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Serum Proteins Modified by Neutrophil-Derived Oxidants as Mediators of Neutrophil Stimulation

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Reactive oxygen intermediates (ROI) released during inflammation may act as important mediators of neutrophil effector functions. The objective of this investigation was to evaluate the influence of ROI generation on neutrophil adhesion molecule regulation and degranulation. Induction of the neutrophil oxidative burst via Fcγ receptor cross-linking was accompanied by up-regulation of neutrophil surface CD11b, CD35, and CD66b only in the presence of selected serum proteins, such as purified human C4, C5, or human serum albumin (HSA). Scavenging of ROI attenuated protein-dependent receptor regulations. Moreover, exogenous hydrogen peroxide was effective to increase neutrophil CD11b expression in a protein-dependent way. HSA exposed to neutrophil-derived ROI displayed signs of oxidative modification in terms of carbonyl formation. Such modified HSA transferred to resting neutrophils bound readily to the cell surface and effected receptor modulation as well as cellular spreading. In contrast, neither native HSA nor HSA protected against oxidation by the tocopherol analog Trolox exhibited agonistic properties. In conclusion, we demonstrate that neutrophil-derived ROI modify selected serum proteins, which, in turn, act as proinflammatory mediators of neutrophil stimulation.


The primary role of polymorphonuclear neutrophil granulocytes (PMN) is the elimination of invading bacteria in infection. Neutrophil extravasation to the interstitium as the site of inflammation is the prerequisite for PMN defense function. This is initiated by selectin-dependent PMN rolling along the endothelial lining, followed by firm PMN adhesion and transendothelial diapedesis mediated by binding of β2 integrins to endothelial intercellular adhesion molecules (1). The principal neutrophil integrin, CD11b/CD18 (Mac-1, αMβ2), is crucially involved in neutrophil aggregation and also acts as phagocytic complement receptor type 3 (2). Ligand binding may potentiate neutrophil effector functions such as respiratory burst and degranulation (3). The multifunctional nature of CD11b/CD18 is reflected by its subcellular location in PMN. Aside from constitutive plasma membrane expression, this receptor is preformed and stored in secretory vesicles, gelatinase granules, and specific granules (4). This guarantees a controlled stepwise translocation to the cell surface upon cell stimulation, because neutrophil exocytotic organelles follow a certain rank order of mobilization. First, secretory vesicles are mobilized on PMN activation to fuse with the cell membrane, followed by gelatinase granules, specific granules, and azurophilic granules on continuing or increased cell stimulation (5). Only CD11b/CD18 molecules present on or translocated to the cell surface are then transiently activated to enable avid ligand binding and adhesion (3). Because CD11b/CD18 is central to neutrophil effector functions, its cell surface up-regulation serves as well-established marker of neutrophil activation (6, 7).

Phagocytosis via Fcγ or complement receptors by PMN is usually accompanied by the formation of a spectrum of aggressive oxidants initiated by the action of the neutrophil NADPH oxidase (8). The reactive oxygen intermediates (ROI) released are the highly reactive superoxide anion, its dismutation product hydrogen peroxide (H₂O₂), probably the aggressive hydroxyl radical, and products of the myeloperoxidase (MPO)-H₂O₂-halide system, such as hypochlorous acid (HOCl) and chloramines (9). These oxidants are secreted intracellularly into the forming phagosome, as well as into the extracellular PMN environment (8), resulting in both microbial killing and tissue damage (10).

Various oxidants were found to modulate the expression of neutrophil adhesion molecules and opsonic receptors and to increase PMN adhesion to endothelial cells (11–13). In addition, ROI are able to activate complement factor C5 in a nonenzymatic way, generating either C5b-like activity without C5 cleavage (14) or anaphylatoxins (15) depending on the ROI species studied. Moreover, ROI are implicated in the pathogenesis of a variety of conditions, such as adult respiratory distress syndrome (ARDS) (16), atherosclerosis (17), chronic obstructive pulmonary disease (18), and Ab therapy- and hemodialysis-associated side effects (19, 20). Reduced plasma antioxidant status (21) and protein oxidation correlating with either PMN infiltration or tissue MPO content (22–26) was observed in patients suffering from inflammatory disorders. However, it is not known whether oxidized proteins are simply a consequence of increased ROI burden during inflammation or also contribute to neutrophil effector functions.

The present study was designed to evaluate the role of PMN-derived ROI as mediators of PMN activation by inducing a strong neutrophil oxidative burst using an in vitro model for FcγR cross-linking. We were able to demonstrate for the first time that...
activated PMN, via released ROI, modify a number of selected serum proteins, which, in turn, stimulate PMN adhesion molecule regulation, degranulation, and neutrophil spreading.

