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C1q-independent activation of neutrophils by immunoglobulin M-coated surfaces[1]

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Running Title: C1q-independent activation of neutrophils by IgM

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[1] No benefit of any kind will be received either directly or indirectly by the authors.

Abstract: Neutrophil granulocytes are known to rapidly adhere and undergo frustrated phagocytosis upon contact with immunoglobulin and/or complement protein opsonized artificial surfaces. In this study, we examined the relation between serum protein deposition and human neutrophil activation on hydrophobic glass and silicon model surfaces that were coated with immunoglobulin G or M (IgG/IgM), both initiators of the classical complement pathway. Protein adsorption from normal human serum (NHS) was quantified with null-ellipsometry combined with antibody techniques. The neutrophil oxygen radical production was registered by luminol-amplified chemiluminescence (CL) and the morphology, as well as changes in the content of filamentous actin (F-actin), was documented by fluorescence microscopy. Complement factor 3 (C3) bound to both IgG- and IgM-coated surfaces, but surprisingly C1q was found only on IgG-coated surfaces. Both immunoglobulins triggered complement dependent neutrophil activation. However, CL and F-actin accumulation were found sensitive to the presence of C1q in serum only at the IgG-coated surface. We suggest that spontaneously adsorbed IgM activates the complement system and interacts with neutrophils by C1q-independent mechanisms.

Keywords: complement activation; immunoglobulins; actin cytoskeleton; neutrophil respiratory burst; protein adsorption

INTRODUCTION

It is well established that artificial surfaces often activate both the cellular and humoral constituents of the inflammatory response upon contact with body fluids (reviewed by Tang and Eaton).[1] Biomaterials may rapidly recruit leukocytes *e.g.* neutrophil granulocytes, a phenomenon likely mediated by cascade reactions following the spontaneous host plasma protein adsorption. The plasma protein layer composition is greatly influenced by relatively simple biomaterial characteristics,[2-4] and different surface wettabilities provoke different behavior in adherent neutrophils.[5-8] The neutrophil activation involves different antimicrobial mechanisms including oxygen radical production[9, 10] and release of proteolytic enzymes,[11] and excessive neutrophil activation at surfaces may potentially impair the host defense to subsequent infections.[12]

Two abundant and commonly adsorbed plasma proteins, IgG and fibrinogen, are both potent activators of adherent neutrophils, although by different mechanisms.[13] The complement system opsonizes surfaces and takes active part in bacterial and cellular lysis and the immune complex clearance,[14] but its impact for acute inflammatory response to surfaces has been questioned.[15] Nevertheless, the system is of importance for the maintenance of leukocyte adhesion to larger non-self surfaces, such as blood-contacting biomaterials *in vitro*.[16, 17] The early studies on complement activation at the blood – material interface demonstrated a major role for the alternative activation pathway for the opsonization of in particular nucleophilic artificial surfaces, but the impact of the classical activation pathway is not yet well understood. However, it was recently demonstrated that the classical pathway is of importance also at nucleophilic surfaces[18-20] and IgG, IgM, C1q and C3 have all been observed at the interface between blood and several artificial surfaces.[18, 21-23] In the initiation part of the classical complement activation pathway, the F_c-parts of two surface-bound and adjacent IgG (not IgG_4) or one IgM molecule interact with the globular heads of the C1q (11S-Protein) molecule.[14, 24, 25] The soluble monomeric immunoglobulins, especially IgG, have only a weak affinity for C1q, but when surface-bound or aggregated immunoglobulins form two or three salt bridges with each globular head of C1q.[24, 26] The C1q-binding site of IgG is located in the C_H2

domain and IgM is suggested to bind C1q in the C_H3 domain.[14] C1r and C1s then interacts with their binding sites located in the collagen region of the C1q molecule and the first classical pathway convertase is thereby assembled, and is now able to cleave and activate C4 and C2. These, in turn, form a C3-convertase, and cleaved C3 may form another C3-convertase that is common to all three known complement activation pathways.[25]

The aim of the present study was to compare the effects of adsorption on two recognized classical complement activators, IgG and IgM, by means of subsequent spontaneous serum protein deposition and neutrophil inflammatory response. The experiments were performed using a surface model system that was previously described. [6-8]

MATERIALS AND METHODS

Reagents, sera and buffers

The reagents were of at least analytical grade and delivered from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Normal human serum (NHS) was prepared from whole blood of several donors according to standard procedures, cooled on melting ice and immediately frozen below -70°C. Pooled NHS was used to study protein adsorption and different single donor NHS were used in the neutrophil experiments. C1q-depleted serum and purified C1q were purchased from the Quidel Corp. (San Diego, CA, USA) and the complement cascade in the NHS was inhibited by heat-inactivation for 30 minutes at 56°C. Immobilization of immunoglobulins were performed in Phosphate-buffered saline (PBS; 10 mM sodium hydrogen phosphate, 10 mM potassium dihydrogen phosphate and 0.15 M sodium chloride, pH 7.3). Studies on serum adsorption were performed in Veronal-buffered saline (5 mM 5,5'-dietylbarbituric acid, the corresponding sodium salt and 145 mM sodium chloride, pH 7.4) supplemented with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (VBS^{2+}). All buffers used in the protein adsorption experiments contained 0.25 mM sodium azide. Isolated neutrophils (see below) were resuspended in Krebs-Ringer phosphate buffer supplemented with 10 mM glucose, 1.5 mM $MgSO_4$ and 1.1 mM $CaCl_2$, pH 7.3 (KRG, free from sodium azide).

