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P Eggleton, ... , K B Reid, A I Tauber

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Research Article

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Identification of a gC1q-binding Protein (gC1q-R) on the Surface of Human Neutrophils

Subcellular Localization and Binding Properties in Comparison with the cC1q-R

Paul Eggleton,^{*§} Berhane Ghebrehwet,[‡] Kedarnath N. Sastry,^{*} John P. Coburn,^{*} Ken S. Zaner,^{*} Kenneth B. M. Reid,[§] and Alfred I. Tauber^{*}

^{*}The William B. Castle Hematology Research Laboratory, Boston City Hospital and the Departments of Pathology and Medicine, Boston University School of Medicine, Boston, Massachusetts 02118; the [‡]Departments of Medicine and Pathology, State University of New York, Stony Brook, New York 11790; and [§]Medical Research Council Immunochemistry Unit, Department of Biochemistry, University of Oxford, OX1 3QU, United Kingdom

Abstract

Human neutrophils have multiple C1q-binding proteins. Direct ligand-binding studies with the globular domain of C1q and two-dimensional Western blot analysis revealed two gC1q-binding proteins (gC1q-R): a 33,000 M_r protein (pI 4.5) mainly in the neutrophil plasma membrane and an 80,000–90,000 M_r protein (pI 4.1–4.2) located mainly in the granules. Direct binding studies showed that C1q bound to this higher molecular weight protein under physiological conditions. In contrast, anti-cC1q-R antibody, which recognizes a protein binding to collagenous tails of C1q, detected only a 68,000 M_r protein in the plasma membrane. Both the 33,000 and 68,000 M_r receptors appear early on the surface of differentiating HL-60 cells. On mature neutrophils, surface expression of both C1q receptors was evident, but no upregulation was observed upon stimulation. Phorbol myristate acetate treatment of neutrophils downregulated both the receptors from cell surface, and significant amounts of soluble gC1q-R were in cell media supernatants, suggesting receptor shedding or secretion. gC1q-R, unlike cC1q-R, did not bind to other C1q-like ligands, namely mannose binding protein, surfactant protein-A, surfactant protein-D, or conglutinin under normal ionic conditions, suggesting a greater specificity for C1q than the "collectin" type receptor (cC1q-R). Rather, gC1q-R only bound purified C1q, and the binding was enhanced under low ionic conditions and in the absence of calcium. The role of C1q receptor shedding and its biologic consequence remain to be defined, but may contribute to the diversity of C1q-mediated responses observed in many cell types. (*J. Clin. Invest.* 1995; 95:1569–1578.) **Key words:** complement receptors • subcellular fractionation • neutrophil granules • C-type lectins • two-dimensional electrophoresis

Introduction

C1q is present in the blood as a complex with two proenzymes C1r₂ and C1s₂ as C1 (1). Upon activation of C1, the proenzymes

Address correspondence to Alfred I. Tauber, M.D., Boston University School of Medicine, S-Building-Room 301, 80 E Concord St., Boston, MA 02118. Phone: 617-638-5630; FAX: 617-638-5636.

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C1r and C1s are activated, releasing C1q in a form which can readily form immune complexes or interact with various blood cells. The structure of C1q is complex and consists of six globular heads each attached to a short collagen triple helix. The globular head of C1q has been shown to be involved in the initiation of the complement cascade by binding to the Fc region of immunoglobulin (2). The presence of receptors for C1q on leukocytes was first suggested in 1972 (3). Many cell types have been described in which C1q binds to their cell surface, including platelets (4), neutrophils, monocytes (5), eosinophils (6), fibroblasts (7), endothelial cells (8), Raji cells (9), smooth muscle, and epithelial cells (10). The binding of C1q to these cells is thought to be via the collagen-like domain of the protein. A collagen C1q-receptor (cC1q-R)¹ was purified from Raji cells in 1984 (9) and was found to be a single chain, acidic glycoprotein with a molecular mass of ~ 60,000–68,000 D and present on the cell types above. Recently, a heavily glycosylated surface membrane protein of ~ 126,000 M_r present on monocytes, neutrophils, and on the monocyte-like cell line U937 has also been demonstrated to interact with the collagen-like region of C1q (11). These data suggest that there may be more than one C1q-binding protein on the surface of leukocytes.

In 1989, a receptor which binds to the globular heads of C1q (gC1q-R) with high affinity was discovered on the surface of diploid fibroblasts (12). This receptor has been shown recently to be present on Raji cells, platelets (13), and eosinophils (6). The receptor was purified from Raji cells and cloned from a B lymphocyte cDNA library and was found to be a highly acidic protein with three potential N-linked glycosylation sites and migrates with an approximate molecular mass of 33 kD (13). In this study, we detected gC1q-R on mature human neutrophils and gradual expression on HL-60 cells after 6 d of differentiation. In neutrophils, complement receptors can be categorized into one of two groups, those which are upregulated from intracellular stores upon cell activation (e.g., CR1 and CR3) (14, 15), and those which are present on the plasma membrane surface, whose levels are reduced upon cell stimulation (e.g., C5a receptor and cC1q-R) (16, 17). We therefore examined the subcellular distribution of the gC1q-R protein recognized by polyclonal anti-gC1q-R antibodies and by direct ligand-binding studies. Neutrophil plasma membranes, cyto-

1. *Abbreviations used in this paper:* cC1q-R, collagen domain C1q receptor; CR, complement receptor; Dx, dextran sulfate; gC1q-R, globular domain C1q receptor; HRP, horseradish peroxidase; MBP, mannose-binding protein; MFI, mean fluorescent intensity; PVDF, polyvinyl difluoride; RA, retinoic acid; rgC1q-R, recombinant soluble form of gC1q-R; SP, surfactant protein; TBS, Tris-buffered saline.

plasm, and granules prepared by nitrogen cavitation and differential centrifugation were analyzed by SDS-PAGE, two-dimensional electrophoresis, Western blotting analyses, and binding studies using either antibodies against ligands or biotinylated ligands. The data suggest that a 33,000 M_r gC1q-binding protein is located mainly in the plasma membrane, with lesser amounts detectable in the primary granule and cytosolic fraction as a highly acidic protein. Furthermore, an 80,000–90,000 M_r acidic protein was also recognized by anti-gC1q-R antibodies in the granule fractions and may be a precursor of the final membrane-bound protein. A recombinant soluble form of gC1q-R (rgC1q-R) has been engineered recently (13) and was used in this work to study the binding specificity and affinity of gC1q-R for free and aggregated C1q under normal and low ionic conditions. We also examined the ability of the purified rgC1q-R protein to bind to human mannose-binding protein (MBP), surfactant proteins A and D (SP-A and SP-D), and conglutinin, each of which show structural similarity to C1q.

Methods

Generation and purification of recombinant gC1q-R. rgC1q-R was generated by expression in *Escherichia coli* and purified as previously described (13).

gC1q-R and cC1q-R antibody production. Polyclonal rabbit antibodies to human gC1q-R and an 18-amino acid gC1q-R peptide were prepared as described by Ghebrehiwet et al. (13). Rabbit polyclonal antibodies were raised against highly purified cC1q-R isolated from Raji cells, as described in detail elsewhere (18). Both antibodies were stored at a concentration of 1 mg/ml in Tris-buffered saline (TBS).

