and inflammation. Previously, using cultured murine fibroblasts [31], mast cells [11], and eosinophils [12], it has been shown that human C1q can induce chemotaxis across species.

One of the aims of the present study was to further characterize the nature of the interaction of C1q with human neutrophils with specific reference to chemotaxis. Specific studies were carried out to determine which region of C1q interacts with the neutrophil. Native C1q can be conveniently divided by proteolytic cleavage into two distinct fragments: an N-terminal region that contains the collagen-like sequence, cC1q, and a C-terminal globular head region, designated as gC1q. Our results support the notion that the collagen-like region of C1q mediates neutrophil chemotaxis and peaks with a cC1q concentration of 100 nM; a second response peaks with 1 nM cC1q concentration and presents a biphasic cell migration similar to that observed for C1q chemotaxis by mast cells [11].

gC1qR has been recently cloned and sequenced [8], but does not have a transmembrane-spanning domain nor a consensus site for a glyco-phosphoinositol anchor. It is, therefore, possible that the protein is attached to the cell surface through association with other membrane-spanning proteins. It has been observed on the surface of neutrophils [7]. Monoclonal antibodies raised against human gC1qR have served as a useful tool in defining the presence of gC1qR on the neutrophil surface under different states of cell activation. A panel of monoclonal antibodies against gC1qR reacted weakly to human neutrophils in suspension as detected by flow cytometry. However, when cells become adherent, the monoclonal antibodies react strongly to gC1qR on the neutrophil surface, as examined by immunofluorescence [26]. Independent workers [32], using an IgM monoclonal antibody raised against gC1qR, were unable to detect gC1qR at all on the surface of neutrophils in suspension. These conflicting results may reflect the different epitopes recognized by the different monoclonal antibodies, as well as differences represented by the class of immunoglobulin generated. However, the IgG monoclonal antibodies used in this study showed much stronger reactivity when neutrophils were adherent to glass coverslips. This suggests that some of neutrophils' antigenic sites may be cryptic on cells in suspension but become exposed when the cells are adherent. It is possible that the appropriate adhesion or activation of the cells induces a conformational change in the surface-bound gC1qR, exposing the appropriate antigenic sites. Alternatively, cell adhesion may lead to increased secretion of gC1qR that binds to the outer surface of the cell. The results of this study suggest that direct binding of C1q to the cell surface is enhanced in the presence of extracellular gC1qR, which in turn enhances C1q-mediated chemotaxis. In this respect, gC1qR was observed to occur naturally on the surface of adherent neutrophils and appeared to enhance C1q binding to the cells. Whether the surface gC1qR came from an intracellular or extracellular source is uncertain, since gC1qR is soluble and a secreted form of gC1qR has been detected from a number of cell lines [32]. When additional gC1qR was added, more C1q bound to the cells in a dose-dependent manner. Furthermore, the chemotactic potency of C1q was increased up to seven-fold in the presence of gC1qR, but this was to some respect dependent upon the individual concerned. The mechanism of the enhanced C1q-induced chemotaxis seen in the presence of gC1qR may be due to the fact that gC1qR can simultaneously bind to neutrophils and to the globular heads of C1q, thereby enhancing C1q binding to the neutrophils. This, in turn, could provide the stronger chemotactic signal. The tetrameric characteristics of both native and recombinant forms of gC1qR lend support to this scenario. As gC1qR is tetrameric, it is potentially tetravalent, and it may