Isolation of neutrophils

Peripheral human polymorphonuclear neutrophil granulocytes were isolated for each separate experiment from newly drawn heparinized whole blood of non-medicated volunteering donors (apparently healthy). In principle, the isolation procedure utilizes the methodology described by Böyum.[27] Only Ca^{2+}-free buffers and suitable plastic devices were used during the separation. More specifically, whole blood was carefully layered on top of a separation liquid consisting of one part of Lymphoprep (Nycomed Pharma AS, Oslo, Norway) layered over four parts of Polymorphprep (Nycomed Pharma AS) and centrifuged for 40 minutes at 480 x g at room temperature. The upper band containing mononuclear cells was discarded and the lower band containing neutrophils was harvested. The separation liquid was washed off through 10 minutes at 480 x g in room tempered PBS. The erythrocyte contamination was then eliminated by brief hypotonic lysis in ice-cold distilled water followed by washing of the cells twice at 200 x g at 4°C in KRG. The isolated cells showed excellent viability, low platelet contamination and were counted in a Coulter Counter ZM Channelyser 256 (Coulter-Electronics Ltd., Luton, UK) and kept on melting ice until pre-warming to 37°C immediately before the experiments.

Surfaces

Silicon wafers (Okmetic LMTD, Esbo, Finland) cleaved into 5 x 10 mm pieces in the (100) crystal direction, 76 x 26 mm Microscope Slides (Menzel-Gläser, Braunschweig, Germany, 72.2% SiO_2) or 40 x 8 mm glass test tubes (Assistent KHG, Sondheim-Rhön, Germany, approximately 75% SiO_2) were cleaned for 5 minutes at 80°C in distilled (Milli-Q) water, hydrogen peroxide (30% v/v) and NH_4OH (25% v/v), 5:1:1 parts respectively.[28, 29] The surfaces were then rinsed ten times in distilled water and incubated for another 5 minutes at 80°C in distilled water, hydrogen peroxide and hydrochloric acid (37% v/v), 6:1:1 parts respectively. The SiO_2-surfaces were thereby hydrated and accordingly hydrophilic (water/surface static contact angle, θ_w, $< 10°$, estimated with a Ramé-Hart NRL Model 100 goniometer, USA). The hydrated surfaces were washed in distilled water and water was removed by rinsing in technically pure ethanol. The surfaces were then transferred to xylene (Merck, Darmstadt, Germany) and methylated during 5 minutes at room temperature in 1% (v/v)

dichlorodimethylsilane ($Cl_2(CH_3)_2Si$, DDS) in xylene. Finally, the hydrophobic ($\theta_w > 90°$) surfaces were rinsed in ethanol, xylene and, again, ethanol to remove traces of silane. The hydrophobic preparations were stored in ethanol and used within two weeks.

Immunoglobulin adsorption

The hydrophobic surfaces were washed in PBS and a monolayer of normal human IgG (prepared from pooled plasma, Gammaglobulin 165 mg/ml, Pharmacia & UpJohn, Stockholm, Sweden) or human IgM (from pooled serum, Sigma product no. I8260) was spontaneously adsorbed by over-night incubation in 0.1 mg/ml protein in PBS at room temperature. Immediately before experiments, non-adsorbed immunoglobulins were washed off by two gentle rinsings in PBS. The protein layer thickness' were 36 Ångströms ($\pm2Å$; $1Å = 1 \times 10^{-10}$ m) for IgG and 45Å ($\pm2Å$) for IgM. Since the IgM stock solution contained sodium azide (that potentially interferes with the CL method),[30, 31] control experiments were carried out where the azide was removed. Small-volume dialysis of IgM in Slide-A-Lyzer (Pierce Chemical Company, Rockford, IL) cassettes (10,000 D MWCO membrane) was performed at 4°C against PBS with three buffer changes in between. Dialyzed and non-dialyzed IgM elicited similar neutrophil responses.

Ellipsometry

The protein adsorption during 5 minutes from serially diluted serum to hydrophobic silicon surfaces was analyzed by a combination of null-ellipsometry with antibody-techniques. After recording of the ellipsometric angles Δ and ψ at the minimum intensity after reflection at the surface of elliptically polarized laser light (HeNe $\lambda = 6328Å$) at 70° angle of incidence, the surface film thickness was deduced using the McCrackin evaluation algorithm.[32] The 5-minute serum adsorptions were performed at 37°C and the ellipsometric measurements performed in air (organic film refractive index $n_{film} = 1.465$) using a Rudolf Research AutoEl III (NJ, USA). The SiO_2 and DDS background (approximately 20Å) was subtracted and the thickness values converted to amount of mass per unit area according to de Feijter *et al*.[33] The compositions of the adsorbed protein films were characterized by analysis of the thickening of the adsorbed layers after incubation of the serum-coated surfaces for 30 minutes in VBS^{2+} containing 1:50 (v/v) dilutions of specific polyclonal antibodies (immunoglobulin-

fractions of polyclonal A 0062 rabbit anti-human C3c, A 0136 anti-C1q, A 0426 anti-IgM, specific for

μ-Chains and A 424 anti-IgG, specific for γ-Chains, Dakopatts, Glostrup, Denmark). A similar and

more extensive study on protein adsorption during the activation of complement by IgG and IgM was

recently published by Tengvall *et al.*[34]

Chemiluminescence (CL)