Purification of C1q. Hemolytically active C1q (Sigma Immunochemicals, St. Louis, MO) was homogeneous as assessed by SDS-PAGE on a 5–15% gel under reducing conditions, and three distinct bands of 30 kD or less were observed. C1q gels were blotted and probed with antifibronectin to ensure no contaminating fibronectin was present, and finally, linear sucrose gradient centrifugation (5–31%) confirmed the presence of a single nonaggregated C1q protein suspension. However, immediately before use, C1q was centrifuged at 14,000 g for 15 min as a further precaution to eliminate possible aggregates of C1q. In some experiments, C1q aggregates were prepared by mixing equal amounts (wt/wt) of C1q and dextran sulfate (mol wt, 15,000) at pH 7.4. Aggregate formation was instantaneous as detected by 90° light scattering in a SLM 8000C (SLM Instruments, Urbana, IL) fluorometer at 450 nm.

Purification of lectins. Human SP-A was purified from alveolar proteinosis patients by the method of Wright et al. (19), graciously provided by Dr. V. Shepherd and Dr. Z. Chroneos (Vanderbilt University, Nashville, TN), and was shown to be free of other major protein contaminant by SDS-PAGE analysis on a 10% gel. Rat SP-D was a gift of Dr. E. C. Crouch (Washington University, St. Louis, MO) and was isolated from the 10,000 g supernatant of bronchoalveolar lavage from silicotic rats, while human SP-D was isolated from the supernatant of lavage obtained from patients with human alveolar proteinosis. SP-D was purified from both samples by sequential saccharide affinity chromatography on maltosyl-agarose and gel filtration on 4% agarose (A15M; Bio-Rad Laboratories, Richmond, CA). Both preparations appeared as a 43-kD protein on SDS-PAGE under reducing conditions. The procedures for purification have been published previously (19–21). Recombinant human MBP was a generous gift of Dr. R. A. B. Ezekowitz (Harvard Medical School, Cambridge, MA) and was purified by the previously published method (22). The purity of the eluted protein was assessed on an 11% SDS-PAGE. Conglutinin was a gift from Dr. Y. M. Lee (Applied Immune Sciences, Menlo Park, CA) and was isolated from blood plasma by incubation with zymosan and then purified by HPLC ion exchange chromatography (Bio-Gel DEAE-5-PW; Bio-Rad Laboratories) (23). The homogeneity of the purified pro-

tein was checked by reducing SDS-PAGE, and a single band of 43,000 M_r was observed.

Preparation of HL-60 cells, neutrophils, and subcellular fractions. HL-60 cells were maintained at 5×10^5 cells/ml in RPMI medium supplemented with L-glutamine, containing 20% vol/vol heat-inactivated fetal bovine serum and 200 U/ml penicillin and streptomycin. After maintaining the culture for 1 wk in a humidified atmosphere of 95% air/5% CO₂, cells were stimulated to differentiate toward a neutrophil lineage by addition of RPMI containing 1×10^{-6} M retinoic acid (RA). Cells were sampled at 0, 1, 3, 6, and 8 d after RA induction. Human neutrophils were isolated with prior consent from normal donors by erythrocyte sedimentation in dextran citrate, followed by hypotonic lysis and Ficoll-Paque gradient centrifugation, as described previously (24). The isolated cells (95% pure) were then suspended in PBS without calcium or magnesium, pH 7.4, and maintained at 4°C. In experiments requiring stimulated neutrophils, cells were prewarmed to 37°C for 5 min before stimulation with either FMLP (10^{-7} M) or PMA (100 ng/ml) for 15 min. The cells were then washed and resuspended in the appropriate buffer. Trypan blue was used to monitor the integrity of the plasma membrane of neutrophils before and after stimulation. Neutrophil membranes and subcellular fractions were prepared from isolated cells disrupted by nitrogen cavitation, and the subcellular fractions were separated into bands by density gradient centrifugation on Percoll gradients. Each band was assayed for marker enzymes to their respective homogeneity by previously published methods (25): the α band (primary granules) by myeloperoxidase, the β band (secondary granules) by vitamin B₁₂-binding proteins, and the γ -fraction (plasma membrane) by alkaline phosphatase. Each fraction was then stored in liquid nitrogen until required.

Western blots of neutrophil membrane and subcellular fractions. Neutrophil subcellular fractions or plasma membrane (5–40 μ g each) were suspended in sample buffer (62 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue with 5% 2-mercaptoethanol) and incubated for 4 min at 100°C and then applied to individual lanes of a 5–15% polyacrylamide gel; electrophoresis was performed in TBS at a constant current of 35 mA on 8 cm \times 7 cm gels. Molecular weight standards were purchased from Bio-Rad Laboratories or Sigma Immunochemicals and were run simultaneously. The separated proteins were then electrotransferred onto polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-P; Millipore Corp., Bedford, MA) using a Semiphor transfer unit. After saturation of the nonspecific sites with 5% non-fat dry milk/TBS overnight, the proteins were probed with 1:250 dilutions of (a) rabbit anti-human cC1q-R, (b) mouse anti-human CD15, (c) mouse anti-human CR3, or (d) 1:500 diluted rabbit anti-human gC1q-R in TBS/milk overnight at 4°C. The strips were then washed in TBS/0.4% Tween 20 and incubated with 1:1,000 appropriate second IgG antibody peroxidase conjugate. After extensive washing, the immunoblots were exposed to enhanced chemiluminescence (ECL) immunoassay substrate reagent (Amersham International, Buckinghamshire, United Kingdom) for 1 min, and the membranes were then exposed to x-ray film (XAR-film; Eastman Kodak Co., Rochester, NY). Ligand blot experiments were performed as described above, except the neutrophil subcellular proteins were first exposed to free C1q (5 μ g/ml) or biotinylated gC1q (400 ng/ml) overnight in TBS/milk under physiological or low ionic strength conditions before probing the blots with 1:1,000 diluted mouse anti-human C1q (Quidel.Q, San Diego, CA), followed by 1:2,000 diluted goat anti-mouse horseradish peroxidase (HRP)-conjugated second antibody or 1:1,000 dilution of streptavidin-HRP conjugate (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) for biotinylated ligands, followed by chemiluminescence assay. Biotinylation of purified, collagenase-resistant globular domain of C1q (gC1q) was done essentially as described earlier, with the only modification being the use of NHS-LC-biotin (Pierce, Rockford, IL) instead of NHS-SS-biotin (18).

Two-dimensional electrophoresis. Isoelectric focusing and SDS-gel electrophoresis was performed as described by O'Farrell (26). First dimension isoelectrofocusing gels were cast in glass tubes (75 \times 1.5 mm). An equal volume of granule or cytoplasm extraction mixtures