essentially cross-link C1q, enhancing its binding to a putative C1q receptor on the cell surface as a result of C1q being presented as a multivalent array. No such enhancement of chemotaxis was observed when cC1q was presented with gC1qR. This suggests the gC1qR is binding to the globular heads of whole C1q and enhancing chemotaxis by allowing closer association of the collagen-like regions with a cC1q-binding protein or receptor. Moreover, gC1qR has recently been shown to complex with cC1qR (calreticulin homologue), which is often detected on the membrane surface of activated cells [15]. The fact that gC1qR selectively enhances chemotaxis induced by the whole C1q molecule, but not C1q fragments, also provides evidence that gC1qR, and not endotoxin or another bacterial contaminant, is specifically responsible for the enhanced chemotaxis observed with whole C1q. The specificity of gC1qR on C1q-mediated chemotaxis is further substantiated in experiments in which preincubation of gC1qR with gC1q abolished the enhanced chemotaxis, suggesting gC1qR/C1q interaction may play a prominent role in stimulating cell migration. The fact that polyclonal anti-C1q fab fragment antibodies failed to inhibit C1q-mediated chemotaxis suggests the C1q–C1q receptor sites are not blocked. Alternatively, the antibody–C1q complexes may simply act as a potent stimulant recognized by C1q or other inflammatory receptors. For example, anti-cC1qR fab fragments from systemic lupus erythematosus patients have been shown to activate human neutrophils [27]. Different cellular activities, such as chemotaxis, degranulation and respiratory burst, are mediated by distinct pathways of signalling. Also, the activity of chemokines and other chemoattractants is the outcome of a complex cascade that depends on the cell type, the ligand, the structure and the configuration of the receptor, the G-proteins involved, and the different enzymes that are available to be activated in a given cell type. The activation of the receptors by specific ligands results in coupling to G-proteins, followed by a cascade of events that leads to specific cellular responses. The G-proteins consist of a large gene family coding for at least 16 α, 4 β and multiple γ subunits [33]. Most of the reactions induced by chemotactic substances are PT-sensitive, but some activities are shown to be PT-resistant. The best-characterized signal-transduction pathway of G-protein-coupled receptors starts with ligand binding, followed by activation of a heterotrimeric G-protein. An exchange occurs in the α subunit of the G-protein from a GDP- to a GTP-bound state, resulting in a dissociation of the α subunit from the $\beta\gamma$ subunits. The free α subunit can activate both phospholipase C (PLC) β1 and PLC β2, whereas the free $\beta\gamma$ complex activates preferentially PLC β2. The activation of PLCs results in the hydrolysis of PtdIns(4,5)P_2 to generate two second messengers; Ins(3,4,5)P_3 and diacylglycerol. In general, PLC activation as well as stimulation of various second messengers and inositol phosphate participate in a number of functional responses. There is evidence that chemotaxis results from PLC activation and release of intracellular Ca^{2+} [34], whereas other reports indicate that protein kinase C activation or an increase in intracellular Ca^{2+} is not always essential for a migratory response or for actin polymerization [35,36].

In the light of this information, we therefore investigated whether C1q-mediated chemotaxis was also sensitive to inhibition by PT. C1q-mediated chemotaxis of neutrophils did appear to be sensitive to PT treatment. This is in contrast with a superoxide burst mediating a C1q receptor of 125 kDa, which was not inhibited by PT [17]. In neutrophils, some receptor-coupled mechanisms also lead to PtdIns3 kinase activation downstream of GTPase-coupled receptor interaction [37]. Therefore, specific inhibitors of PtdIns3 kinase, such as LY294002 [29] and wortmannin [28], were used to examine if the C1q-mediated chemotaxis occurs downstream to the PtdIns3 kinase signalling pathways. Treatment of intact human neutrophils with LY294002 or wortmannin before exposure of the cells to the chemotactic gradient resulted in complete inhibition of C1q-mediated chemotaxis. The lack of chemotaxis strongly suggests the involvement of G-proteins and PtdIns3 kinase in propagating the C1q-induced signal for chemotaxis.