The intra- and extracellular generation of oxygen free radicals over time from neutrophils

interacting with the methylated and protein coated glass tube surfaces was studied by luminol-

amplified chemiluminescence (CL) in a regularly calibrated six-channel Biolumat (LB 9505 C,

Berthold Co., Wildbaden, Germany), essentially as described by Wetterö *et al.*[8] All measurements

were performed at 37°C in KRG, with the various sera added 5 minutes before horseradish peroxidase

(4 U/ml), luminol (5-amino-2,3-dihydro-1,4-phtalazinedione, 50 μM) and cells (1 x 10^6/ml) were

introduced. The 5-minute NHS pre-incubation time was chosen because at this time period (i) classical

pathway activation had started but not the alternative pathway; (ii) if the immunoglobulins binds C1q,

then this protein is maximally accessible to react, as observed through the maximal deposition of anti-

C1q at this time period.[34] The measurements were commonly performed for 30 or 60 minutes, but

occasionally control experiments were continued for 3 hours. The experiments were performed at least

in duplicate and the samples were analyzed in different channels for each new repeat. The C1q-

depleted NHS was reconstituted with a high, but still physiologically relevant [25] dose of C1q (180

μg/ml) at least 3 hours before the serum was exposed to surfaces.

Neutrophil morphology and quantification of F-actin

The neutrophils were allowed to interact with methylated and immunoglobulin-coated glass

slides placed at an approximate angle of 70° relative to the horizontal plane in beakers containing

sample buffers and sera. After 30 minutes of incubation, the slides with adherent cells were removed to

a fixation buffer (4% ice-cold paraformaldehyde in PBS) for 30 minutes. The fixed samples were then

washed twice in PBS and stained for F-actin through 30 minutes of incubation in a solution containing

0.6 μg/ml bodipy phallacidin (Molecular Probes, Leiden, The Netherlands) and 100 μg/ml

lysophosphatidylcholine in PBS at room temperature and protected from light. The stained samples

were washed twice in PBS and mounted in Dako fluorescence mounting medium (Dakopatts, Glostrup, Denmark) containing anti-fade agent. The slides were studied and the fluorescence documented as positive color pictures with a Zeiss Axioscope equipped with a MC 100 microscope camera (Carl Zeiss, Oberkochen, Germany). The mounted positive images were scanned using a Nikon (Japan) LS-2000 Scanner and Nikon Scan 2.2 software. All editing of the scanned images was performed using the Canvas 5.0.1 software (Deneba Systems, Inc., Miami, FL). The F-actin content in single, adherent neutrophils was quantified by registration of the fluorescence in a cytofluorometer.[35] In brief, cells were observed and individual neutrophils of varying morphology selected in the light-microscopy mode of a Leitz MPV II microscope photometer connected to a computer system. For each sample, 30 or 50 individual cells per slide were excited for < 0.8s and the fluorescence recorded. The background fluorescence values were routinely subtracted.

Data analysis

The data were assumed normally distributed and the results are presented as arithmetic means ± standard errors of the means (SEM). The statistical evaluation was performed using the paired (if nothing else is mentioned) or non-paired, two-tailed Student's *t*-test (Microsoft Excel 97). Three levels of significance were used ($p < 0.05$, 0.01 or 0.001).

RESULTS

Protein Adsorption

Both IgG- and IgM-coated hydrophobic silicon surfaces acquired a significant deposition of serum proteins after 5 minutes of exposure to diluted NHS (Fig. 1A) and the immunoglobulin-coated surfaces bound their respective specific polyclonal antibody at low serum concentrations (not shown) indicating that the C1q- and F_c-reptor binding F_c-regions of the pre-adsorbed immunoglobulins were at least initially accessible to both humoral and cellular interactions. The binding of the specific antibodies against adsorbed complement proteins varied with the NHS dilution (Fig. 1B). Anti-C3c

bound to both IgG- and IgM-coated surfaces after 5 minutes of incubation when the NHS concentration exceeded 1%. C1q was detectable only at the IgG-coated surface for NHS concentrations ranging between 1% to 10%. This C1q-deposition at IgG monolayers was inhibited when Ca^{2+} in NHS was chelated with EGTA and when C1q-depleted serum was used instead of NHS.[34] Surprisingly, anti-C1q did not bind to serum layers at any dilution on IgM-coated surfaces. In order to investigate if the absence of C1q on immobilized IgM could influence the activation of neutrophils, further experiments were performed with neutrophils that interacted with similarly treated glass surfaces.

Neutrophil respiratory burst

In a previous study we showed that the oxygen radical production from neutrophils that interact with clean methylated hydrophobic surfaces in KRG was amplified by approximately 15% after coating with IgG.[8] Upon interaction with the IgM-coated surfaces, the CL was instead lowered by 52% (p < 0.001). The CL response at the non-coated hydrophobic surface was lowered by approximately 20% in the presence of 5% NHS in the medium during the experiments. When the commercial C1q-depleted serum was reconstituted with C1q, it induced effects similar to those obtained with the NHS. C1q-depleted serum, however, showed only 37% of the CL activity compared to C1q-repleted serum (p < 0.01) at non-coated surfaces. As previously reported,[8] the neutrophil radical response was again very potent when heat-inactivated serum was exposed to the non-coated hydrophobic surface.