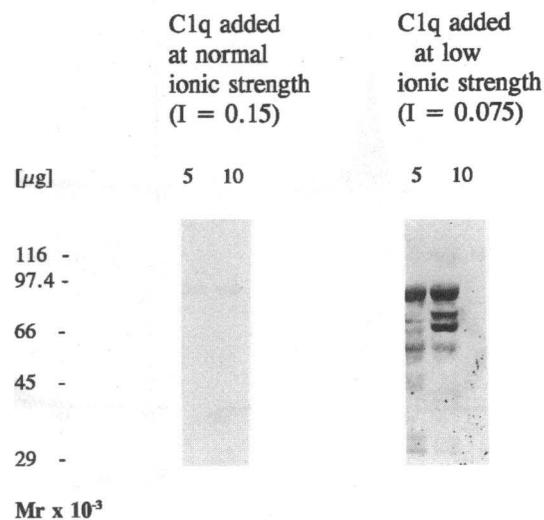
was prepared in sample buffer (to give a final concentration of 9.5 M urea, 2% Nonidet P-40, 5% 2-mercaptoethanol, 2% ampholytes [1.6% pH 3.0–10, and 0.4% pH 5.0–7.0; Bio-Rad Laboratories]) and layered on the gel (maximum 20 μg of protein), and isoelectric focusing was carried out for 12–14 h at 400 V with pre-electrofocusing. After isoelectric focusing, the gels were equilibrated by gentle rocking at room temperature for 5 min in 1 ml of Tris-HCl/SDS equilibration buffer (Millipore Corp.). Gels were immediately electrophoresed in the second dimension on a gel apparatus (SE220; Hoefer Scientific Instruments, San Francisco, CA). Isoelectric focusing gels are layered on top of 5–15% SDS-PAGE gels with a layer of 1% agarose between the two gel types. The gel was electrophoresed at 35 mA/slab for \sim 3 h, and then proteins were electrotransferred onto PVDF transfer membrane using a Semiphor (TE 70; Hoefer Scientific Instruments, San Francisco, CA) transfer unit for 2 h. The blots were then probed with anti-gC1q-R peptide as described above for Western blot analysis. The isoelectric point calibration of two-dimensional gels was estimated by running a parallel isofocusing gel with each isoelectric focusing experiment loaded with standard proteins of known molecular weight and pI (two-dimensional SDS-PAGE standards; Bio-Rad Laboratories). Gels were run in the second dimension and stained with 0.25% Coomassie blue in 45% methanol/10% acetic acid and destained with 10% acetic acid/50% methanol. The molecular weight standards consisted of conalbumin, 76,000 (pI 6.0, 6.3, 6.6); BSA, 66,200 (pI 4.98, 5.07, 5.18); actin, 43,000 (pI 5.47, 5.50, 5.53); GAPDH, 36,000 (pI 8.3, 8.5); carbonic anhydrase, 31,000 (pI 5.9, 6.0); and trypsin inhibitor, 21,500 (pI 4.5).

Flow cytometry. Resting, FMLP-, and PMA-stimulated neutrophils were placed in reagent A (PBS without Ca^{2+} or Mg^{2+} , containing 0.1% vol/vol heat-inactivated human serum) for 15 min, then whole heat-inactivated serum for another 15 min to block remaining nonspecific sites. Neutrophils were then placed in heat-inactivated serum-containing rabbit polyclonal antibodies against either cC1q-R or gC1q-R (diluted 1:50 from 1 mg/ml stocks), nonimmune IgG (negative control), or CD15 (positive control) for 30 min at 4°C. After two washes in reagent A, cells were labeled with FITC anti-rabbit F(ab')₂ IgG over a 30-min period, then washed and fixed in 1% paraformaldehyde in PBS. Flow cytometry was performed on a FACScan® analyzer (Becton Dickinson, Mountain View, CA) running on Lysis II software. cC1q-R and gC1q-R expression was estimated by monitoring the mean fluorescent intensity (MFI) differences.

Enzyme-linked immunosorbent assay. Microtiter plate wells were coated with either C1q, lectin proteins (all at 500 ng/well), or 100- μl volumes of cell suspension media recovered from 10^6 nonstimulated and stimulated cells. BSA acted as a negative control protein. All proteins were prepared in sodium carbonate buffer (pH 9.6) unless stated and allowed to bind to the wells for 14 h at 4°C. Nonimmune rabbit IgG acted as a nonspecific immunoglobulin. Plates were washed in PBS/Tween, and nonspecific binding sites were blocked with 5% milk/100 mM glycine. rgC1q-R ($-1 \mu\text{g}/\text{ml}$) was added to each well for 2 h at 37°C and then washed three times before anti-gC1q-R or anti-gC1q-R peptide was added to each well for 1 h at 37°C. The wells were washed again and 1:1,000 dilution HRP-conjugated anti-rabbit polyclonal IgG was added. After three additional washes with PBS/Tween 20, 150 μl of 3,3',5,5'-tetramethyl benzidine (TMB)-peroxidase substrate was added to each well, and the color was developed for 20 min. The reaction was terminated by adding 2 N H_2SO_4 , and the plate was read at 450 nm.

Superoxide production by HL-60 cells. Monitoring of HL-60 differentiation was performed by assessing the ability of RA-treated cells to generate superoxide as measured by the O_2^- dismutase-inhibitable reduction of ferricytochrome *c* as previously described (17). Briefly, 10^6 HL-60 cells were exposed to either FMLP (10^{-6} M) or PMA (100 ng/ml, final concentration) at 37°C, and superoxide production was monitored over a 30-min period in a UV/VIS double beam spectrophotometer (model 559; Perkin-Elmer Corp., Norwalk, CT), and the rate of O_2^- generation was expressed as nanomoles of O_2^- per minute per 10^7 cells calculated from an extinction coefficient for reduced cytochrome *c* of 21.2×10^3 (mol/liter) $^{-1}$ cm^{-1} .

A. Identification of C1q - binding proteins



B. Identification of cC1q-R and gC1q-R

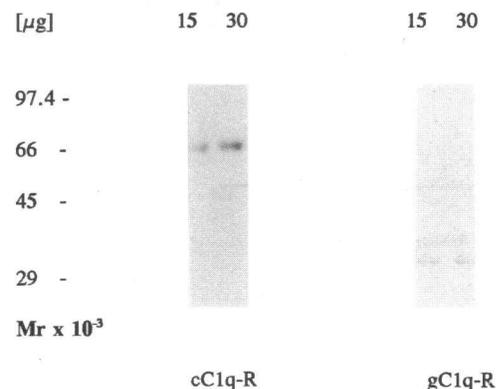


Figure 1. Ligand blot of free C1q (5 $\mu\text{g}/\text{ml}$) binding to neutrophil membrane proteins separated under reduced conditions on a 5–15% SDS-PAGE gel and blotted onto PVDF membrane under (A) physiological ($I = 0.15$) and low ($I = 0.075$) ionic strength conditions. (B) Western blot of a 33-kD gC1q-R and the 68-kD cC1q-R identified as neutrophil membrane proteins.

Results

Identification of cell surface proteins from human neutrophils that bind to C1q. Plasma membrane proteins from nonstimulated neutrophils were separated under reducing conditions on 5–15% SDS-PAGE gels and blotted onto PVDF membranes. C1q in milk/TBS at physiological strength and half physiological strength buffer was applied to the blot overnight. Fig. 1 A shows that C1q binds very weakly to a single protein of apparent molecular weight of 90,000 under physiological conditions. Under low ionic strength conditions, C1q binds strongly to a number of other membrane proteins with apparent molecular weight of 90,000; 75,000; 68,000 and 60,000; in addition, two lower molecular weight proteins of 33,000 and 30,000 also bind to C1q. Separate blots of the neutrophil membrane proteins shown in Fig. 1 B were probed with polyclonal antibodies raised against two purified C1q-binding proteins, the cC1q-R, which

