Characterization of the Human Neutrophil C1q Receptor and Functional Effects of Free Ligand on Activated Neutrophils

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The partial characterization and expression of the C1q receptor (C1q-R) in relation to other complement receptors present on the surface of neutrophils has been examined, as well as the effects of free C1q on cell function. A polyclonal anti-C1q-R antibody recognizes a 68-kD neutrophil surface protein. C1q-R expression was not upregulated upon warming, priming, or exposure to FMLP, but decreased after exposure to phorbol myristate acetate (PMA), because of shedding potentially available for binding to C1q-R. The physiologic complex with C1r2 and Clq was upregulated from intracellular pools after cell stimulation by PMA. No evidence of intracellular pools of C1q-R was found, as assessed by immunoblotting of subcellular fractions. But C1q-R appeared to be expressed early in cell differentiation, was detected on undifferentiated HL-60 cells, and like CR3 expression, increased upon 5 days differentiation towards a neutrophil lineage. However, C1q-R expression decreased upon additional culture, whereas CR3 expression continued to increase. A large variation in the percentage of peripheral cells expressing C1q receptors in donors was observed, ranging from 13% to 100%, contrasting with CR3 receptors that exhibited less variability. Interactions between free monomeric C1q and neutrophils were also studied. Incubation of stimulated neutrophils with 10 to 100 μg/mL C1q resulted in a further increase in CR3 expression and adherence to albumin-coated surfaces. Staphylococci opsonized with low quantities of C1q (0.1 to 1 μg/mL) mediated a moderate and sustained respiratory burst in neutrophils, whereas a burst of similar magnitude was generated only with free C1q at concentrations 10- to 100-fold higher. Stimulation was only partially inhibited if cells were first treated with anti-C1q-R antibody, suggesting other C1q binding proteins may be present on the cell surface. In summary, neutrophil C1q receptor is approximately 68-kD, exhibits varying expression on different subjects, and is not upregulated from intracellular stores on exposure to soluble stimuli. Stimulated, but not resting, neutrophils selectively respond to raised levels of free C1q, resulting in altered cell function and enhanced CR3 receptor expression. These studies thus suggest complex roles for C1q in neutrophil function.

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C1q is a 460-kD molecular weight glycoprotein that circulates in the plasma as a calcium (Ca²⁺)-dependent complex with C1r2 and C1s (C1q). Upon activation of C1, C1r, and C1s are disassembled by C1 inactivator, leaving complex-bound C1q with its collagenous region exposed and potentially available for binding to C1q-R. The physiologic levels of free monomeric C1q available for binding to its receptor is uncertain. C1q not only binds avidly to antigen-antibody complexes, but presumably has nonspecific associations with denatured proteins, soluble, altered lgs and other polyanionic substances. A wide range of values for C1q in human serum have been reported. Total serum C1q levels measured by radial immunodiffusion (RID) of about 200 pg/mL have been reported, with a range of 34 to 246 μg/mL. However, under high ionic conditions that aid in the dissociation of C1q from immune aggregates or soluble altered lgs, a lower mean concentration of 136 μg/mL has been reported. Upon reevaluation of the RID technique by Ziccardi and Cooper, a value of 70 μg/mL was obtained. No value for free C1q has been made, but Ziccardi and Cooper hypothesized that relatively small amounts of free monomeric C1q are present in serum. Sjöholm et al. have suggested between 9% and 30% of C1q in a free state in normal serum. Irrespective of methodology, free C1q has been found to be elevated in various pathophysiologic conditions.

Specific receptors for human C1q (C1q-R) are expressed on the surface of B lymphocytes, monocytes/macrophages, endothelial cells, fibroblasts, platelets, and neutrophils. Most of these early findings were based on radioligand binding studies of a putative cell surface receptor for C1q. C1q has been found to be capable of releasing superoxide and other free radicals from the cell. Previous studies have shown that homogeneous monomeric free C1q binds only to a small number of neutrophils under physiologic conditions, and monomeric 125I-C1q binding specifically to the C1q-R is reduced by 74% under physiologic conditions. However, when C1q is bound to latex beads or immobilized on plastic, it apparently binds to the neutrophil under normal ionic strength conditions, as will C1q bound to pathologically relevant compounds such as immune complexes, viruses, and lipopolysaccharides. It is believed such binding may lead to some structural alteration in the C1q molecule that facilitates binding of the ligand to its receptor. In contrast, Erdei has shown that free unbound C1q binds strongly to pre-B-cell lymphomas, cell lines of myeloid origin, and to ~50% of mature B-cell lymphomas, showing that physical alteration in the structure of the ligand may not always be necessary for binding.