Materials and Methods

Reagents

Tiron, 1-methionine, sodium azide, p-aminobenzohydrazide, sodium hypochlorite (NaOCl), PMSF, eglin C fragment 60–63 methyl ester, gelatin, 3-amino-1,2,4-triazole, 1-chloro-2,4-dinitrobenzene, mercaptosuccinate, FITC-conjugated human serum albumin (HSA), FMLP, and PMA were purchased from Sigma (St. Louis, MO). FMLP and PMA were dissolved in DMSO at 10−2 and 10−3 M, respectively, and stored at −20°C. Methoxy- succinyl-Ala-Ala-Pro-Val chloromethylketone and Z-Gly-Leu-Phe chloromethylketone were obtained from Enzyme Systems Products (Livermore, CA), and apocynin and Trolox were obtained from Aldrich (Milwaukee, WI). Reduced glutathione (GSH) was obtained from ICN (Costa Mesa, CA), hydrogen peroxide (H₂O₂) was obtained from Merck (Darmstadt, Germany), and 2,7'-dichlorofluorescin-diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR). DCTH-DA was dissolved in ethanol at a concentration of 2.5 mg/ml and stored in the dark at −20°C. Complement component C1q-, C3-, and C5-depleted human serum as well as purified human C4, C4a, C4b, C5, C5a, C6, C7, and factor H were obtained from Calbiochem (La Jolla, CA), as was high purity nonhumanized HSA. Purified anti-C6 and anti-C7 mAb was purchased from Qiudel (San Diego, CA). In some experiments, human C4, C5, C6, and C7 from Qiudel and highly purified anti-HSA (II Dyn 610 hemolysate, Marburg, Germany) and nondenatured HSA (ICN) were used to compare different protein preparations. Recombinant soluble CR1 (sCR1) was a gift from Carolyn Petley (AVANT Immunotherapeutics, Needham, MA). Pefabloc SC was purchased from Roche (Mannheim, Germany). The murine IgG2a mAb Orthoclone OKT3 recognizing the T cell-specific CD3 Ag not expressed by PMN was obtained from Ortho Pharmaceutical Corp. (Raritan, NJ). FITC-conjugated mouse IgG1, IgG2a, IgG2b, or IgG3 mouse myeloma proteins; medium control; NHS (100 μl); and 0.75 μg/ml PMA (10−3 M) were added to the incubation wells. Generally, microtiter plates were incubated at 37°C for 30 min. Supernatants were transferred to resting PMN and calibrated against a standard curve established by mixing known protein concentrations.

Cell preparations

Neutrophils were isolated from heparinized peripheral blood of healthy donors using Lymphoprep (Nycomed, Oslo, Norway) density gradient centrifugation. After separation, all further isolation steps were performed on ice. Erythrocytes were removed from the pellet by isotonic lysis with ammonium chloride buffer (157 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA). After washing twice, PMN were resuspended in ice-cold Dulbecco’s PBS containing calcium and magnesium (Life Technologies, Vienna, Austria) and preincubated with the respective agent at 4°C before final addition of PMN. In experiments with the MPO inhibitors sodium azide (0.01–1 mM) or p-aminobenzohydrazide (0.05–2.5 mM), the antioxidants apocynin, Tiron, GSH, PBS (10–250 mM), and Trolox or the protease inhibitors Pefabloc SC (0.4–10 mM), PMSF (0.5–5 mM), methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone and Z-Gly-Leu-Phe chloromethylketone (each at 20–360 μM), and eglin C fragment (5–100 μM) were preincubated with the respective agent at 4°C for 15 min before being added to the incubation wells. Generally, microtiter plates were incubated in a 37°C water bath for 30 min. Then reactions were stopped by transferring the microtiter plates back onto melting ice. PMN suspension (100 μl) was mixed with 20 μl of the respective FITC-conjugated mAb and left on ice for 45 min. In experiments with RBC subsequent lysis of RBC was performed with ADG lysis (Coulter, Miami, FL). For supernatant transferal studies PMN were first incubated with medium control. In experiments with immobilized IgG2a (IMIG; OKT3, unless otherwise indicated), extracellular ROI formation was assessed according to the method of Rosenkranz et al. (28). Purified PMN (2.5 × 10⁵) and 0.75 μg/ml DCFA-DA were incubated in duplicate with IMIG or immobilized IgG1, IgG2a, IgG2b, or IgG3 mouse myeloma proteins; medium control; PMA (10−3 M) or FMLP (10−5 M) at 37°C. After 30 min microtiter plates were read on a Cytofluor 2300 fluorescence concentration analyzer (Millicore, Vienna, Austria) using 485-nm excitation and 530-nm emission wavelengths. After background subtraction, production of ROI was expressed as stimulation index by the formula: x-fold increase = FLUOstim/FLUOcontrol, where FLUOstim is the developed fluorescence intensity per well with cells incubated in the presence of immobilized Ig, PMA, or PMA and FLUOcontrol is the fluorescence intensity per well with cells incubated with medium control.