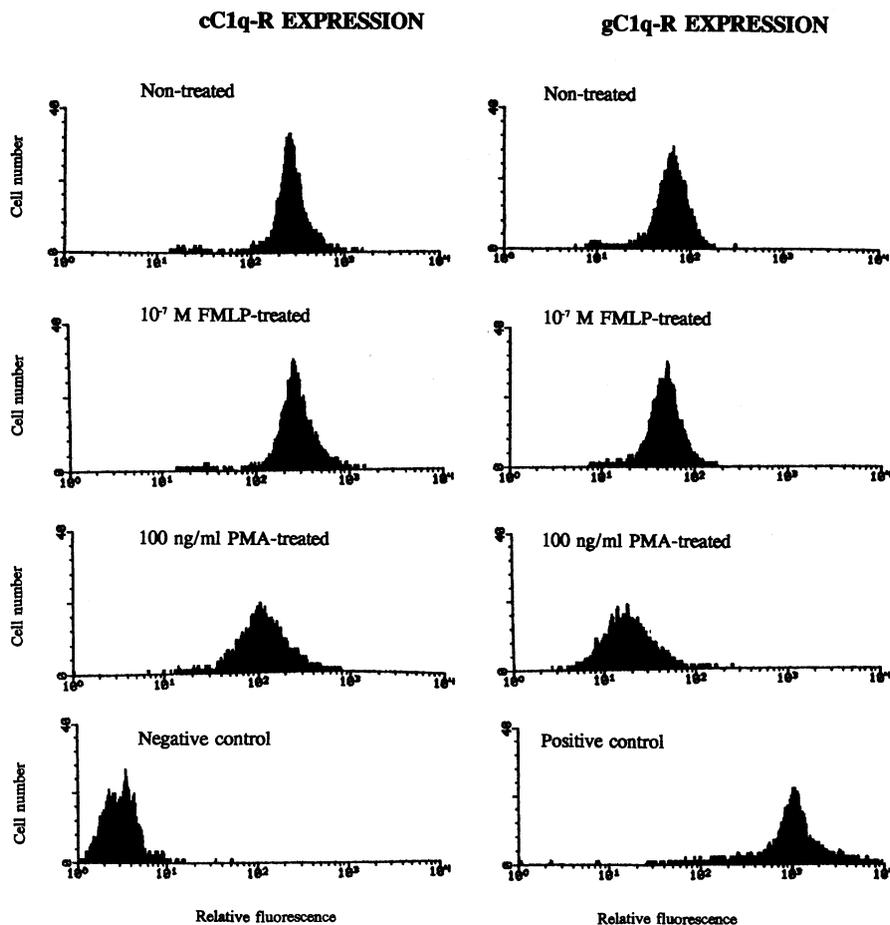


Figure 2. Distribution of cC1q-R and gC1q-R on human neutrophils as assessed by flow cytometry. Cells were left unstimulated or stimulated with FMLP or PMA as described in Methods. Positive control cells were incubated with CD15 and negative control cells with nonimmune rabbit IgG. Mean fluorescence intensity is plotted on a log scale.

has been shown previously to bind to the collagen domain of C1q, and the gC1q-R, which has a high affinity for the globular head region of the C1q protein. The antibodies identified proteins of 68,000 and 33,000 M_r , respectively. To confirm the expression of these two C1q-binding proteins on the plasma membrane of intact cells, nonstimulated neutrophils and neutrophils stimulated with PMA (100 ng/ml) or FMLP (10^{-7} M) for 15 min at 37°C were then placed on ice, labeled with either 1:50 dilution anti-cC1q-R or anti-gC1q-R, and then stained with 1:100 dilution anti-rabbit FITC-labeled antibody. Fig. 2 shows the cell surface expression of each C1q receptor. The intensity of staining was less for gC1q-R compared with cC1q-R, regardless of cell treatment, but this may simply reflect differences in the affinity or titer of antibody used. The MFIs for cC1q-R and gC1q-R were under resting conditions (277 vs 66), FMLP-stimulated (266 vs 51), or after PMA treatment (130 vs 26), respectively. Both cC1q-R and gC1q-R expression decreased 2.5-fold after stimulation with PMA.

Phenotypic expression of gC1q-R and cC1q-R was also studied in comparison with other complement receptors (Table I) on the surface of differentiating HL-60 cells. Throughout the 8-d test period, control cells treated with nonimmune IgG and stained with FITC-labeled (Fab')₂ secondary antibodies consistently gave low MFI values (MFI = 5–6 units). In contrast, staining for gC1q-R was evident on undifferentiated cells (day 0, MFI = 12.3) and progressively increased on the cell surface during differentiation of the cells with RA, while cC1q-R appeared after 6 d. Low levels of CR3 and CR1 receptors were

present on undifferentiated cells and steadily increased after 2 and 3 d of differentiation, respectively.

Detection of soluble cC1q-R and gC1q-R in neutrophil suspension media after stimulation. Neutrophils were suspended at a concentration of 10^7 cells/ml in PBS with Ca^{2+} and Mg^{2+} , prewarmed to 37°C for 5 min, and then stimulated with FMLP (10^{-7} M) or PMA (100 ng/ml) for an additional 15 min. Neutrophils were pelleted in Eppendorf tubes at 2,000 g for 2 min, then cell suspension media (PBS) was recovered and recentrifuged at 16,000 g for 30 min at 4°C to remove any precipitous material. ELISA analysis of the cell suspension media using anti-gC1q-R and anti-cC1q-R detected very little cC1q-R in the cell suspension media from both resting and FMLP-stimulated neutrophils (Table II). Both cC1q-R and gC1q-R were detected by ELISA in the cell suspension media of cells treated with PMA.

Subcellular analysis to identify localization of C1q-binding proteins in the neutrophil. To determine if either gC1q-R or cC1q-R was located in a subcellular store, preparations of primary and secondary granules and the cytosolic fractions from neutrophils were studied by blotting PVDF membranes containing the subcellular fractions with polyclonal rabbit anti-cC1q-R, anti-gC1q-R peptide polyclonal test antibodies, and control antibodies against CR3 and CD15. The gC1q-R peptide antibody recognized three proteins of different molecular weight, two bands of 200,000 and 33,000 M_r were identified in the cytosolic fraction, and a band of 80,000–90,000 M_r was located in the secondary granule fraction (Fig. 3). In contrast,

Table 1. Effect of RA Differentiation of HL-60 Cells on CR and CR Expression

Days of differentiation	Receptor expression (MFI)					O ₂ ⁻ (nmol/min/10 ⁷ cells)	
	cC1q-R	CR1	CR3	gC1q-R	Control	FMLP-stimulated	PMA-stimulated
0	7.2	7.5	13.4	12.3	5.9	0.00	0.00
1	6.6	9.7	26.8	9.00	5.9	0.00	0.00
3	6.3	12.5	60.5	22.0	6.1	0.00	0.00
6	16.7	10.2	78.9	24.7	3.4	14.2	11.8
8	17.4	14.3	87.5	27.1	6.1	16.6	23.7
Neutrophil	141	26.0	163.0	38.0	5.4	28.4	42.6

O₂⁻, superoxide production.

no subcellular localization of the membrane-bound cC1q-R of 68,000 M_r was identified. Control proteins CR3 and CD15 which are known to be present in the secondary granules of neutrophils were both evident within these granules. To determine which of the three identified proteins actually binds to C1q, a ligand blot of the subcellular fractions was performed under normal and low ionic strength conditions, in which a separate blot was incubated with C1q (5 μg/ml) overnight and then probed with an anti-C1q monoclonal antibody. C1q was observed to bind only to a band of 80,000–90,000 M_r and appeared to be at the exact same position as the protein recognized by the anti-gC1q-R peptide antibody under normal ionic conditions (Fig. 4). MBP (5 μg/ml) was also used as a ligand-binding probe, but failed to bind to the 80,000–90,000 M_r secondary granule protein.

The cell surface gC1q-R has been estimated to be very acidic with a pI of ~ 4.1. To further characterize the intracellular C1q-binding proteins, two-dimensional gel electrophoresis was performed on the secondary granule and cytosolic protein fractions, which were then transferred to PVDF membranes and probed with anti-C1q-R peptide. A single dominant protein of 80,000–90,000 M_r was observed on the secondary granule protein blot (Fig. 5 A). Based on molecular weights and isoelectric range estimates which were run simultaneously, the pI of the

Table II. Detection of Soluble cC1q-R and gC1q-R Released from Neutrophils during Stimulation

Conditions	Absorbance at 450 nm	
	gC1q-R (n)	cC1q-R (n)
0°C	0.37±0.11 (7)	0.007±0.010 (3)
37°C	0.45±0.14 (7)	0.008±0.010 (3)
10 ⁻⁷ M FMLP*	0.61±0.18 (7)	0.001±0.001 (3)
100 ng/ml PMA	0.98±0.09 [‡] (7)	0.032±0.010 [§] (3)

[‡] Levels of soluble gC1q-R in cell suspension media from PMA-stimulated cells were significantly different compared with unstimulated cells (37°C) at confidence levels of P < 0.001 as determined by the Student's t test. [§] Levels of soluble cC1q-R in cell suspension media from PMA-stimulated cells were significantly different compared with unstimulated cells (37°C) at confidence levels of P < 0.001 as determined by the Student's t test. * Values for both gC1q-R and cC1q-R in media from FMLP-stimulated cells and nonstimulated cells maintained at 4°C were not significantly different from prewarmed cells.