When the IgM-coated surfaces were exposed to 5% NHS 5 minutes prior to the addition of cells, an amplification by 344% (*p* < 0.05, compared with the IgM-surfaces without NHS exposure) of the neutrophil respiratory burst activity was observed. The response was slow with a single peak after 15-25 minutes, and approached the base levels within an hour (Fig. 2A). The IgM/NHS-triggered neutrophil response was reduced by 61% (*p* < 0.05) when heat-inactivated NHS was used instead. IgM-coated surfaces that were incubated in C1q-depleted or reconstituted sera showed almost identical CL responses, indicating that C1q was not essential for the IgM-triggered response (Fig. 2B). Control experiments with IgM-coated hydrophilic tubes showed no difference in the neutrophil radical generation between non-heated NHS and heat-inactivated serum, indicating that the above response depended on the adsorbed IgM (proteins are easily displaced on hydrophilic surfaces).[2] IgG-coated

hydrophobic surfaces induced a massive neutrophil oxygen radical generation after NHS was exposed to the surface.[8] The response was biphasic with peaks after approximately 10 and 20 minutes (essentially like the reconstituted serum in Fig. 2C) and sensitive to the presence of C1q in serum. The second peak disappeared when the NHS was heat-inactivated or depleted on C3. Blocking of neutrophil F_c- and complement receptors was performed prior to contact with the serum-exposed IgG-coated surfaces. Only minor effects were observed when monoclonal antibodies specific for the receptors were used, suggesting that C1q, IgG and C3 triggered the CL in synergy. However, approximately 75% of the CL response could be quenched when the complement receptor 3 was blocked with an inhibitory peptide.[8]

The integrated CL response from the neutrophils after 30 minutes of contact with complement-opsonized IgG-coated surfaces was clearly lower (38%; $p < 0.01$) when C1q was absent (Fig. 3, left). Both the primary and secondary peaks were reduced (Fig. 2C). However, the neutrophil respiratory burst activity on IgM-surfaces was more or less insensitive to the presence of C1q for 30 minutes (Fig. 3, right) or 1-3 hours of measurement (not shown). In order to further characterize the CL response, neutrophils that interacted with IgG- or IgM-surfaces were exposed to a second stimulus, 0.1 μM of the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) that was added after 30 minutes of cell - surface interaction. We observed then that fMet-Leu-Phe induced a CL response on both surfaces and the responsiveness to fMet-Leu-Phe was higher in serum environments (not shown).

Neutrophil adhesion and cytoskeletal F-actin

Several studies have shown a relationship between occupation of phagocytosis and adhesion receptors, reorganization of the actin cytoskeleton and activation of the NADPH-oxidase in neutrophils.[36, 37] We found that the NHS-coated IgM-surfaces attracted only half the number of neutrophils, compared to IgG/NHS-surfaces (Table 1). No significant differences regarding the number of adherent cells were registered in the absence or presence of C1q in serum, on either IgG- or IgM-surfaces. On serum-opsonized IgG-coated surfaces, neutrophils exhibited an extensive spreading including accumulation of F-actin in moving peripheral lamellipodia and focal adhesion points (Fig.

4A), and when the serum was depleted on C1q the spreading and actin polymerization was slightly reduced (Fig. 4B, Fig. 5). Cells that adhered to serum-opsonized IgM-surfaces, with or without C1q, displayed less pronounced spreading (Fig. 4C) and F-actin accumulation than in lamellipods and focal contacts on IgG-coated surfaces (Fig. 5).

DISCUSSION

The present study indicates that adsorbed IgG- and IgM-monolayers both activate the complement system, the former dependent and the latter independent on C1q. Thus, the C3 surface-opsonizations occur through partly different mechanisms. The difference is surprising since it is expected that IgG-coated surfaces are less C1q-dependent due to known direct covalent associations between IgG and C3.[38, 39]

The radical production induced by NHS was higher on IgG- than on IgM-coated surfaces since neutrophils, at least initially, may have interacted with both surface-localized immunoglobulins and complement proteins, among them C1q and C3. In contrast to IgG, IgM is not a ligand for neutrophil F_c-receptors [40-42] and consequently IgM is a weak opsonin. Furthermore, IgG, C1q and C3 may activate both non-adherent and adherent neutrophils either through independent or synergetic stimulation of F_c- and complement receptors.[7, 8, 37, 43-51] It is therefore reasonable to assume that blood cells interact with immunoglobulin-fixed C1q, since the collagen-like region of C1q is known to interact with cell-surface receptors and the globular heads of C1q bind to IgG or IgM. The total neutrophil CL response on IgG/NHS-surfaces was affected when C1q was depleted from NHS, indicating that the classical activation pathway was involved. Hence, we suggest that the initial phase of the biphasic[8] CL response on IgG/NHS-surfaces depend on F_c- and, possibly, C1q-receptor stimulation by surface-denatured IgG and fixed C1q since: (i) this early part of the CL profile was not observed on IgM/NHS-coated surfaces; (ii) the first part was relatively insensitive to heat-inactivation of NHS[8] (iii) the stimulation corresponded in time roughly with the response triggered by IgG in serum-free media; and; (iv) neutrophils in KRG containing NHS were more activable by a second

fMLP-stimulation than without NHS, suggesting that NHS enhances the oxygen radical production induced by ligation of cell surface receptors (e.g. F_c-receptors). The second phase of the CL response on the IgG/NHS-surfaces was probably complement-receptor dependent, as demonstrated by the heat-inactivated serum and by the use of various complement-depleted sera. In support for this, the entire CL response on IgM/NHS-surfaces seemed to be triggered by complement-dependent factors since the heat-inactivation markedly decreased the response and the CL correlated in time with the complement-dependent latter part on the IgG/NHS-surface.