Determination of surface receptor expression

Isolated neutrophils (2.5 × 10⁵) were added to each well with or without IMIG in the presence of medium control; NHS; His; C1q-, C3-, or C5-depleted serum (final concentration, 25%); a purified serum protein; or a protein-activated NHS. The latter was prepared by incubating NHS (200 μl) with 14 mg of the known complement-activating dialyzable material cuprophan at 37°C for 30 min (29). All incubations were performed in duplicate and prepared on ice to synchronize PMN and/or C activation. In some experiments sCR1 (30 μg/ml), autologous RBC (in PMN-RBC ratios of 1:100 and 1:1000), l-methionine (1–50 mM), H₂O₂ (0.05–10 mM), or NaOCl (0.1–100 μM) was added to wells at 4°C before final addition of PMN. In experiments with the MPO inhibitors sodium azide (0.01–1 mM) or p-aminobenzohydrazide (0.05–2.5 mM), the antioxidants apocynin, Tiron, GSH, DMSO (10–250 mM), and Trolox or the protease inhibitors Pefabloc SC (0.4–10 mM), PMSF (0.5–5 mM), methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone and Z-Gly-Leu-Phe chloromethylketone (each at 20–360 μM), and eglin C fragment (5–100 μM) were preincubated with the respective agent at 4°C for 15 min before being added to the incubation wells. Generally, microtiter plates were incubated in a 37°C water bath for 30 min. Then reactions were stopped by transferring the microtiter plates back onto melting ice. PMN suspension (100 μl) was mixed with 20 μl of the respective FITC-conjugated mAb and left on ice for 45 min. In experiments with RBC subsequent lysis of RBC was performed with ADG lysis (Coulter, Miami, FL). For supernatant transferal studies PMN were first incubated with or without IMIG in the presence or the absence of native HSA or FITC-conjugated HSA for 30 min, followed by centrifugation in the cold at 600 × g for 6 min. Supernatants were transferred to resting PMN and incubated at 37°C for 30 min, and surface receptor expression or PMN-associated FITC-HSA fluorescence was determined as described above.

Determination of complement activity

The total hemolytic complement activity was assessed by testing the ability of the respective sera to lyse 50% of a standard suspension of sheep erythrocytes coated with optimal concentrations of rabbit IgM. This includes the entire classic activation pathway as well as the terminal sequence (CH50 assay). The quality of C1q-, C3-, and C5-depleted sera used and the efficacy of the heat-inactivating procedure as well as of sCR1 (30 μg/ml)-mediated inhibition of enzymatic C cascade activation in NHS were confirmed by showing absolute nonreactivity in CH50 assays.

Protein-associated carbonyl measurement

The HSA carbonyl content was estimated in supernatants of PMN incubated with or without IMIG using an ELISA as previously described (30). Dinitrophenylhydrazine (Sigma)-reactive carbonyls were detected by anti-DNP rabbit antiserum (Sigma) and a peroxidase-conjugated mouse anti-rabbit Ig mAb (Sigma) (31). Development was performed with p-phenylenediamine as a blank. Samples were read against a blank consisting of NHS without HSA and calibrated against a standard curve established by mixing varying proportions of DNP-HSA (Sigma) with native HSA while maintaining a constant protein concentration.
Assessment of neutrophil shape change

Neutrophils were incubated with or without IMIG in the presence or the absence of HSA and Trolox at 37°C for 30 min. Cell-free supernatant (75 μl) were added in duplicate to resting PMN (6.25 × 10⁴ in 25 μl) and incubated in gelatin-coated wells (1 mg/ml gelatin for 3 h at 37°C, followed by washing three times with PBS) of 96-well flat-bottom culture clusters (Costar) at 37°C with occasional agitation. After 30 min PMN morphology was examined by phase contrast microscopy.

Statistics

Statistical analysis for the evaluation of a possible difference in neutrophil receptor expression, HSA-FITC binding, and protein-associated carbonyl formation after PMN stimulation under different conditions was performed by using Student’s r test. Dose-response data were compared by ANOVA using a randomized block design. Comparison of different doses vs the untreated condition was performed using Dunnett’s test. All results are expressed as the mean ± SD and were considered statistically significant at p < 0.05.

Results

Immobilized IgG2a stimulates the PMN oxidative burst

As previously demonstrated (19), the neutrophil oxidative burst is strongly activated by murine monomeric IgG2a in a FcγR-dependent way. Similarly, IgG2a immobilized to microtiter plates to mimic immune complexes markedly induced ROI formation by purified PMN (Fig. 1). This effect was due to the murine IgG2a nature of IMIG, because immobilized IgG2a mouse myeloma protein activated the PMN oxidative burst to the same extent, in contrast to murine IgG1, IgG2b, or IgG3.

Neutrophil receptor regulation by immobilized IgG2a is serum dependent

In acute inflammation, PMN adhesion and extravasation are preceded by activation and up-regulation of neutrophil surface CD11b (1). Thus, CD11b modulation is considered a reliable marker of cellular activation (6, 7). Incubation of isolated PMN with IMIG in the presence of NHS, but not PBS, resulted in significant CD11b up-regulation (Fig. 2A). Previously, in vivo and in vitro OKT3/T cell immune complexes (32, 33) as well as plastic-adsorbed murine IgG2a (34) were reported to activate the complement system. Moreover, in experiments performed without PMN, CH50 assays revealed a strong C activation by IMIG in NHS, but not in HIS, compared with uncoated wells (data not shown). To define the contribution of the enzymatic C cascade to NHS-dependent neutrophil CD11b up-regulation by IMIG, we applied HIS devoid of enzymatic C activity, which, however, resulted in receptor regulations comparable to the NHS milieu (Fig. 2A). Also using C1q-, C3-, or C5-depleted sera instead of NHS did not attenuate IMIG-induced neutrophil CD11b regulation (data not shown). Moreover, addition of sCR1 (30 μg/ml) that inhibits activation of both the classical and alternative pathways of C (35) reduced CD11b modulation in NHS by not more than 30%, showing no effect in HIS incubations (Fig. 2A). Consequently, neutrophil CD11b up-regulation by IMIG depended on the presence of serum constituents without an absolute need for enzymatic C activation.