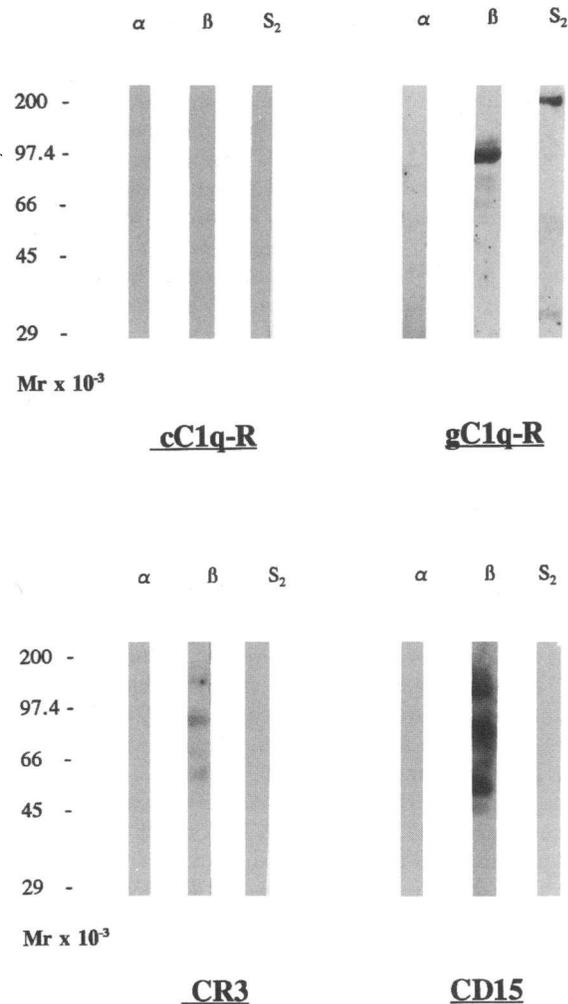
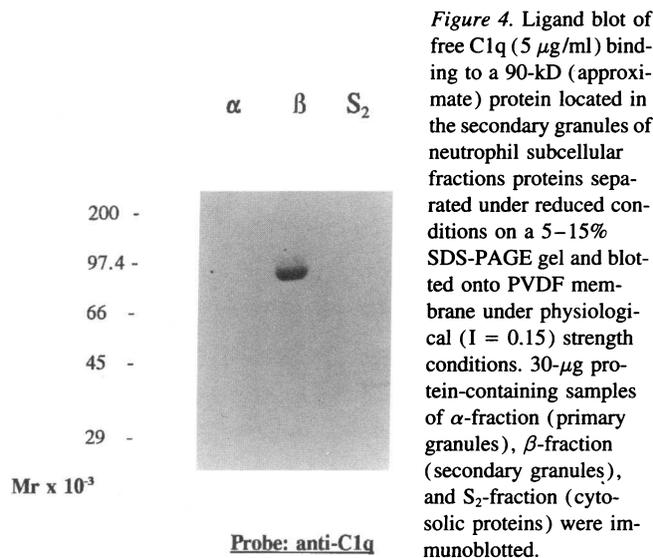


Figure 3. Western blots of gC1q-R, cC1q-R, CR3, and CD15 of neutrophil subcellular fractions. Neutrophils were disrupted by nitrogen cavitation, fractionated over discontinuous Percoll gradients, separated by SDS-PAGE in a 1–15% polyacrylamide gel under reducing conditions, transferred to PVDF membranes, and immunoblotted with either anti-gC1q-R, anti-cC1q-R, anti-CR3, or anti-CD15 as detailed in Methods. 30-μg protein-containing samples of α-fraction (primary granules), β-fraction (secondary granules), and S₂-fraction (cytosolic proteins) were immunoblotted.



protein was estimated to be 4.3 or less and therefore consistent with the calculated pI for the 33,000 M_r membrane version of the protein isolated from Raji cells. The cytosolic proteins (fraction S_2) also underwent two-dimensional gel electrophoresis. The original bands of 33,000 M_r focused as two spots of 33,000 and 28,000 M_r , this latter protein being a breakdown product of the 33,000 M_r protein. Both proteins have a pI of 4.5 (Fig. 5 B), the 200,000 M_r protein which reacted with anti-gC1q-R in the Western blot did not appear on the two-dimensional blot and was most likely a protein with nonspecific binding activity for the anti-gC1q-R peptide antibody.

The collagenase-resistant, globular domain of C1q (gC1q) was next examined for its ability to bind directly to proteins from neutrophil subcellular fractions blotted onto PVDF membranes. As shown in Fig. 6 A, the biotinylated gC1q used for binding studies was pure and showed appropriate size expected after loss of collagen domain from C1q. Biotinylated gC1q bound a 33,000 M_r protein in both α - and γ -fractions and an 80,000–90,000 M_r protein in α -, β -, and cytoplasmic fractions (Fig. 6 B).

rgC1q-R (33,000 M_r) binds to C1q and lectins to varying degrees. The cC1q-R has been found to bind to not only C1q but also to three structurally similar ligands, MBP, conglutinin, and lung SP-A, which share similar collagenous domains but dissimilar globular domains. C1q (250 ng) and a number of test lectins (human MBP, human SP-A, human and rat SP-D, and bovine conglutinin), together with BSA which acted as an irrelevant protein, were immobilized to plastic microtiter wells overnight at 4°C in sodium carbonate buffer. Purified gC1q was added for 2 h under low or normal ionic strength, the wells were then washed, and antibodies raised against both the purified protein and 18-amino acid peptide were used to detect gC1q-R binding. The results shown in Fig. 7 indicate that under normal ionic conditions C1q binds significantly to gC1q-R ($P < 0.01$). In addition, gC1q-R also appears to bind moderately to SP-D and SP-A. Previous cell-binding studies have shown that C1q binding to other C1q-binding proteins is enhanced under low ionic strength conditions, and as expected under such conditions, gC1q-R binding to C1q, SP-A, and SP-D is further enhanced significantly ($P < 0.05$).