The results of the present study substantiate the hypothesis that C1q is a potent chemoattractant. It is becoming evident that both the globular head region and collagen-tail region of C1q work in concert with gC1q- and cC1q-binding proteins. From the evidence in this study, it would appear that cC1q is important in the actual mechanism of chemotaxis. The precise binding proteins on the neutrophil cell surface involved in C1q-cell mediated chemotaxis are still unknown. Although C1q, kininogen and factor XII are bound with high affinity by purified gC1qBP/gC1qR [38-40], the gC1qBP cannot be expected to serve as a surface receptor for these molecules since it is located intracellularly on normal endothelial cells [41] and this is also probably the case in other cell types. However, it is possible that when cells are stressed, or undergoing apoptosis, that gC1qR may be found on the cell surface and thus be able to bind C1q and modulate binding of C1q to the cell surface. This would be consistent with the report that it is not possible to detect gC1qBP on Raji cells using FACS analysis, unless the cells were first permeabilized with saponin [32]. Like gC1qBP, the cC1qR/calreticulin may find its way to the cell surface of cells in the form of apoptotic 'blebs' [42] and this mechanism, or the existence of some isoform of calreticulin, may also be responsible for the observed binding of C1q to cells.

This work was supported by the Medical Research Council of Great Britain (K. B. M. R. and P. E.), Burroughs Wellcome Research Travel grant, the American Heart Association-N.Y. Affiliate (0001) and the American Cancer Society (IM771) (B. G.).

REFERENCES

1 Porter, R. and Reid, K. B. M. (1979) Adv. Protein Chem. **33**, 1–71
2 Gewurz, H., Ying, S.-C., Jiang, H. and Lint, T. E. (1993) Behring Inst. Mitt. **93**, 138–147
3 Ying, S.-C., Gewurz, A. T., Jiang, H. and Gewurz, H. (1993) J. Immunol. **150**, 169–176
4 Guan, E. G., Burgess, W. H., Robinson, S. L., Goodman, E. B., McTigue, K. J. and Tenner, A. J. (1991) J. Biol. Chem. **266**, 20345–20355
5 Malhotra, R., Willis, A. C., Jensenius, J. C., Jackson, J. and Sim, R. B. (1993) Immunology **78**, 341–348
6 Eggleton, P., Ghebrehiwet, B., Coburn, J. P., Sastry, K. N., Zaner, K. S. and Tauber, A. I. (1994) Blood **84**, 1640–1649
7 Eggleton, P., Ghebrehiwet, B., Sastry, K. N., Coburn, J. P., Zaner, K. S., Reid, K. B. M. and Tauber, A. I. (1995) J. Clin. Invest. **95**, 1569–1578
8 Ghebrehiwet, B., Lim, B. L., Peerschke, E. I. B., Willis, A. C. and Reid, K. B. M. (1994) J. Exp. Med. **179**, 1809–1821
9 Peerschke, E., Moon, A., Lim, B.-L., Reid, K. and Ghebrehiwet, B. (1995) FASEB J. **9**, A807
10 Bobak, D., Gaither, T., Frank, M. and Tenner, A. J. (1987) J. Immunol. **138**, 1150–1156
11 Ghebrehiwet, B., Kew, R. R., Gruber, B. L., Marchese, M. J., Peerschke, E. I. B. and Reid, K. B. M. (1995) J. Immunol. **155**, 2614–2619
12 Kuna, P., Iyer, M., Peerschke, E. I. B., Kaplan, A. P., Reid, K. B. M. and Ghebrehiwet, B. (1996) Clin. Immunol. Immunopathol. **81**, 48–54
13 Kishore, U., Sontheimer, R. D., Sastry, K. N., Zappi, E. G., Hughes, G. R. V., Khamashta, M. A., Reid, K. B. M. and Eggleton, P. (1997) Clin. Exp. Immunol. **108**, 181–190
14 Korb, L. C. and Ahearn, J. M. (1997) J. Immunol. **158**, 4525–4528
15 Ghebrehiwet, B., Lu, P. D., Zhang, W., Keilbaugh, S. A., Leigh, L. E., Eggleton, P., Reid, K. B. M. and Peerschke, E. I. (1997) J. Immunol. **159**, 1429–1436
16 Crawford, N. and Eggleton, P. (1991) Biochem. Soc. Trans. **19**, 1048–1055
17 Goodman, E. B. and Tenner, A. J. (1992) J. Immunol. **148**, 3920–3928
18 Harvath, L. and Aksamit, R. R. (1984) J. Immunol. **133**, 1471–1476
19 Tenner, A. J. and Cooper, N. R. (1981) J. Immunol. **126**, 1174–1179