Single serum proteins mediate PMN activation by immobilized IgG2a

Next, we investigated PMN activation by IMIG in the presence of isolated serum proteins. Upon PMN stimulation in the presence of either C4 (50 μg/ml), C5 (70 μg/ml), HSA (150 μg/ml), or HIS (25%), highly significant up-regulation of CD11b was observed (Fig. 2, B and C). This effect was dose dependent (C4 or C5, each at 17–150 μg/ml; HSA, 17–1000 μg/ml), whereas no significant changes were found in the absence of protein or with C6, C7, factor H (each at 17–150 μg/ml), or gelatin (17–450 μg/ml). The combined use of C4, C5, and HSA showed an additive effect with respect to neutrophil receptor modulation (data not shown). Experiments performed with purified C4, C5, C6, C7, and HSA from different sources yielded similar results (data not shown). In the absence of IMIG, none of the purified proteins was able to induce significant receptor modulation.

Up-regulation of CD11b may signify mobilization of secretory vesicles, gelatinase granules, and specific granules (4). To better characterize the degranulation process, we also assessed the surface expression of the activation Ags CD35 (36) and CD66b (37) as markers for exocytosis of secretory vesicles and specific granules, respectively (4). As illustrated in Fig. 3, these two granule markers showed a parallel pattern of up-regulation by IMIG in the presence of C4, C5, HSA, or HIS. Considering the known rank order of granule mobilization (4, 5), not only secretory vesicles and specific granules, but also gelatinase granules, appeared to have fused with the plasma membrane. These results demonstrated that selected serum proteins were involved in neutrophil stimulation.

C4- and C5-mediated PMN stimulation by immobilized IgG2a does not involve known complement split product activity

Next, we wondered whether the observed neutrophil receptor modulations with purified C4 or C5 were due to the action of known complement cascade-derived agonists possibly formed during PMN stimulation by IMIG. Incubation of isolated PMN with purified split product C4a or C4b (at 0.03–1 mM or 2–50 μg/ml, respectively) did not influence neutrophil CD11b expression (data not shown). Therefore, a contribution of these two candidates to IMIG-induced C4-dependent PMN activation was ruled out.

C5a is known to stimulate neutrophil receptor modulation and chemotaxis (38). After binding of C5a to its receptor, CD88, the C5a/CD88 complex is rapidly internalized, transiently diminishing neutrophil CD88 surface expression (39). To clarify whether any C5a activity is generated from native C5 when incubated together with PMN and IMIG (15), we performed C5a receptor kinetics studies. As shown in Fig. 4, we used PMA (10⁻⁷ M; FMLP (10⁻⁶ M); IMIG in the presence of PBS, C5 (70 μg/ml), or HSA (150 μg/ml); and CD88-specific PMN activators (purified C5a and eu- rophan-activated NHS containing C5a) and monitored neutrophil...
CD88 surface expression. As expected, ligand binding of CD88 resulted in marked CD88 down-regulation, whereas with all other stimuli, including IMIG in the presence of purified C5, CD88 expression was elevated compared with that in unstimulated cultures. These data demonstrated that neutrophil receptor modulation by IMIG and C5 was not brought about by C5a activity.

Neutrophils store considerable amounts of C6 and C7 that can be released during cell stimulation (40). Theoretically, PMN activation by IMIG in the presence of C5 could lead to C5b activity (14) and subsequent formation of hemolytically inactive C5b67, which was recently reported to be a chemotactic agonist of PMN.

FIGURE 2. Neutrophil CD11b regulation by IMIG. A, Expression of CD11b was determined after stimulation of PMN by IMIG in the presence of PBS, NHS (25%), or HIS (25%) at 37°C for 30 min. Furthermore, cultures were set up in the absence (■) or the presence (■■) of the complement inhibitor sCRI (30 μg/ml). Data are expressed as the percent increase in mean fluorescence (mean ± SD of at least four independent experiments) compared with that in cultures in the absence of IMIG. The p values shown (***, p < 0.001) indicate levels of significance for differences to cultures in PBS milieu. B, Effect of different serum proteins on neutrophil CD11b regulation by IMIG. Expression of CD11b was determined after stimulation of PMN by IMIG in the presence of PBS, C4 (50 μg/ml), C5 (70 μg/ml), HSA (150 μg/ml), C6 (150 μg/ml), or C7 (150 μg/ml) at 37°C for 30 min. Stimulation of PMN by FMLP (10⁻⁶ M) served as a positive control. Data are expressed as the percent change in mean fluorescence (mean ± SD of at least five independent experiments) compared with that in cultures in the absence of IMIG. The p values shown (***, p < 0.001; n.s., not significant) indicate levels of significance for differences to cultures in PBS milieu. C, Data from one representative experiment are shown. Shaded profiles represent isotype control staining. Stimulation of PMN by FMLP serves as a positive control (medium control, dotted profile; FMLP, black profile). In histograms designated PBS, C4, or HSA, dotted profiles show staining in the absence of IMIG, and black profiles show staining in the presence of IMIG.