The actin filament system and associated receptors become activated during the contact with artificial materials.[36, 52, 53] Others and we have shown previously that the complement-triggered neutrophil radical response depends on a dynamic actin cytoskeleton.[7, 8] Both the phagocytosis process and the regulation of the NADPH-oxidase require the action of the actin cytoskeleton, which may explain the extensive actin polymerization obtained on the IgG/NHS-surfaces. The C1q-triggered neutrophil oxidative response is especially potent when C1q is surface-bound[45] and C1q also enhances the phagocytosis process.[47] The obvious conclusion in the present study is that IgG-coated, but not IgM-coated, surfaces bind and expose C1q during the early activation phase, thereby presenting C1q to neutrophils at the blood-material interface.

IgG is much more abundant in plasma than IgM (8-17 versus 0.05-2 mg/ml)[3] and as it is a smaller molecule it diffuses more rapidly to surfaces. On the other hand, rapidly adsorbed proteins can be covered by other proteins or be replaced, thereby becoming less accessible to the later arriving platelets and leukocytes. Consequently, a potential role for the large and slowly diffusing acute-phase IgM molecule during biomaterial-related neutrophil activation is possible. In conclusion, both IgM- and IgG-coated hydrophobic surfaces become readily opsonized by C3, the former in a C1q-independent manner. This may affect the regulation of the neutrophil inflammatory response at artificial surfaces in contact with body fluids.

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CAPTIONS

Table 1. Characterization and quantification of adherent neutrophils on serum-opsonized immunoglobulin G or M (IgG/IgM) pre-coated hydrophobic glass slides. The C1q-dependency for the adhesion was investigated. The cells were counted in at least 10 separate microscopy views (original magnification x 400) on each slide, and prepared in duplicate for 3 donors. For further details regarding the slides, see captions Fig. 4-5.

Figure 1.

(A) Deposition of normal human serum (NHS) at various concentrations, after 5 minutes, at 37°C, onto surfaces that were pre-coated with a monolayer of immunoglobulin G (IgG) or M (IgM). The NHS was diluted in VBS^{2+} and rinsed with pre-warmed buffer before the ellipsometric measurement (n = 5; ± SEM).

(B) Deposition of specific polyclonal anti-C1q or anti-C3c to surfaces in (A).

Figure 2. Chemiluminescence profiles for IgG- and IgM pre-coated surfaces that were exposed/not exposed to 5% serum for 5 minutes prior to the introduction of neutrophils (1 x 10^6/ml). The illustrations in A-C shows examples from different donors on the total generation of oxygen free radicals from isolated neutrophils interacting with the immunoglobulin-coated hydrophobic glass surfaces at 37°C in KRG buffer, with or without native or C1q-depleted serum added 5 minutes before the cells. The counts per minute (CPM) of chemiluminescence were recorded in a Biolumat in presence of luminol (50 μM) and extra peroxidase (4U/ml). The response at non-coated hydrophobic surfaces is described in the text and elsewhere[8].

(A) Immunoglobulin M (IgM) pre-coated surfaces were exposed or not exposed to normal human serum (NHS). In control experiments the complement in the NHS was inhibited by heating to 56°C for 30 minutes.

(B) IgM-coated surfaces exposed to NHS depleted on complement factor 1q (C1q). In control experiments, the depleted serum was reconstituted with 180 μg/ml C1q. Note the relative insensitivity to C1q for the radical generation.

(C) As (B) but IgG-coated surfaces.

Figure 3. Neutrophil respiratory burst in serum-opsonized hydrophobic glass tubes pre-coated with immunoglobulin G (IgG) or M (IgM). The figures show the 30-minute integrated chemiluminescence values generated from 1×10^6/ml neutrophils in the presence of 50 μM luminol and 4U/ml horseradish peroxidase, with or without 5% normal human serum (NHS) depleted on complement factor 1q (C1q). In control experiments, the depleted serum was reconstituted with 180 μg/ml C1q. The experiments were performed at least in duplicate with neutrophils from 8 separate donors in a six-channel Biolumat at 37°C in KRG buffer. (CPM = counts per minute, bars illustrates the SEM).

Figure 4. Neutrophil morphology displayed after 30 minutes of interaction with immunoglobulin pre-coated hydrophobic glass slides that were exposed to different sera for 5 minutes. The neutrophils were subsequently fixed in 4% paraformaldehyde, permeabilized with lysophosphatidylcholine and the filamentous actin (F-actin) was stained with bodipy-phallacidin. Positive images were documented using a Zeiss Axioscope and an MC 100 microscope camera, developed at ASA 3200 and scanned. Images A-C were treated identically during edition. Original magnification x 630.

(A) Immunoglobulin G coated surfaces exposed to 3% complement factor 1q (C1q) depleted normal human serum (NHS) that was reconstituted with 180 μg/ml C1q, followed by the addition of neutrophils.

(B) As in (A) but with C1q-depleted serum.

(C) Immunoglobulin M coated surfaces exposed to 3% C1q-depleted NHS that was reconstituted with 180 μg/ml C1q. The morphology was similar on surfaces exposed to C1q-depleted serum.