Effect of calcium and C1q-aggregation on C1q-binding to gC1q-R. Using an ELISA technique, the effect of calcium on

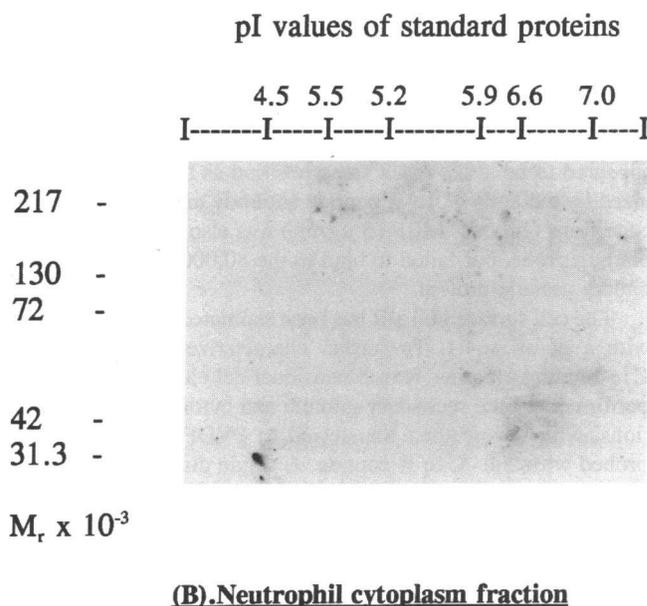
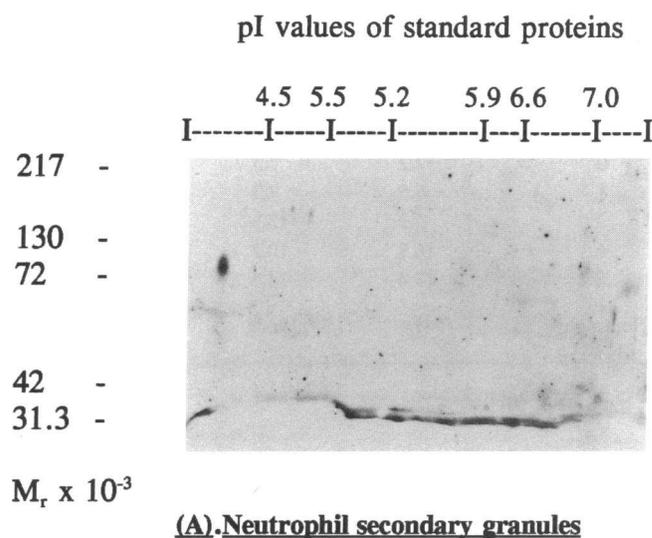


Figure 5. Two-dimensional isoelectric focusing and SDS-PAGE of proteins from the cytosolic fraction and secondary granules probed with anti-gC1q-R peptide. The spots identified by Arabic numerals indicate the labeled proteins most consistently confirmed in several experiments.

the ability of gC1q-R to bind monomeric C1q is shown in Fig. 8. Ligand binding to gC1q was greatest at low ionic strength, and under these conditions the presence or absence of calcium made no difference to binding. However, under normal ionic conditions, the presence of calcium resulted in a threefold decrease in binding of C1q to gC1q-R. Dextran sulfate (Dx) is a polyanion which has the ability to form aggregates with C1q at physiological pH. C1q was mixed with Dx in a wt/wt ratio of 1:1 and allowed to aggregate. As shown in Fig. 9, aggregate formation was pH-dependent and instantaneous. Monomeric and C1q:Dx aggregates were then immobilized and gC1q-R-binding was assessed under normal and low ionic strength conditions (Fig. 9). No enhancement of the binding of gC1q-R

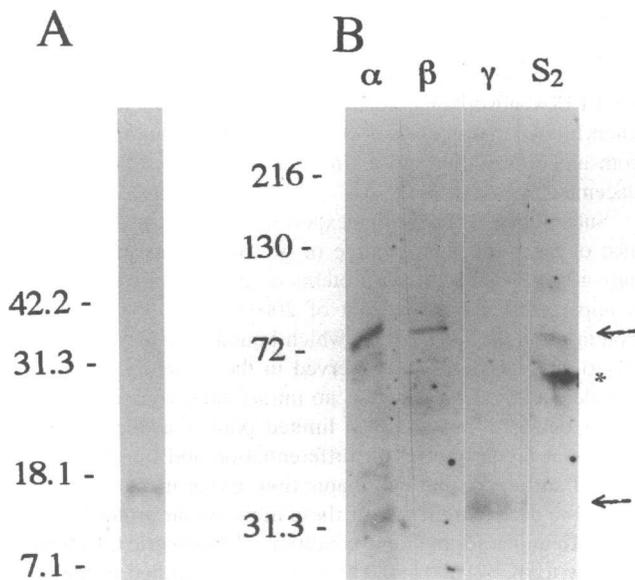


Figure 6. Direct ligand-binding studies using globular domain of C1q. (A) 10% SDS-PAGE of biotinylated gC1q used for binding studies. Approximately 200 ng of the biotinylated gC1q was electrophoresed under reducing conditions, transferred to PVDF membranes, and detected using streptavidin-HRP conjugate followed by chemiluminescence assay. (B) Binding of biotinylated gC1q to blots of various human neutrophil subcellular fractions indicated, prepared as described under legend to Fig. 3 and Methods. The numbers to the left are $M_r \times 10^{-3}$. The arrows indicate the 80,000–90,000 and 33,000 M_r proteins. *, artifact.

to aggregated C1q was observed under normal or low ionic conditions.

Discussion

In this study, the gC1q-R was identified as one of several C1q-binding proteins associated with the neutrophil plasma membrane surface which range in size from 90,000 to 30,000 M_r . In a previous study, and under different SDS-PAGE conditions, an additional C1q-binding protein of 120,000 M_r has also been observed (11). gC1q-R surface expression during differentiation was examined, as was the subcellular localization of gC1q-R and its spontaneous and stimulated release, together with its in vitro binding characteristics to C1q and serum lectins. Monomeric C1q binds to a number of neutrophil membrane proteins under low ionic conditions (Fig. 1 A), which is consistent with the findings of others (27), and is indicative that some conformational change in C1q is normally required before C1q will bind under physiological conditions. Polyclonal antibodies were raised against two of these membrane C1q-binding proteins that had been purified previously from the human Raji lymphoblastoid cells (13, 28). One of these proteins, the cC1q-R, binds to the collagen region of C1q (29), while gC1q-R has a greater affinity for the globular head of C1q (12). The amino terminus of the gC1q-R is reported to be L(R)A(H)TDGD-KAFVDFLSDEIKEE (R) KIQ, (13) the underlined sequence was used for the generation of a synthetic peptide and antipeptide antibody used in this study. The cC1q-R amino terminus is completely different and is comprised of EPAVYF-KEQFLDGDG (30). Anti-cC1q-R antibody recognized a pro-

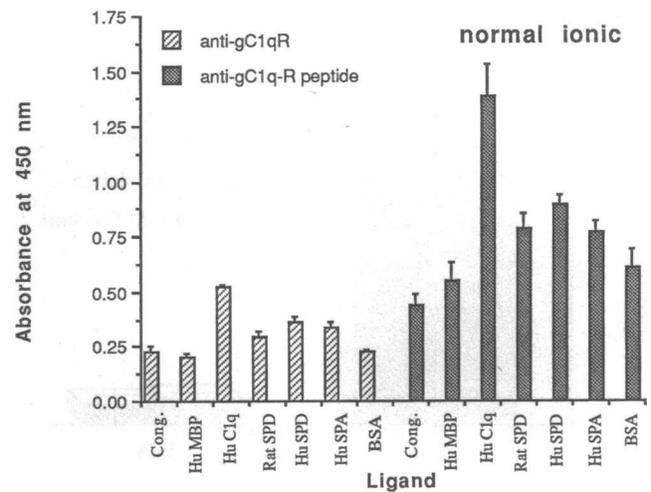


Figure 7. Binding of gC1q-R and gC1q-R peptide to immobilized C1q and various lectins under physiological and low ionic strength conditions. Each data point represents a mean of three separate experiments.

tein of 68,000 M_r on the plasma membrane of neutrophils, whereas anti-gC1q-R detected a protein of 33,000 M_r (Fig. 1 B). Both proteins corresponded to neutrophil plasma membrane proteins of a similar relative migration which were shown to bind to C1q (Fig. 1 A). Our data demonstrate that both cC1q-R and gC1q-R are present on the cell surface of mature nonstimulated neutrophils.