FIGURE 3. Effects of different serum proteins on neutrophil CD35 (■) and CD66b (■) regulation by IMIG. Receptor expression was determined after stimulation of PMN in the presence of PBS, C4 (50 μg/ml), C5 (70 μg/ml), HSA (150 μg/ml), or HIS (25%) at 37°C for 30 min. Stimulation of PMN by FMLP (10⁻⁶ M) served as a positive control. Data are expressed as the percent change in mean fluorescence (mean ± SD of at least four independent experiments) compared with that in cultures in the absence of IMIG. The p values shown (***, p < 0.001) indicate levels of significance for differences from cultures in the PBS milieu.

FIGURE 4. Effects of different PMN activators on neutrophil CD88 regulation. Expression of CD88 was determined after stimulation of PMN by cuprophan-activated NHS (acNHS; 25%), C5a (250 and 500 nM), PMA (10⁻⁷ M), FMLP (10⁻⁶ M), or IMIG in the presence of PBS, C5 (70 μg/ml), or HSA (150 μg/ml) at 37°C for 30 min. Data are expressed as the percent change in mean fluorescence (mean ± SD of at least three independent experiments) compared with that in unstimulated cultures.
(41). However, because IMIG-induced C5-dependent neutrophil CD11b up-regulation was neither reduced by anti-C6/anti-C7 mAb nor increased upon addition of purified C6 and C7 (data not shown), it appeared unlikely that C5b(67) was operative in our setting.

Scavenging of ROIs inhibits protein-dependent neutrophil receptor regulation by immobilized IgG2a

Because oxidants induce a multitude of conformational alterations in proteins (14, 42–44), we were interested whether IMIG-induced PMN-derived ROI had some role in protein-dependent neutrophil receptor modulation. Antioxidants such as catalase or glutathione contained within erythrocytes are known to break down PMN-derived H2O2 (45) and to prevent neutrophil HOCl and hydroxyl radical formation (46) and ROI-mediated apoptosis (27). We sought to attenuate the oxidative stress in the environment of IMIG-stimulated PMN by using autologous RBC at different PMN/RBC ratios. This markedly reduced/abolished HIS-, HSA-, and C4-dependent neutrophil CD11b regulation (Fig. 5A). In contrast, no significant inhibition of neutrophil CD11b regulation was seen when RBC with inactivated antioxidant systems (see Materials and Methods) were used, indicating that ROI were involved (data not shown). Similar results were obtained for C5-dependent reactions and with regard to CD66b. In parallel experiments the presence of RBC dose-dependently increased FMLP-stimulated neutrophil CD11b modulation (data not shown). FMLP induces PMN-derived ROI that terminate its chemotactic activity (47). In the presence of RBC, FMLP appeared to be protected from FMLP-induced ROI to mediate a more pronounced neutrophil CD11b up-regulation, confirming the strong antioxidant effect of RBC.

Potent antioxidants, such as the NADPH oxidase inhibitor apocynin (48), the superoxide anion scavenger Tiron (13), and the main intracellular antioxidant GSH (49), significantly inhibited IMIG-induced protein-dependent CD11b and CD66b regulations, as exemplified by studies with HSA (Fig. 5B). In the presence of Trolox, a water-soluble tocopherol analog known to prevent oxidative modification of proteins (50), an even more pronounced inhibition of HIS-dependent CD11b and CD66b up-regulation by IMIG was observed (Fig. 5B). These data suggested that HSA was protected from oxidative attack, which, in turn, resulted in reduced PMN activation. Interestingly, neither the hydroxyl radical scavenger DMSO nor the HOCI and chloramine scavenger l-methionine (44) influenced IMIG-induced C4- and HSA-dependent neutrophil CD11b and CD66b regulations (data not shown). Inhibition of MPO to prevent H2O2 consumption and formation of chlorinated ROI was reported to increase cumulative H2O2 levels of stimulated PMN (45). Importantly, the application of MPO inhibitors sodium azide (45) and p-aminobenzohydrazide (51) did not decrease but, rather, slightly increased protein-dependent PMN receptor regulations in IMIG incubations (data not shown). Taken together, these results pointed toward H2O2 as an operative ROI species in this experimental setting.

Hydrogen peroxide mediates serum protein-dependent and -independent neutrophil receptor modulation

In a further series of experiments reagent H2O2 was added to isolated PMN. In the absence of protein, H2O2 mediated CD11b up-regulation in a dose-dependent fashion. The presence of C4 or HSA significantly augmented this action of H2O2 and brought about considerable neutrophil receptor modulation even at low H2O2 concentrations that were ineffective in protein-free milieu (Fig. 6). Similar results were obtained with regard to CD66b (data not shown). These direct H2O2-mediated effects were independent from the action of PMN-contained MPO and the possible generation of chlorinated oxidants, because addition of the MPO inhibitor sodium azide (1 mM) remained without effect (data not shown). Moreover, exposure of PMN in the presence or the absence of HSA or HIS to different concentrations of NaOCl did not result in significant neutrophil CD11b modulation (data not shown). These results identified H2O2 as an essential element for serum protein-dependent PMN activation, comparable to neutrophil receptor modulations by IMIG.
Stimulation of neutrophils by immobilized IgG2a leads to oxidative protein modification

Because proteins are preferred targets for ROI (43, 52), we sought to clarify whether oxidative protein modification actually occurred in PMN cultured with IMIG and protein. Protein-associated carbonyls were determined by ELISA to estimate oxidative damage (30, 42). Supernatants from PMN stimulated by IMIG in the presence of HSA contained markedly elevated carbonyls compared with controls. In contrast, when the tocopherol analog Trolox (50) was present during PMN activation, carbonyl levels in supernatants were only minimally increased (Fig. 7A). This was clear evidence that oxidative modification of protein was brought about by IMIG-induced PMN-derived ROI.