Figure 5. Quantification of the filamentous actin (F-actin) in individual neutrophils that adhered to serum-opsonized (3%) immunoglobulin M (IgM) or G (IgG) coated methylated glass slides. The neutrophils were fixed after 30 minutes (4% paraformaldehyde), permeabilized (lysophosphatidylcholine) and the F-actin stained with bodipy-phallacidin. The results demonstrate the
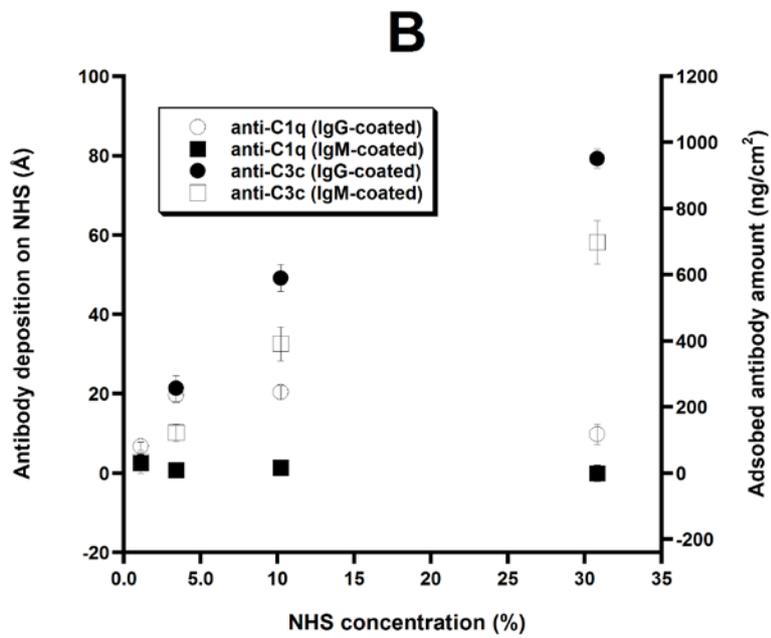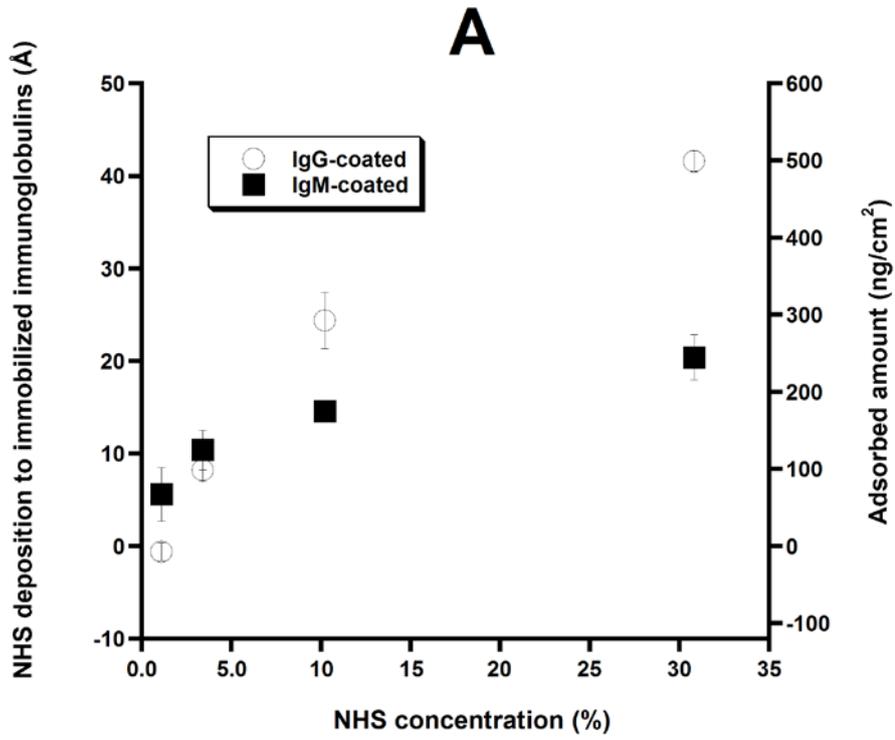
effect following reconstitution with 180 µg/ml complement factor (C1q) into the C1q-depleted normal human serum. The zero-level indicates fluorescence after interaction with C1q-depleted serum. All experiments were performed in duplicate and 30 or 50 cells were analyzed for each slide. The results are averaged from 4 separate donors and the bars indicate the SEM. The change in fluorescence on IgG-, but not on IgM-surfaces, after the C1q-reconstitution was significant ($p < 0.05$; non-paired).
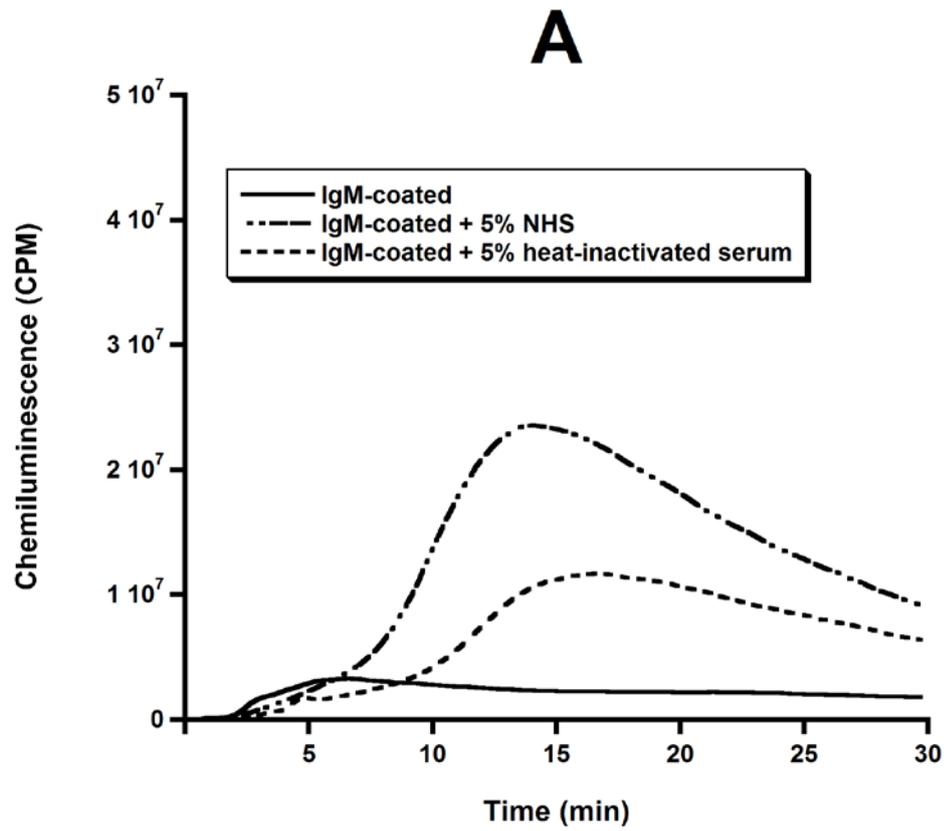
TABLE 1