The present study was designed to provide further molecular characterization of the C1q-R and determine its distribution on human neutrophils. In addition, we have reevaluated the interaction of monomeric C1q on resting cells, as typical of previous studies, but also examined, for the first time, the
effect of free Clq on the function of stimulated neutrophils under physiologic conditions.

MATERIALS AND METHODS

Materials. Dulbecco's phosphate buffered saline (PBS) with and without calcium and magnesium (10 mM); FMLP, phenol myristate acetate (PMA), superoxide dismutase, ferricytochrome-c, human serum albumin, diisopropyl fluorophosphate, Phosphatidylcholine-β-acetyl-γ-O-alkyl (platelet-activating factor; PAF), Dextran (500 KD molecular weight) were purchased from Sigma Chemical Co (St Louis, MO). Ficol-Hypaque was obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Monoclonal antibodies (MoAbs) to CR1 (CD35) was purchased from Becton Dickinson (Mountain View, CA), CD15, IgG-1, and IgG-2a were obtained from Sigma and antibody purified from an OKMI cell line was a gift of Dr M. Wilkinson (Hunterian Institute, London, UK). Fluorescein isothiocyanate (FITC)-labeled rabbit and mouse antihuman IgG and IgM were purchased from Jackson Immunoresearch Laboratories (West Grove, PA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis and sucrose-gradient ultracentrifugation of Clq preparations. Hemolytically active Clq was purchased from Sigma. The preparations were examined by SDS-PAGE under reduced conditions in the presence of 4 M urea, to ensure homogeneity of the preparation. As expected, three distinct bands of 30 KD or less were observed. In addition, Clq gels were immunoblotted and probed with antiheparinase to ensure no contaminating fibronectin was present in the commercially purchased Clq samples. Linear sucrose gradients of 5% to 31% (w/vol) sucrose were prepared in PBS in a mixing chamber with a constant volume. Commercially purified samples of Clq, IgG, IgM, and anti-Clq in 200 μL volumes were applied to 11.5-mL gradients and centrifuged at 41,000 rpm at 4°C for 18 hours using a TH641 Sorvall rotor in a Sorvall OTD55B ultracentrifuge. Two hundred microliter fractions were collected by puncturing the bottom of the tube with an ISCO piercing mechanism (Lincoln, NE); samples were obtained by pressing of the gradient from the base of the gradient with 50% (w/vol) sucrose. Protein content was monitored using an on-line absorbance monitoring apparatus (280 nm) attached to a fraction collector.

Isolation and preparation of neutrophils and HL-60 cells. Whole blood was taken from consenting healthy volunteers into sodium citrate, and neutrophils were isolated by erythrocyte sedimentation in dextran citrate, followed by hypotonic lysis and Ficol-Hypaque density centrifugation as previously described.24 The isolated neutrophils were then resuspended in PBS without calcium and magnesium at 4°C until required, then they were suspended in the appropriate buffer. Neutrophil membranes were prepared from isolated, unstimulated neutrophils disrupted by nitrogen bomb cavitation and the subcellular fractions separated into bands by density centrifugation on Percoll gradients. Each band was assayed for marker enzymes to assume their respective homogeneity by previously published methods:25 the α band (primary granules) by myeloperoxidase, the β band (secondary granules) by vitamin B12-binding protein, and the γ-fraction (plasma membrane) by alkaline phosphatase. Other workers have shown the presence of intracellular vesicles that also contain alkaline phosphatase; therefore, care is required to avoid cross-contamination of each fraction, or further separation by free-flow electrophoresis can eliminate the problem.26 Each fraction was then stored in liquid nitrogen. The γ band, which represents the plasma membrane, was analyzed by SDS-PAGE on 5% to 15% acrylamide gels from both resting and 10−6 M FMLF-stimulated cells. HL-60 cells obtained from the American Type Culture Collection (ATCC) were maintained at 1 x 10^6 cells/mL in RPMI medium supplemented with 10% fetal bovine serum and 200 U/mL penicillin and streptomycin. After maintaining the culture for 1 week in a humidified atmosphere of 95% air/5% CO₂, cells were stimulated to differentiate towards a neutrophil lineage by addition of RPMI containing 1.3% dimethyl sulfoxide (DMSO). Cells were sampled at 0, 5, and 7 days after DMSO induction.