Oxidatively modified HSA binds to the neutrophil surface

Protein oxidation may lead to altered specific functional activity (14, 44), protein unfolding (42), or new binding properties of oxidized proteins, as is the case with oxidized low density lipoprotein binding to the macrophage scavenger receptor implicated in atherogenesis. In our experimental setting only oxidatively modified HSA, but not native HSA, was able to mediate PMN activation. To investigate the binding behavior of HSA, we prepared supernatants of PMN incubated with or without IMIG in the presence of FITC-conjugated HSA. These supernatants were incubated with resting PMN. Enhanced HSA-FITC binding was only noted when supernatants from IMIG-stimulated PMN were applied (Fig. 7B). In contrast, very low neutrophil binding of HSA-FITC from Trolox-supplemented supernatants was observed. Thus, oxidative modification of HSA was essential for HSA binding to PMN as a probable basic condition for the mediation of PMN stimulation.

Additional experiments were performed to investigate the agonistic effects of oxidized HSA. Supernatants from IMIG-stimulated PMN containing HSA transferred to resting PMN induced significant CD11b and CD66b up-regulation. However, no such reaction was evoked by protein-free supernatants or by supernatants containing HSA that had been protected against oxidation by Trolox (Fig. 8). Trolox not already present during supernatant preparation but added later to resting PMN and stimulatory HSA-containing

FIGURE 6. Influence of hydrogen peroxide on neutrophil CD11b expression. Expression of CD11b was determined after stimulation of PMN with different concentrations of H$_2$O$_2$ in the presence of PBS (□), C4 (50 µg/ml, ■), or HSA (150 µg/ml, ▬) at 37°C for 30 min. Data are expressed as the percent change in mean fluorescence (mean ± SD of at least six independent experiments) compared with that in cultures in the absence of H$_2$O$_2$. The $p$ values shown (***, $p < 0.001$; **, $p < 0.01$; n.s., not significant) indicate levels of significance for differences from cultures in the absence of H$_2$O$_2$.

FIGURE 7. A, Generation of HSA-associated carbonyls by IMIG-stimulated PMN-derived oxidants. Carbonyl content was assessed in cell-free supernatants from PMN stimulated by IMIG in the presence of HSA (150 µg/ml) and in the absence or the presence of Trolox (150 µM) at 37°C for 30 min. Data are expressed as the percent increase in HSA-DNP equivalents (mean ± SD of at least five independent experiments) compared with that in cultures in the absence of IMIG. The mean absolute levels of HSA-associated carbonyls in supernatants from cultures with unstimulated PMN in the absence or the presence of Trolox were 54.6 ± 10.3 or 54.2 ± 4.2 ng/ml HSA-DNP equivalents, respectively. The $p$ value shown (**, $p < 0.01$) indicates the level of significance for differences from cultures in the absence of IMIG. B, Binding of HSA-FITC to resting PMN depends on prior modification by PMN-derived oxidants. Cell-free supernatants from PMN stimulated by IMIG (37°C, 30 min) in the presence of HSA-FITC (300 µg/ml) and in the absence or the presence of Trolox (150 µM) were incubated with resting PMN at 37°C for 30 min, and cell-associated fluorescence was determined. Data are expressed as the percent increase in mean fluorescence (mean ± SD of four independent experiments) compared with that in cultures in the absence of Trolox. The $p$ value shown (***, $p < 0.001$) indicates the level of significance for differences from cultures in the absence of Trolox.
supernatant did not interfere with receptor modulation (data not shown). In contrast, heat-denatured (70°C, 30 min) HSA added to resting PMN did not influence CD11b expression (data not shown). These data demonstrated that oxidative modification of HSA was the key element in IMIG-induced HSA-dependent neutrophil receptor modulation and degranulation.

The morphology of resting PMN exposed to supernatants from PMN that had been incubated with IMIG in the presence or the absence of HSA changed in parallel with CD11b/CD66b expression. Only supernatants containing oxidatively modified HSA were effective to elicit neutrophil spreading on gelatin-coated microtiter plates. Supernatants from PMN incubated either only with IMIG or HSA or in the presence of Trolox did not affect the shape of resting PMN (Fig. 9). Activation-triggered PMN adhesion to and spreading on gelatin are mediated via β2 integrins (53, 54) and require these receptors to be in a high avidity state (3). Consequently, these findings indicated that oxidatively modified HSA, in the absence of additional stimuli, caused structural CD11b/CD66b up-regulation and also suggested integrin activation in resting PMN.

**Proteases appear not to be involved in protein-dependent neutrophil receptor regulation by immobilized IgG2a**

Oxidation of proteins was reported to facilitate proteolytic susceptibility (43, 55). Therefore, IMIG-induced PMN-derived proteases cathepsin G or elastase (56) could possibly degrade oxidized serum proteins and contribute to the observed PMN activation. However, in experiments with IMIG and HSA or C4, no significant influence of the protease inhibitors Pefabloc SC or PMSF or of cathepsin G- (Z-Gly-Leu-Phe chloromethylketone) and elastase-specific inhibitors (methoxysuccinyl-Ala-Ala-Pro-Val chloromethylketone and eglin C fragment) on CD11b modulation was detected (data not shown).