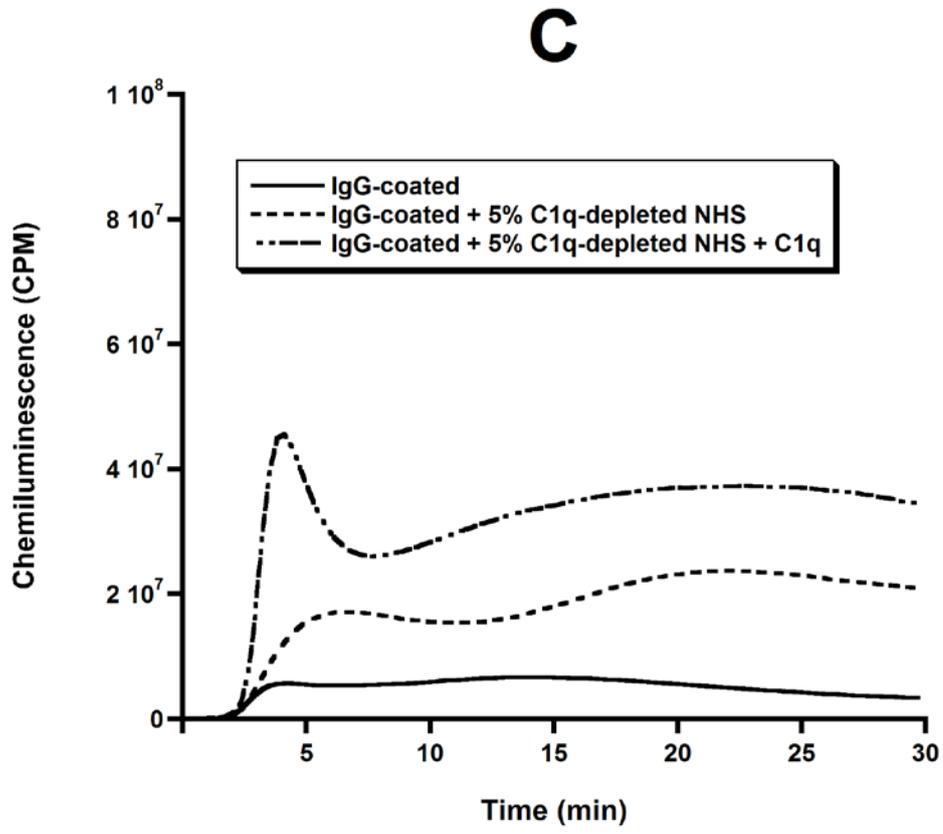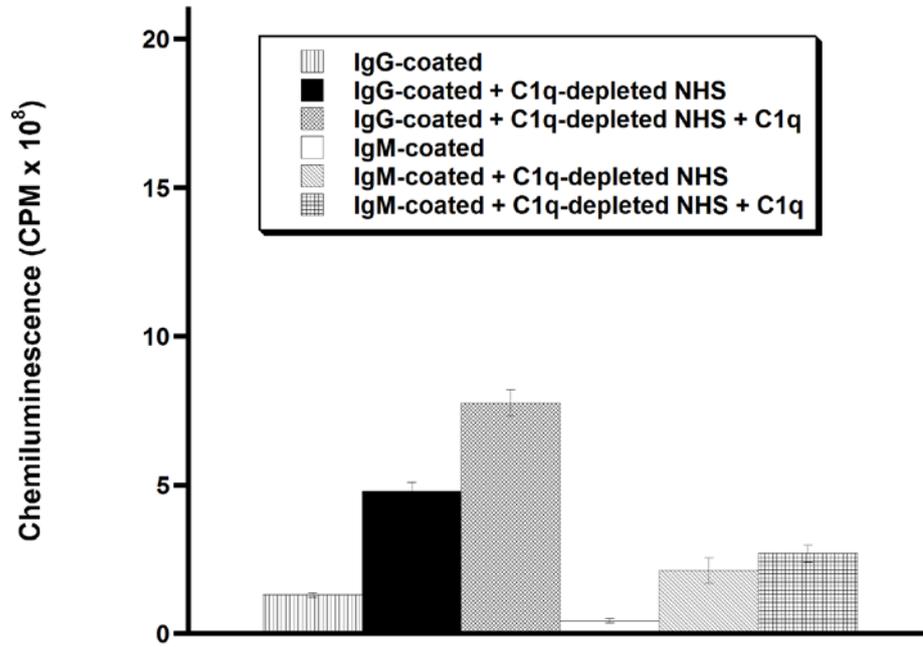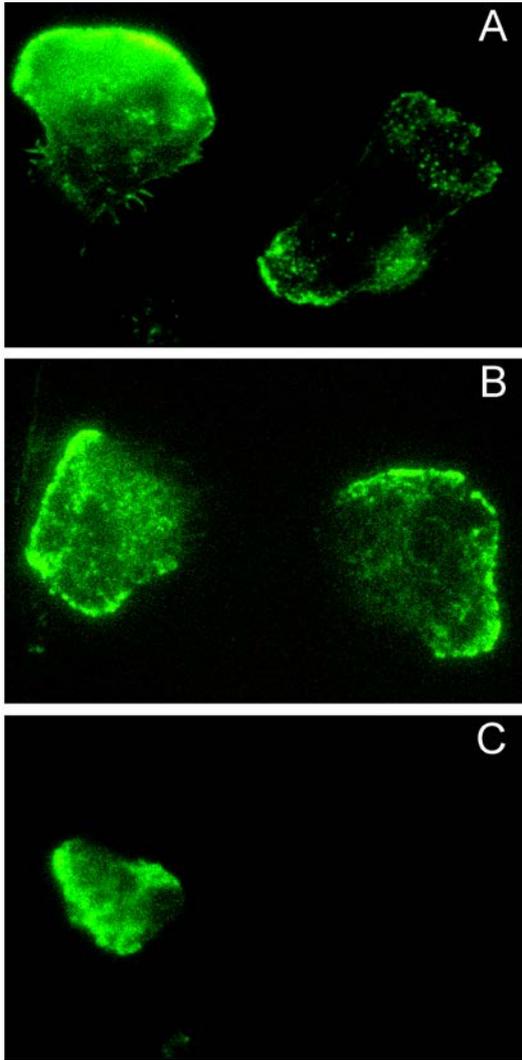| Surfaces	Relative number of neutrophils	Morphology
IgG-coated + C1q-depleted serum reconstituted with C1q	223%	Very spread, perifer accumulation of F-actin (Fig. 4A)
IgG-coated + C1q-depleted serum	256%	Most cells show extensive spreading but a little less pronounced F-actin accumulation than above (Fig. 4B)
IgM-coated + C1q-depleted serum reconstituted with C1q	128%	Moderately spread (Fig. 4C)
IgM-coated + C1q-depleted serum	100% (=CONTROL)	Moderately spread (Fig. 4C)