Western blot of Clq-R. The two polyclonal antibodies raised against purified Clq-R originating from Raji cells used in these studies for immunoblotting and fluorescence-activated cell sorting (FACS) analysis were prepared as previously described.27 Either 5, 10, 20, or 40 μg of each neutrophil subcellular samples were suspended in sample buffer (62.5 mmol/L TRIS-Cl, pH 6.8, 2% SDS, 5% glycerol, and 0.01% bromophenol blue with 5% 2-mercaptoethanol)28 and incubated for 4 minutes at 100°C and then applied to individual lanes of a 5% to 15% polyacrylamide gel; electrophoresis was performed in TRIS-buffered saline (TBS) at a constant current of 35 mA on an 8 x 7-cm gel. Molecular-weight standards purchased from Sigma, were run simultaneously. The separal proteins were then electro-transferred onto polyvinyl difluoride (PVDF) transfer membrane (Immoblin-P; Millipore Corp, Bedford, MA) using a Semiphror transfer unit. After saturation of the nonspecific sites with 5% nonfat dry milk-TBS overnight, the proteins were probed with a 1:30 dilution of rabbit antihuman Clq-R antibody in TBS-5% milk overnight or 1:250 dilution of mouse antihuman CD15 MoAb or 1:250 dilution of CR3 in TBS-5% milk for 1 hour at 37°C. The strips were then washed in 20 mmol/L TRIS-HCl pH 7.5, 0.14 mol/L NaCl containing 0.4% Tween-20 (TBS-Tween) and then incubated for 1 hour in an antirabbit or antimouse peroxidase conjugate IgG antibody diluted 1:1000 in TBS-Tween with 5% milk. After washing five times in TBS-Tween, the immunoblots were exposed to ECL immunosassay substrate reagent (Amersham International plc, UK) for 1 minute to detect any signal, and then the membranes were exposed to x-ray film (XAR-Ilm, Eastman-Kodak, Rochester, NY).

Analysis of Clq-R, CR1, and CR3 expression on neutrophils by flow cytometry. Human neutrophils (1 x 10⁶) were resuspended in PBS with calcium and magnesium (pH 7.4) and were incubated at 37°C for 15 minutes with either PMA (100 μg/mL), FMLP (10−7 M) or PBS. The cells were then immediately washed in 4°C PAB (phosphate buffered saline without Ca²⁺ and Mg²⁺) and 0.1% bovine serum albumin (BSA), 0.02% sodium azide, and 1% human heat inactivated serum), before incubation with antibodies raised against Clq-R, CR1, or CR3 prepared in the same buffer. In addition to the experiments outlined above, neutrophils in some experiments were incubated with the above stimuli at 4°C as well as at 37°C, before addition of the above antibodies. In other experiments, free Clq (100 to 1 μg/mL) was also incubated with the neutrophils under resting and stimulated conditions before addition of the antibodies. All cells were resuspended in 100-μL volumes of the different antibodies tested prepared at the following concentrations: in the case of CR1, 0.3 μg/100 μL; CR3, 5 μg/100 μL; Clq-R, 5 μg/100 μL; CD15, 0.4 μg/mL; and mouse isotype (0.4 μg/100 μL) controls IgG1 and IgG2a, 0.4 μg/100 μL. Cells were incubated for 20 minutes, washed twice with cold PAB, and pelleted by centrifugation. The pellets were resuspended in 100 μL of either FITC goat-antirabbit IgG or FITC goat-antimouse IgG (1 μg/mL) for 30 minutes at 4°C. Cells were then washed twice with cold PAB and fixed with 1% paraformaldehyde. Fluorescent staining was assessed using a FACSScan flow cytometer (Becton Dickinson) and 5,000 events were acquired. Data analysis was performed on the population of fluorescent cells, and subtracting out the autofluorescence.
ence of an un stained control to determine the mean fluorescent intensity (MFI) of positive cells for each antibody of interest.