**Discussion**

In this study we demonstrate that ROI are also able to indirectly mediate PMN activation via oxidative modification of selected serum proteins to conformations that bind to and activate PMN. Because prominent ROI release is a common feature of many pathological conditions, this protein-dependent pathway of PMN activation may represent an enhancer mechanism of inflammation.

In recent years data have accumulated on the contribution of ROI to the development of various disease states, such as ARDS (16, 21), atherosclerosis (17), chronic obstructive pulmonary disease (18), sepsis (24), or side effects of hemodialysis (20, 29) and mAb treatment (19). The therapeutically used anti-CD3 mAb OKT3 strongly induces the neutrophil oxidative burst in a strictly IgG2a-dependent FcγR-mediated fashion (19). It was speculated that this massive ROI release could be involved in the frequently occurring first dose effects of OKT3, characterized by adhesion activation of PMN and sequestration in the pulmonary microvasculature, pulmonary compromise, and neutropenia (32, 33, 57).

In this investigation FcγR cross-linking by IMIG was used as model for immune complex-mediated PMN activation. As shown by comparison with different mouse myeloma isotypes, IMIG strongly stimulated the oxidative burst by virtue of its IgG2a properties. In contrast to the release of ROI, a marked CD11b up-regulation by IMIG was seen only in the presence of serum. The enzymatic complement cascade appeared not to be essential for receptor modulation, because the presence of HIS devoid of enzymatic activity, or human sera depleted of single C components resulted in similar CD11b up-regulation. Moreover, the application of sCR1 reliably inhibiting C3/C5 convertases of both the classical and alternative pathway of C activation effected only minor inhibition of CD11b up-regulation in NHS incubations. Interestingly, both the time range and extent of neutrophil CD11b up-regulation in our experiments were comparable to reactions found in OKT3-treated patients (57).

**FIGURE 9.** Oxidatively modified HSA mediates neutrophil spreading on gelatin-coated surfaces. Supernatants of PMN stimulated by IMIG (37°C, 30 min) in the presence of HSA (300 μg/ml) under the influence of Trolox (150 μM) were incubated with resting PMN at 37°C for 30 min. Photomicrographs of PMN exposed to supernatants from PMN incubated with HSA alone, IMIG and HSA, or IMIG and HSA in the presence of Trolox from one representative experiment of four are shown.
Obviously, serum constituents were essential for IMIG-induced PMN receptor modulation. Indeed, the presence of purified serum protein C4, C5, or HSA was found to suffice to mediate neutrophil activation by IMIG. With each of these proteins, we observed a marked IMIG-induced CD11b up-regulation suggestive of PMN adhesive activation and significantly increased surface CD35 and CD66b expression as markers for exocytosis of secretory vesicles and specific granules, respectively (4). This was due to individual protein structure, as the presence of other proteins, such as C6, C7, factor H, or gelatin, tested over a wide concentration range, was not able to induce significant neutrophil receptor modulation by IMIG. Notably, C4 and C5 are very similar in sequence and overall organization, as are C6 and C7 (58). Additionally, the $M_i$ of effective proteins could play a modifying role, as smaller HSA had to be applied at higher concentration to produce PMN reactions of equal magnitude, compared with the bigger molecules C4 or C5, already effective at lower concentrations.

Interestingly, whether a protein displayed stimulating properties in our experimental setting did not correlate with the adhesive propensity of PMN to the individual native protein, as deduced from studies by DiScipio and coworkers (59), who reported on spontaneous adhesion of PMN to factor H and rather low binding to native C6, HSA, C5, and C7 (in descending order of affinity). In our assays, C4, C5, and HSA did not significantly modulate PMN receptor expression in the absence of IMIG. Therefore, factors released by IMIG-activated PMN appeared to be responsible for conformational changes in native C4, C5, and HSA, enabling PMN to recognize altered proteins, which then activated signaling pathways responsible for receptor modulation.

We provided evidence that no known C4 or C5 split product activity was responsible for PMN stimulation in the presence of purified C proteins. Because HSA was also very effective, we assume that these proteins acquire their activating potential via a common mechanism. Oxidants released from IMIG-stimulated PMN were promising candidates for protein modification to an activatory conformation, similar to C5-dependent PMN activation via ROI induced by the dialyzer material cuprophan (29). The profile of ROI species released is characteristic for individual pathways of neutrophil oxidative burst activation, with immobilized Ig inducing particularly large amounts of $H_2O_2$ (60). Different ROI species released by cuprophan- or IMIG-stimulated PMN (possibly predominantly HOCI vs predominantly $H_2O_2$) could explain differential serum protein requirements (C5 vs a number of selected proteins, including C4, C5, and HSA) for neutrophil receptor modulation.