Figure 1

Figure2

C
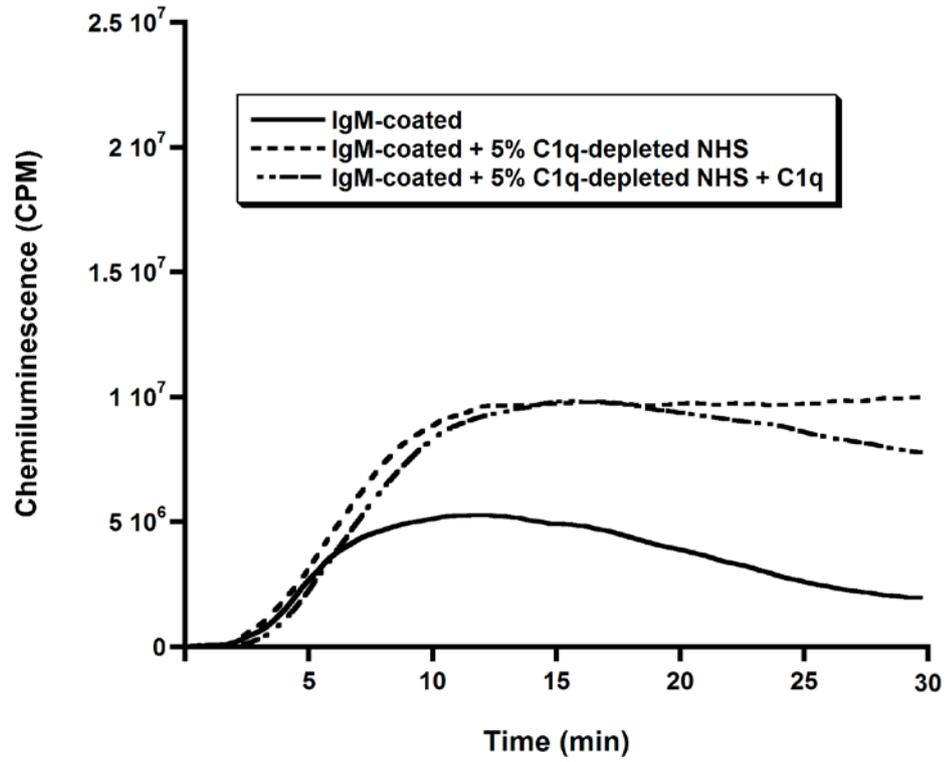
Figure 3

Figure 4

Figure 5