The phenotypic expression of cC1q-R and gC1q-R was studied using the human promyelocytic HL-60 cell line. When HL-60 cells were maintained in a undifferentiated state, CR3 expression was detectable in low levels (Table II), suggesting CR3 is expressed at an early stage of development. Induction of HL-60 cells for 3 d with RA (1 μ M) led to additional increases in CR3 expression and the detection of CR1 expression. After 6 d of differentiation, surface expression of both gC1q-R and cC1q-R was detectable, at the same time that the cells were

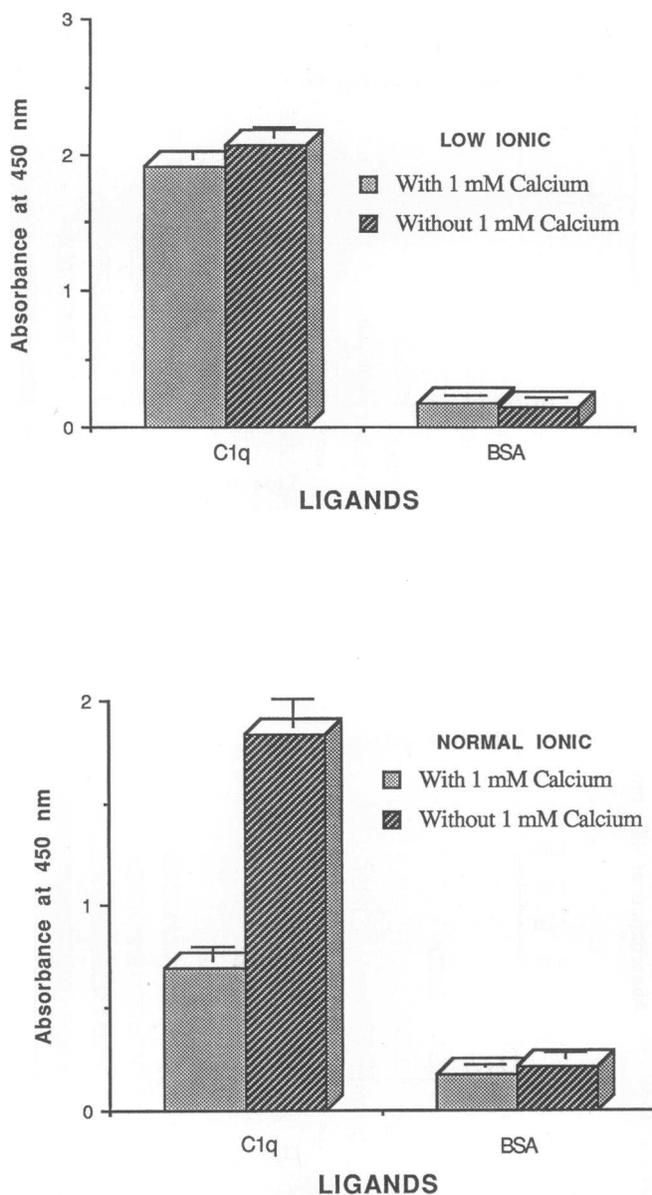


Figure 8. Binding of gC1q-R to C1q and influence of ionic strength or calcium on binding assessed by ELISA, as detailed in Methods.

capable of generating superoxide in response to both FMLP and PMA (Table I). After 8 d, cC1q-R expression no longer increased, while CR1, CR3, and gC1q-R levels continued to increase and superoxide production continued to rise.

When mature neutrophils were stimulated with PMA, both cC1qR and gC1q-R expression were downregulated by three-fold (Fig. 2), while FMLP treatment failed to alter surface expression of either of these two proteins. Cell viability was maintained throughout these experiments and checked by microscopic examination, trypan blue exclusion, and the ability of the cells to produce a respiratory burst. To determine if the reduction in fluorescence was due to shedding or secretion of the protein from the cell, an ELISA was used to measure soluble protein levels of cC1q-R and gC1q-R in the cell suspension buffer (PBS) before and after stimulation. Very small increases in cC1q-R levels in the cell suspension media of PMA-stimu-

lated cells were seen compared with resting cell levels (Table I), suggesting the limited quantities of membrane-associated protein were being displaced from the cell surface. In general, the ELISA absorbance readings for gC1q-R secretion were much higher than those for cC1q-R, possibly due to secretion from an intracellular source in combination with surface displacement.

Subcellular fractionation experiments confirmed the presence of an intracellular source of gC1q-R. Blots probed with anti-gC1q-R detected two proteins in the cytosolic component of apparent molecular weight of 200,000 and 33,000, and a protein of 80,000–90,000 M_r which bound with greatest intensity to anti-gC1q-R was observed in the secretory secondary granules (Fig. 3). In contrast, no intracellular source of cC1q-R was detected, suggesting a limited pool is available which appears at an early stage of differentiation and translocates to the cell surface during cell maturation. Experiments were performed to determine if any of these intracellular proteins could bind C1q under normal ionic conditions. Monomeric C1q bound strongly to the 80,000–90,000 M_r protein identified in the secondary granules and weakly to the 33,000 M_r protein in the cytosolic fraction but did not bind to the 200,000 M_r protein band identified in the same fraction (Fig. 4). These data suggest that this larger protein is either an irrelevant protein which cross-reacts with anti-gC1q-R peptide antibody or an inactive precursor of the 33,000 M_r membrane protein. The former proposal is more likely as the 200,000 M_r protein is not detected on two-dimensional immunoblots of the cytosolic fractions (Fig. 5 B). Additional binding studies also revealed that the 80,000–90,000 M_r antigen recognized by anti-gC1q-R in the secondary granules and which binds avidly to C1q under physiological conditions does not bind to MBP (data not shown) which shares a similar ultrastructure to C1q; therefore the protein is more specific for C1q. This protein, which is also recognized by the same antibodies which bind to the 33-kD protein, most likely shares some common epitopes which may be involved in recognizing the globular head region of C1q. Further analysis by two-dimensional electrophoresis confirmed the existence of a single acidic protein of 80,000–90,000 M_r , of pI 4.1–4.2 recognized by the anti-gC1q-R (Fig. 5 A). Therefore, it is unlikely that the cytosol and membrane gC1q-R 33,000 M_r protein originates from the 80,000–90,000 M_r secondary granule protein, based on the predicted weight of 33,000 D from the published amino acid sequence (13). In addition, unlike CR3, which is found in the secondary granules and is mobilized from granule stores and expressed on neutrophil plasma membranes in greater quantities after PMA or FMLP stimulation, gC1q-R is not upregulated after stimulation with secondary granule degranulating stimuli. In addition, a 33,000 M_r form of gC1q-R is not apparent in the secondary granules, but does appear in the cytosolic fraction. Whether additional processing occurs in a granule constituent of the cytosolic component before mobilization of the final version of the protein to the cell surface remains to be answered. In a previous study, the gC1q-R was found to be a highly acidic protein, with a calculated pI of 4.15. In this study, two-dimensional electrophoresis of cytosolic fraction probed with anti-gC1q-R peptide detected a 33,000 M_r protein (Fig. 5 B) with a pI of 4.5. These results indicate the presence of a C1q-binding protein in the secondary granules with an acidic pI, which shares a common epitope to the 33,000 M_r gC1q-R protein found on the cell membrane and in the cytosol. Use of biotinylated gC1q enhanced the sensitivity of the direct ligand-