Enzyme-linked immunosorbent assay (ELISA). Neutrophils at a concentration of 10^7/mL PBS were incubated at 37°C for 15 minutes in the presence and absence of either FMLP (10^-7 mol/L) or PMA (100 ng/mL). Aliquots of the cell suspension media were then placed in microtitre wells in sodium carbonate buffer (pH 9.6) at 4°C overnight. Plates were washed in PBS/Tween 20 and nonspecific binding sites blocked with 5% milk/100 mmol/L glycine. Polyclonal rabbit antibody against the human Clq receptor (1:200 dilution) was added and allowed to develop for 20 minutes. The reaction was terminated by adding 100 μL 2N H2SO4 and the plate read at 450 nm. Neutrophil adherence to the various protein-coated surfaces was expressed as a percentage of cells adhering in the presence and absence of either FMLP (10^-7 mol/L), PMA, or PBS. After 30-minute incubation at 37°C, nonadherent cells were removed by vigorous washing in PBS and the adherent cells fixed by adding 100 μL of 10% formaldehyde solution for 10 minutes at room temperature. The plates were then washed six times in deionized water and stained by adding 0.85% (wt/vol) NaCl in 25% (vol/vol) ethanol for 10 minutes; excess stain was washed off and the adherent cells solubilized in 550 nm. Neutrophil adherence to the various protein-coated surfaces was expressed as a percentage of cells adhering to nontreatment plastic control wells, which was regarded as 100% adherence.

Statistical analysis. Statistical analysis of the data expressed as mean ± SEM was done by paired and unpaired t-test. The significance threshold was set at P < .05 for all comparisons.

RESULTS

Identification and expression of the neutrophil Clq-R. Western blot analysis of 5, 10, 20, and 40 μg of neutrophil plasma membrane protein (γ fraction) using a 1:30 dilution of polyclonal anti–Clq-R antibody, identified a single immunoreactive band with an apparent molecular weight of 68 kD, under reducing conditions (Fig 2).

To determine if alteration in Clq-R expression on the neutrophil cell surface follows a similar pattern to CR1 and CR3 receptors as assessed by flow cytometry, neutrophils were isolated, maintained at 4°C, and pretreated with selected stimulatory and priming agents as indicated in Tables 1 and 2. After labeling and staining with the various antibodies, the expression of each complement receptor was analyzed.
on a Becton Dickinson FACScan. Cells kept at 4°C for the duration of the experiment displayed a mean fluorescent intensity for Clq-R, CR1, and CR3 of 137 ± 22, 21 ± 2, and 130 ± 20, respectively. Both CR1 and CR3 receptor expression appeared to increase upon warming of the isolated cells to 37°C, consistent with the finding of Van Epps et al32 who showed that both buffy-coat and Ficoll-Hypaque-isolated cells increased these surface receptors to similar degrees. However, the difference in our study was not statistically significant. Furthermore, warming of the cells had no effect on the 68-kD Clq-R expression.

When cells were stimulated with PMA (10 ng/mL), the expression of CR3 was increased by twofold to threefold (P < .05) above cells suspended at 4°C. Only a moderate increase in CR1 was observed upon stimulation with PMA (P < .05), whereas PMA treatment significantly decreased Clq-R expression on neutrophils (Table 1, P < .01). Stimulation of the cells with FMLP (10⁻⁷ mol/L), as expected, led to a twofold enhancement in both CR1 and CR3 expression (data not shown), but failed to affect Clq-R expression in any way. Neutrophils were also treated with the superoxide priming agent, PAF (10⁻⁸ mol/L and 10⁻¹⁰ mol/L), and complement receptor expression analyzed by flow cytometry. Once again, Clq-R expression similarly was not altered by the treatment with PAF. In contrast, 10⁻⁸ mol/L PAF led to an increase in both CR1 and CR3 levels with MFI mean values of 21 and 130 to 34 and 217, respectively (Table 2). FMLP-stimulated O₂ production rates in neutrophils increased from 27 nmoles to 42 nmoles/mL/min/10⁶ cells after pretreatment with 10⁻⁸ mol/L PAF. This enhancing effect on CR1 and CR3 expression and O₂ generation diminished substantially when subpriming levels of PAF (10⁻¹⁰ mol/L) were used. Levels of fluorescence observed by analysis of control cells not treated with primary antibodies, or treated with isotype-matched IgG mouse antibodies together with FITC IgG secondary antibody, were consistently less than 10 U (data not shown).

The mean number of purified neutrophils confirmed by dot-plot analysis and visual examination staining positive for Clq-R was also examined in eight different donors and compared with the number of neutrophils expressing CR3 receptors. Approximately 63% ± 9.6% (mean ± SEM) of neutrophils stained positive for Clq-R. Whereas a greater number of cells expressed CR3 (86% ± 3.6%), there was no significant difference.