On the basis of experiments using a panel of antioxidants with differential ROI specificity, we concluded that mainly $H_2O_2$ was operative in IMIG-provoked PMN activation. Indeed, exposure of PMN to $H_2O_2$ in the absence of protein resulted in a dose-dependent CD11b and CD66b up-regulation, starting at a $H_2O_2$ concentration of 100 $\mu$M. This is compatible with an earlier study that reported on increased neutrophil $\beta_2$ integrin expression by similar amounts of $H_2O_2$ (11). Importantly, the presence of either C4 or HSA considerably triggered $H_2O_2$-dependent CD11b/CD66b up-regulation, especially at $H_2O_2$ concentrations that were too low to modulate receptor expression in the absence of protein. This closely resembled IMIG-activated neutrophil surface receptor dynamics, leading to our speculation that cumulative $H_2O_2$ levels in experiments with IMIG were ranged from 30 to 100 $\mu$M, values that had previously been encountered in close vicinity of stimulated PMN (45, 61).

Our suspicion that PMN activation could be induced by ROI-mediated protein modification was supported by enhanced carbonyl formation in supernatants of PMN incubated with HSA and IMIG (62). The increase in carbonyls under these conditions was abolished by Trolox, a water-soluble tocopherol analog reported to efficiently protect proteins against oxidation (50). Because IMIG-induced carbonyl levels were not as excessively increased as in HOCI-mediated HSA oxidation (63), these data suggested that $H_2O_2$ was the relevant ROI species. A possible proteolytic degradation of oxidized proteins (55) was not involved in protein-dependent PMN activation by IMIG, as judged from studies with a panel of protease inhibitors, including selective elastase and cathepsin G inhibitors.

In parallel with HSA-associated carbonyl formation by IMIG-induced ROI, HSA-FITC exhibited oxidation-dependent binding to PMN. Furthermore, neutrophil CD11b and CD66b up-regulation was seen not only in PMN incubations with ROI-inducing IMIG and HSA, but also in a paracrine setting where supernatants from PMN stimulated by IMIG in the presence of HSA were transferred to unstimulated PMN. Thus, oxidized HSA mediated PMN activation independent from additional stimulation. Supernatant transfer experiments also revealed that oxidized HSA mediated activation-dependent neutrophil spreading on gelatin surfaces. The latter was reported to depend on $\beta_2$ integrins (53, 54), thus arguing for integrin activation in addition to CD11b modulation. As with carbonyl formation, Trolox efficiently inhibited all protein-dependent PMN reactions, provided this agent was present during PMN stimulation by IMIG. These results confirmed the oxidative genesis of protein-dependent PMN activation by IMIG and identified oxidized HSA as a potent stimulatory agonist of resting PMN.

In the course of oxidative protein modification, proteins obviously acquired new binding properties for PMN as a prerequisite for signaling and PMN activation. Methionine sulfoxide as well as protein carbonyl and bitrosine formation, loss of amino acids, and amino acid interconversion have been reported as oxidative protein modifications, leading to changes in overall charge, folding, and hydrophobicity (44, 55, 64). The resultant oxidative denaturation, i.e., alterations of secondary, tertiary, and quaternary structure (55), appears to facilitate subsequent protein binding to PMN (65, 66). In IMIG and $H_2O_2$ experiments, C4, C5, and HSA probably acquired PMN-stimulating conformation by undergoing oxidative denaturation, because proteins not exposed to or protected against ROI were not stimulatory. As denaturing events usually produce largely unpredictable conformational alterations and heterogeneous products, it is difficult to propose probable receptor/ligand interactions that are responsible for protein-dependent PMN activation. Previously, denatured, but not native, HSA was identified as a chemotactic agonist for PMN (65, 67). Denatured HSA effected protease release from PMN and bound to the PMN cell surface in a saturable manner, suggestive of interaction with distinct receptors (68). Indeed, neutrophil CD11b/CD18 and CD11c/CD18 integrins were demonstrated to mediate adhesion to different denatured protein substrates by recognizing common denatured protein epitopes (69). Recognition of oxidatively denatured proteins by integrins could well serve as an explanation for induction of protein-dependent PMN responses by IMIG. We propose that C4, C5, and HSA proteins undergo ROI-mediated conformational alterations and are bound by neutrophil receptors, presumably members of the $\beta_2$ integrin family. Upon modified protein/$\beta_2$ integrin interaction, intracellular signaling may be initiated that could lead to PMN stimulation. Importantly, all protein-dependent PMN reactions observed in this investigation, such as integrin up-regulation, degranulation, and neutrophil spreading, are compatible with $\beta_2$ integrin signaling (70, 71).

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In conclusion, local PMN-induced oxidative alteration of serum proteins may represent a general autocrine and paracrine proinflammatory enhancer mechanism for PMN activation and accumulation at the site of inflammation. Microvascular stasis conditions, neutrophil burst induction, and resultant serum protein oxidation may augment neutrophil extravasation via adhesion molecule regulation. Similarly, serum proteins exuded into the interstitium could be modified by locally produced oxidants and may direct extravascular neutrophil movement toward the center of inflammation. The biological relevance of oxidative protein modification awaits future in vivo elucidation. However, it could be reflected by the recent discovery of highly elevated levels of oxidized proteins in various pathological conditions, including ARDS (26), sepsis, major trauma (24), uremia (63), asthma (23), bronchitis (22), and prematurity (25). On the basis of our results, we propose that oxidatively modified serum proteins may be not only a manifestation of collateral damage by PMN-derived ROI (10), but also proinflammatory mediators of neutrophil stimulation.

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References