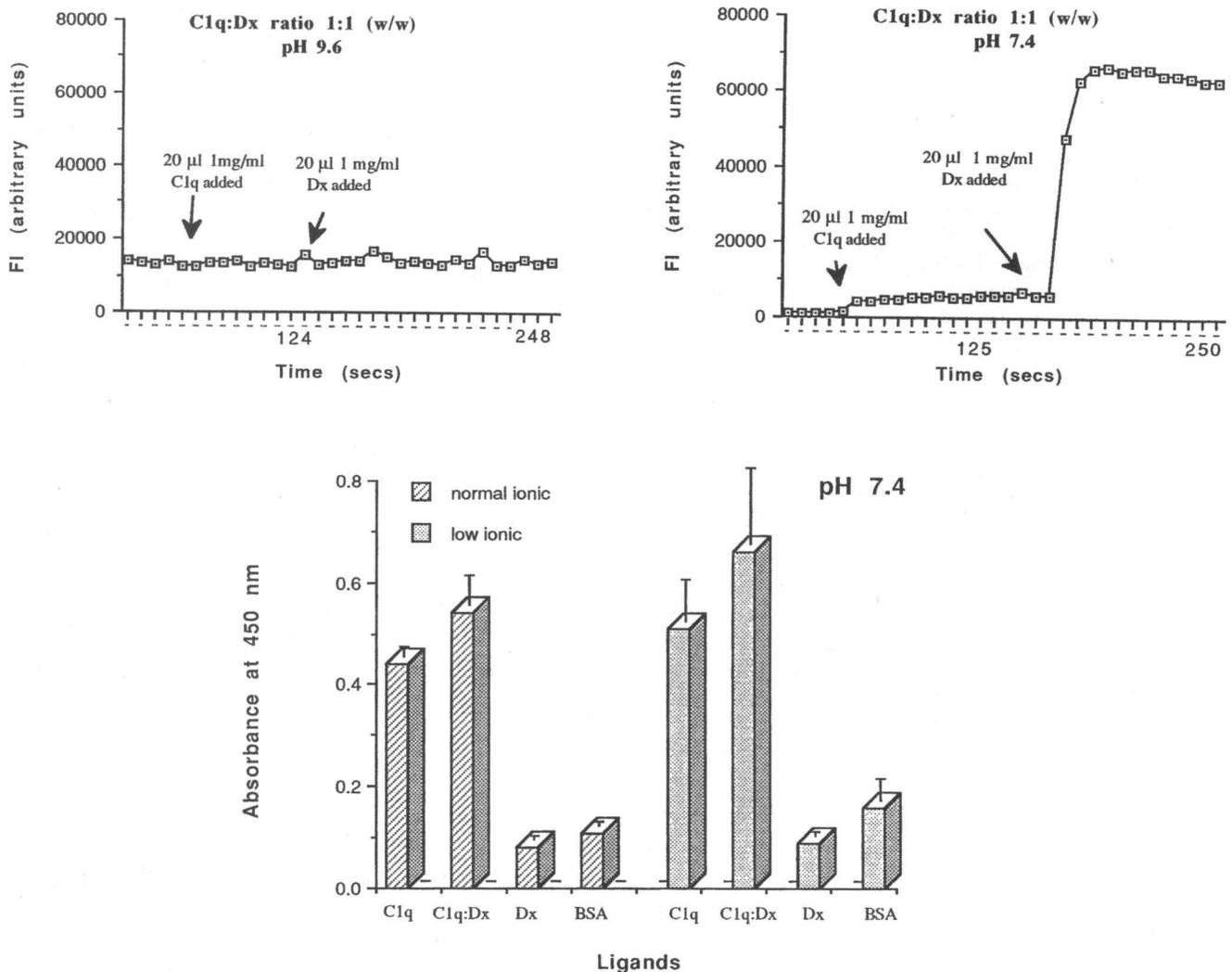


Figure 9. Effect of aggregation on binding of gC1q-R to C1q at physiological ($I = 0.15$) and low ($I = 0.075$) ionic strength. C1q was mixed with dextran sulfate in a ratio of 1:1 (wt/wt) at either pH 9.6 or 7.4. Aggregate formation was monitored in a fluorometer by 90° light scattering. Fluorescence intensity is in arbitrary units. Aggregates were immobilized to microtiter wells overnight, and gC1q-R binding was assessed by ELISA.

binding assay and is perhaps the reason for our ability to detect gC1q-R in more fractions (Fig. 6), as compared with earlier studies using antibodies to gC1q-R. Biotinylated gC1q allowed the detection of an 80,000–90,000 M_r protein in α - and S_2 fractions of neutrophils, which was not detectable in Western blots (Fig. 3).

The cC1q-R has been reported by Malhotra et al. (31) to bind not only to C1q, but also to three other structurally similar ligands, namely MBP, conglutinin, and SP-A via a collagenous region common to all of the proteins. The gC1q-R has a higher affinity for the globular domain of C1q (12). As serum- and lung-associated lectins also have a globular domain, we were prompted to examine its possible interaction with these lectins as well as C1q. ELISA analysis demonstrated that gC1q-R binds to C1q strongly and to SP-A and SP-D more moderately under normal ionic conditions; under the less stringent low ionic binding conditions, gC1q-R binding to these proteins was enhanced further (Fig. 7). However, as the amino acid sequences of the globular domains of SP-A and SP-D are completely divergent from that of C1q and bind to carbohydrates and peptides, respec-

tively (32), the moderate binding seen may be nonspecific and based on common physical features of the surfactant proteins and C1q (33). Our binding studies performed under low ionic conditions confirmed previous reports that such conditions can enhance C1q interaction with C1q-binding proteins (34). Unlike other C1q receptors isolated from leukocytes, gC1q-R does not bind to MBP nor conglutinin under any test conditions. This indicates that gC1q-R does not bind to C1q via a common binding domain shared by proteins of similar ultrastructure as is the case for the cC1q-R (Table II) (31). Although C1q binds to gC1q-R under normal ionic strength conditions, the presence of physiological levels of calcium reduces binding of gC1q-R to C1q (Fig. 8). This observation may partially explain why only ~5–10% of neutrophils stain positive for C1q after incubation with monomeric C1q under physiological conditions (5). However, when C1q is bound to Latex beads or bound to pathologically relevant compounds such as immune complexes, virus, and lipopolysaccharides (35) via the globular domain, some structural alteration in the C1q molecule is believed to occur facilitating binding of C1q to the cC1q-R-type receptors. In

this study, no increase or decrease in binding was observed when C1q was complexed with dextran (Fig. 9), which suggests that, unlike cC1q-R–C1q interactions which have been reported to be enhanced under these same conditions, the gC1q-R interaction may play a different role within the broad scheme of C1q–C1q-R interactions. The results of this report raise two important questions: (a) what functional role does soluble gC1q-R play in the inflammatory repertoire?; and (b) what is the nature of the 80,000–90,000 M_r C1q-binding protein present in the secondary granules that binds to C1q under physiological conditions and is recognized by the anti-gC1q-R antibody? Soluble gC1q-R may participate in various other biological functions of neutrophils or other circulatory cells in an aggregated or monomeric form. The release of complement receptors into the circulation facilitates the possibility of new, but as yet, undefined biologic consequences. A recent study has detected elevated levels of soluble CR1 released by human leukocytes in a number of disease states (36). Downregulation of cell surface receptors either by internalization or surface shedding is one way in which cells control their responses to agonists. Shedding of many plasma receptors from cells has been described, but the biochemical processes involved are not well understood. In this study, gC1q-R was found in the cell buffer media of PMA-activated neutrophils. In view of the recent evidence which suggests gC1q-R can mediate chemotaxis and/or chemokinesis (6), the presence and release of soluble gC1q-R during cell activation may constitute a cellular strategy for additional cell recruitment at local inflammatory sites. Further investigations are needed to establish the function of this novel receptor which binds to the globular domain of C1q.

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