Alteration in the expression of Clq-R expression in peripheral neutrophils and undifferentiated and differentiated HL-60 cells. To determine whether the decrease in Clq-R expression from the surface of human neutrophils was caused by shedding of the receptor upon stimulation with PMA, an ELISA assay was used to detect the presence of

Table 1. Expression of Clq-R, CR1, and CR3 Receptors in Stimulated and Unstimulated Neutrophils

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MFI*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clq-R</td>
</tr>
<tr>
<td>0°C</td>
<td>137 ± 22 (3)</td>
</tr>
<tr>
<td>37°C</td>
<td>114 ± 15 (8)</td>
</tr>
<tr>
<td>37°C + PMA</td>
<td>53 ± 13 (7)</td>
</tr>
</tbody>
</table>

* Numbers represent the MFI for each stained population. The MFI for unstimulated cells or FITC-labeled cells were 3.7 ± 0.4 and 3.3 ± 0.8, respectively. Isotype-matched control MoAbs for FITC-IgG1 and IgG2a always gave MFI values of less than 10. Each value represents the mean ± SEM for the number of subjects given in parenthesis.

† Treatment of neutrophils with PMA (10 ng/mL) at 37°C significantly reduced the MFI of Clq-R expression, P < .01.

‡ Warming of the cells did not significantly enhance any complement receptor expression.

§ Treatment of neutrophils with PMA (10 ng/mL) at 37°C significantly enhanced the MFI of CR1 and CR3 receptor expression above 0°C control cell levels (P < .05 and .01, respectively).

Table 2. Effect of PAF Priming on Clq-R, CR1, and CR3 Receptor Expression

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MFI</th>
<th>FMLP-Induced O₂ Production (min/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clq-R</td>
<td>CR1</td>
</tr>
<tr>
<td>None</td>
<td>141 ± 22</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>10⁻⁸ mol/L PAF</td>
<td>137 ± 22</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>10⁻¹⁰ mol/L PAF</td>
<td>131 ± 14</td>
<td>26 ± 6</td>
</tr>
</tbody>
</table>

Neutrophils (10⁶ cells) were incubated at 37°C for 15 minutes with and without PAF. Separate aliquots were assayed for complement receptor expression, or exposed for an additional 15 minutes with 10⁻⁸ mol/L FMLP and superoxide assayed. The results represent the mean of three separate experiments ± SD.
The data are expressed as absorbance values at 450 nm representing the relative amount of receptor released/10^6 cells, given as mean ± SD from four subjects.

A series of experiments were performed to study the expression of C1q-R with CR3 during HL-60 differentiation. Undifferentiated and differentiated HL-60 cells (towards a neutrophil lineage) were compared. As shown in Fig. 4, C1q receptors were present on the surface of 2-week cultured undifferentiated, nonsuperoxide-producing HL-60 cells (MFI = 34); after 5 days of differentiation with DMSO, the HL-60 cells were able to generate superoxide, and their C1q-R expression increased twofold (MFI = 70). A similar profile was seen with CR3 expression. However after 7 days of culture in the presence of DMSO, the C1q-R expression was observed to diminish to basal levels (MFI = 37), whereas CR3 expression continued to increase.

Subcellular analysis to identify localization of C1q-R in the neutrophil. Neutrophil subcellular fractions were studied by immunoblotting with the polyclonal rabbit anti-C1q-R to see if the receptor could be detected in cytoplasm granules (Fig 5); however, the 68-kD protein was limited to the plasma membrane. In addition, immunoblots of FMLP-stimulated cell membranes were examined, but showed no evidence of an increase in density or subcellular location of the single protein band detected by anti-C1q-R. In contrast, the CR3 was associated with the plasma membrane fraction, but in resting cells was most evident as a large subcellular pool within the specific granules (β-band) and cytoplasm fraction (Sβ band). As expected, the control CD15 antibody identified multiple proteins in the plasma membrane and secondary granule fractions.