20 Hamada, A. and Greene, B. M. (1987) J. Immunol. **138**, 1240–1245
21 Jack, R. M., Lowenstein, B. A. and Nicholson-Weller, A. (1994) J. Immunol. **153**, 262–269
22 Eggleton, P., Wang, L., Penhallow, J., Crawford, N. and Brown, K. A. (1995) Ann. Rheum. Dis. **54**, 916–923
23 Reid, K. B. M. (1981) Methods Enzymol. **80**, 16–25
24 Paques, E. P. and Huber, R. P. H. (1979) Hoppe-Seyler's Z. Physiol. Chem. **360**, 177–182
25 Reid, K. B. M. (1976) Biochem. J. **155**, 5–9
26 Ghebrehiwet, B., Lu, P. D., Zhang, W., Lim, B.-L., Eggleton, P., Leigh, L. E. A., Reid, K. B. M. and Peerschke, E. I. B. (1996) Hybridoma **15**, 333–342
27 van den Berg, R. H., Siegert, C., Faber-Krol, M. C., van Es, L. A. and Daha, M. R. (1997) Exp. Clin. Immunogenet. **14**, 38
28 Arcaro, A. and Wymann, M. P. (1993) Biochem. J. **296**, 297–301
29 Vlahos, C. J., Matter, W. F., Brown, R. F., Traynor-Kaplan, A. E., Heyworth, P. G., Prossnitz, E. R., Ye, R. D., Marder, P., Schelm, J. A., Rothfuss, K. J. et al. (1995) J. Immunol. **154**, 2413–2422
30 Ruiz, S., Henschen-Edman, A. H. and Tenner, A. J. (1995) J. Biol. Chem. **270**, 30627–30634
31 Oiki, S. and Okada, Y. (1988) J. Immunol. **141**, 3177–3185
32 van den Berg, R. H., Prins, F., Faber-Krol, M. C., Lynch, N. J., Schwaeble, W., van Es, L. A. and Daha, M. R. (1997) J. Immunol. **158**, 3909–3916
33 Simon, M. I., Strathmann, M. P. and Gautam, N. (1991) Science **252**, 802–808
34 Devreotes, P. N. and Zigmond, S. H. (1988) Annu. Rev. Cell Biol. **4**, 649–686
35 Sozzani, S., Zhou, D., Locati, M., Rieppi, M., Proost, P., Magazin, M., Vita, N., Van Damme, J. and Mantovani, A. (1994) J. Immunol. **152**, 3615–3622
36 Sham, R. L., Phatak, P. D., Ihne, T. P., Abbound, C. N. and Packman, C. H. (1993) Blood **82**, 2546–2551
37 Stephens, L., Eguinoa, A., Corey, S., Jackson, T. and Hawkins, P. T. (1993) EMBO J. **12**, 2265–2273
38 Lim, B. L., Reid, K. B. M., Ghebrehiwet, B., Peerschke, E. I. B., Leigh, L. E. A. and Preissner, T. K. (1996) J. Biol. Chem. **271**, 26739–26744
39 Herwald, H., Dedio, J., Kellner, R., Loos, M. and Muller-Esterl, W. (1996) J. Biol. Chem. **271**, 13040–13047
40 Joseph, K., Ghebrehiwet, B., Peerschke, E. I. B., Reid, K. B. M. and Kaplan, A. P. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 8552–8557
41 Dedio, J. and Muller-Esterl, W. (1996) FEBS Lett. **399**, 252–258
42 Eggleton, P., Reid, K. B. M., Kishore, U. and Sontheimer, R. D. (1997) Lupus **6**, 564–571

Received 9 September 1997; accepted 8 October 1997