Modulation of CR3 expression by C1q treatment of neutrophils and effect on cell adhesion. C1q has been reported to enhance phagocytosis of IgG-opsonized red blood cells via modulation of Fc receptor function in monocytes. Here, the ability of free soluble C1q to increase C1q-R, CR1, and CR3 expressions on resting and PMA-stimulated neutrophils was examined. Under resting conditions, the presence of free C1q (1 to 100 μg/mL) did not affect the expression of any of the complement receptors as determined by mean fluorescence intensity measurements on the flow cytometer. As expected, exposure of the cells to PMA alone (10 ng/mL) resulted in a mean increase in CR3 MFI from 191 ± 29 (mean ± SEM; N = 5) to 385 ± 75, representing a twofold increase in CR3 expression. The simultaneous addition of 10 and 100 μg/mL free C1q led to further increases in PMA-stimulated expression of CR3 by 24% and 50% (P < .05), respectively. The use of 1 μg/mL C1q or 100 μg/mL human serum albumin did not affect the stimulated level of CR3 expression as determined by flow cytometry. Neither C1q-R or CR1 receptor expression in the presence of PMA was enhanced in the presence of C1q at the concentrations tested.

Because CR3 (CD11b/CD18) is associated with increases in the adherence of cells, the ability of neutrophils to adhere to BSA, fibronectin, and laminin-coated microtitre wells in the presence and absence of free C1q was assessed (Table 3). In the presence of 10 μg/mL C1q, 72% of neutrophils
adhered to the BSA-coated wells, which was similar to the degree of adherence observed when the neutrophils were incubated in the presence of the chemotactic peptide, FMLP, which is known to upregulate the CR3 antigen complex. There was no increase in adherence of neutrophils in the presence of 1 pg/mL Clq above control levels on PBS-treated control wells. Neutrophils allowed to adhere on fibronectin adhered to the BSA-coated wells, which was similar to the level of adherence above PBS-treated control levels.

High doses of free Clq and lower doses of bound Clq induce neutrophil O₂⁻ production. Clq at concentrations of 2.2 μg or less per 5 × 10⁵ latex beads have been reported to stimulate chemiluminescence and the hexose monophosphate shunt, but not O₂⁻ production of neutrophils in suspension. The relative ability of free and bound Clq to stimulate neutrophils has now been reexamined by stimulating cells with either Clq-opsonized S. aureus, opsonized with between 0.01 and 100 μg/mL Clq or free Clq (ranging between 1 and 100 μg/mL). The presence of free Clq alone acts as an activator of O₂⁻ production by neutrophils (Fig 6A). Resting cells generated a basal amount of O₂⁻ (5 ± 1.8 nmoles) during incubation at 37°C for 30 minutes; neutrophils exposed to relatively high doses of free Clq, ie, 10 μg/mL and 100 μg/mL Clq over the same period led to the generation of significantly more O₂⁻ production by the neutrophils: 31 ± 11 and 63 ± 29 nmoles of O₂⁻ 10⁶ cells, respectively (P < .01). One micromgram per milliliter of free Clq did not stimulate an increase in O₂⁻ above basal levels generated by nontreated control cells in any subjects tested. Next, the ability of Clq to enhance the respiratory burst of neutrophils in the presence of bacteria was determined. As shown in Fig 6B, cells exposed to unopsonized bacteria were stimulated sufficiently to generate a mean O₂⁻ production of 26 ± 4 nmoles (mean ± SEM) over 30 minutes/10⁷ neutrophils, whereas neutrophils exposed to bacteria opsonized with fresh human serum generated 75 ± 7 nmoles over the same period. Exposure of neutrophils with bacteria opsonized with as little as 0.1 μg/mL Clq resulted in the generation of 57 ± 11.6 nmoles O₂⁻, which represented a significant increase in the level of O₂⁻ (P < .05) achieved by cells exposed to unopsonized bacteria alone. When neutrophils were treated with anti–Clq-R antibody for 30 minutes at 4°C before the addition of 10 μg/mL free Clq, the generation of O₂⁻ evoked by Clq was partially inhibited. Cells treated with a control IgG antibody (anti-mouse CD15) continued to generate increased levels of O₂⁻ above PBS-treated control cells. Some enhancement of O₂⁻ was also seen when a high concentration of BSA (10 μg/mL) was used, but this proved...
Fig 6. (A) Stimulation of neutrophil $O_2^-$ production by free soluble Clq. Neutrophils were warmed to 37°C and exposed to various concentrations of free soluble Clq and the generation of superoxide recorded after 30 minutes incubation as detailed in Materials and Methods. The results are presented as the mean ± SEM from experiments of three to eight different donors. The rate of $O_2^-$ produced by cells incubated with 10 and 100 μg/mL free Clq was significantly greater than nontreated cells ($P < .01$). (B) Effect of Clq on Staphylococci-stimulated neutrophils. To determine whether the binding site occupied by anti-ClqR was located near the site occupied by Clq on the receptor, nitrocellulose blots of isolated neutrophil membrane were pretreated with free Clq and then immunoblotted with anti–Clq-R. A 68-kD band was not observed under these conditions, suggesting bound ligand was occupying the antibody binding site. As a control, MoAb against Clq confirmed the presence of Clq bound to the neutrophil membrane protein at 68-kD.

**DISCUSSION**

Human neutrophils possess a number of complement receptors: CR1 (C3b/C4b receptor, CD35, 160 to 250 kD) and CR3 (Mac-1, C3bi receptor, CD11b/CD18, 280 kD) are high molecular-weight proteins that are stored in granules and can be mobilized upon cell activation and subsequently internalized. In contrast, the C5a receptor (55 kD) is not upregulated from internal stores upon stimulation, but its presence on the cell surface is reduced after stimulation. Similar expression characteristics were observed with the 68-kD Clq-R protein. The receptor for Clq has some unique features as compared with CR1, CR3, and the C5a receptor. Complement peptides bind specifically to these latter receptors; however, the Clq-R is distributed on a diverse number of cell types, and several forms of the Clq-R have been found. Clq-R is believed to bind a number of different proteins, such as mannann binding protein, lung surfactant protein (SP-A) and bovine conglutinin, which all show a structural similarity to Clq, each having both collagenous and globular domains. These distinct domains may be responsible for different functional messages upon binding to the Clq-R. Clq itself can be found in human serum in three different forms: (1) bound to the C1 complex, (2) bound to immune complexes, or (3) in a free soluble form. It is able to bind to the surface of a number of cell types via the Clq receptor or form complexes or aggregates with other serum proteins in the microvascular environment.

The present study was designed to identify the neutrophil membrane Clq-R, analyze its coexpression in association with other complement receptors under resting and stimulated conditions, and examine the effect of free Clq upon neutrophil function under similar conditions. Western blot
analysis of plasma membrane receptor extracts of human neutrophils by a polyclonal anti-Clq-R showed the presence of a single protein of 68 kD under reducing conditions. Recent evidence by Guan et al.\(^1\) have suggested that Clq, via its collagenous protein of 68 kD, may play a role in modulation of other complement receptor expression, depending on the individual, with a mean ± SEM value of 63% ± 9.6% whereas CR3 expression appeared more uniform, mean 86% ± 3.6% (range, 55% to 99%).

Clq-R expression decreased after treatment of the cells with PMA (Table 1). Other studies using similar concentrations of PMA (5 to 10 ng/mL) as a stimulant, have observed upregulation of both CR1 and CR3,\(^2\) whereas the use of greater concentrations of PMA/phorbol dibutyrate (>10 ng/mL) has resulted in a reduction in CR1, but not CR3 expression.\(^3\) An explanation for the differing responses of various complement receptors in response to phorbol esters is not yet fully understood. Immunoblots of subcellular components of neutrophils failed to detect any granule or cytoplasm stores of Clq-R, making it unlikely that the receptor is internalized or localized in these subcellular compartments. However, Percoll gradient extracts of nitrogen cavitated cells has limited ability to fractionate tertiary and secretory granules that may be a source of the 68-kD membrane protein.\(^4\) Alternatively, reduction in Clq-R surface expression could be caused by shedding of the receptors from the cell surface. This is one mechanism by which CR1 and FeRIII down modulation occurs.\(^5\) As shown in Fig 3, the decrease in Clq-R could be explained by release of the receptor into the cell media upon stimulation. This observation, together with the donor variation in Clq-R expression suggests there may be a limited pool of Clq-R that is not replenished upon release from the membrane. Further insight as to the phenotypic expression of Clq-R was gained from studying the human promyelocytic HL-60 cell line. When HL-60 cells were cultured in an undifferentiated form, both Clq-R and CR3 expression were detectable in low levels (Fig 4), suggesting that the Clq-R receptor like the CR3 receptor is expressed at an early stage of development. Induction of HL-60 cell differentiation of cells for 5 days with DMSO led to a twofold increase in both Clq-R and CR3 expression. Evidence that the cells were differentiating normally, was confirmed by PMA-inducible O\(_2\) generation. Interestingly, after 7 days of differentiation, the Clq-R expression began to diminish, whereas the CR3 expression continued to increase, suggesting expression of Clq-R occurs early in differentiation, and is not further upregulated from an intracellular pool.

The effect of free soluble Clq on neutrophil function under resting and stimulated conditions was performed in normal ionic strength buffer. Incubation of neutrophils in suspension with various concentrations of Clq, with and without PMA for 15 minutes, led to a significant increase in PMA-stimulated upregulation of CR3 expression above levels achieved by PMA alone, suggesting a role for Clq-R in modulation of other complement receptor expression, but only when the cells are in an activated state, as similar concentrations of free Clq added to resting cells did not alter CR3 expression significantly, which is consistent with the recent studies of Goodman and Tenner,\(^7\) who similarly observed that free Clq did not alter CR3 expression on unactivated neutrophils. Increased expression of CR3 receptors is a marker of increased cellular adherence, therefore the adherence of neutrophils to protein-coated surfaces in the presence of free Clq was assessed. Work in our laboratory,\(^8\) and others\(^9\) has shown that adherence of neutrophil to uncoated polystyrene itself triggers a sustained respiratory burst, whereas coating of the wells with BSA can reduce the activation partially, whereas adherence to laminin or fibronectin does not lead to respiratory burst stimulation.\(^10\) However, it should be noted that laminin can stimulate a respiratory burst in suspended cells.\(^11\) Based on these observations, microtiter wells were coated with either BSA to mimic moderate stimulation, or fibronectin or laminin to allow adherence of neutrophils without inducing a respiratory burst. As expected, neutrophils incubated in the presence of FMLP led to a significant increase in adherence above control levels. Clq (10 μg/mL) was also a potent stimulator of neutrophil adherence on BSA-coated wells. In contrast, free Clq at the same concentration did not enhance cell adherence above baseline levels on laminin or fibronectin-coated surfaces. This supported the notion that free Clq can enhance neutrophil adherence in activated cells, but not in resting ones.

Clq-bound to latex is unable to enhance the generation of O\(_2\) in neutrophils in suspension,\(^12\) whereas neutrophils adhering to Clq-coated plastic wells do generate O\(_2\).\(^13\) In our study, the ability of free Clq to stimulate a respiratory burst in both resting and bacteria-stimulated neutrophils was studied. Staphylococci, fully opsonized with fresh serum, generated the greatest respiratory burst, and unopsonized bacteria induced a moderate amount of O\(_2\) production during the 30-minute incubation period, but the response was poor compared with serum or Clq-treated bacteria. A low concentration of free Clq (0.1 μg/mL) used to opsonize Staphylococci was sufficient to significantly enhance the respiratory burst, suggesting that the quantities of free Clq thought to exist in the circulation may be sufficient, in combination with other opsonizing proteins, to activate the neutrophil respiratory burst synergistically. When used alone, 100 to 1,000-fold higher concentrations of free Clq are required to trigger a respiratory burst of similar magnitude. Such increases in free Clq may only arise in inflammatory disease states such as rheumatoid arthritis and primary biliary cirrhosis, where higher levels of Clq have been reported. As observed in other studies,\(^14\) even the ability of surface-bound Clq to evoke a O\(_2\) response by neutrophils from different donors has differed significantly. This may be explained by the number of cells from an individual staining positive for the Clq-R, or alternatively the density of the receptor on the cell surface. Addition of the anti-Clq-R antibody led to a 50% reduction in the O\(_2\) production induced by 10 μg/mL free Clq, suggesting the burst was mediated partially through a Clq–Clq-R interaction. Qualitative evidence that the antibody was directly binding to the site occupied by the Clq...
protein was provided by immunobots of the neutrophil membrane proteins. When Western blots were exposed to Clq before immunoblotting with the anti-Clq-R antibody, the ability of the antibody to detect the 68-kD protein was inhibited, whereas control immunobots confirmed Clq binding to a 68-kD protein. Interestingly, another Clq-binding protein of 126-kD has been isolated from monocytes, neutrophils, and U937 cells that appears to be involved in Clq-mediated enhancement of phagocytosis in monocytes. The presence of other Clq-binding proteins on the cell surface not detected by the antibody used in this study may also play a role in Clq-mediated respiratory burst. In support of this possibility, a marked heterogeneity in the number of neutrophils expressing the 68-kD Clq-R was observed, which is in agreement with previous binding studies performed under nonphysiologic ionic strength conditions, which noted the number of binding sites for Clq per neutrophil varied considerably amongst donors, and which supports the already substantial evidence that neutrophils are heterogeneous with respect to cell membrane proteins and functional responsiveness.

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Characterization of the human neutrophil C1q receptor and functional effects of free ligand on activated neutrophils

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