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LOW-AFFINITY INTERACTION OF FIBRINOGEN CARBOXY-GAMMA TERMINUS WITH HUMAN MONOCYTES INDUCES AN OXIDATIVE BURST AND MODULATES EFFECTOR FUNCTIONS

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The interaction of highly purified, monomeric fibrinogen (Fg) with human monocytes (MO) was investigated. In contrast to commercial Fg, no high-affinity binding of monomeric Fg to MO or mononuclear cells could be demonstrated. MO preincubated with Fg in the presence or absence of Ca^{++} elicited an oxidative burst when triggered with anti-Fg antibodies. Divalency of the antibody and specificity were required, but an intact Fc portion was not. Surface-adsorbed monomeric Fg also promoted an oxidative burst. Evidence is presented that Fg-MO interaction is mediated by the carboxy-γterminus of Fg. MO treated with monomeric Fg or exposed to Fg-coated surfaces show a reduced oxidative burst upon triggering with unrelated stimuli. Thus, MO function may be modulated upon interaction with surface-adsorbed Fg or with fibrin.
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Monocytes (MO) and macrophages were reported to bind fibrinogen (Fg) and/or fibrin (Fb), but the exact binding sites on both Fg/Fb and cells have been controversial (1-8). Recent evidence suggests that Mac-1 (CD11b/CD18, complement receptor type III), a member of the integrin superfamily (9) binding iC3b (10) and factor X of the coagulation cascade (11), serves as a binding site for Fg on both human polymorphonuclear leukocytes and MO (12-14). We showed that the binding event may be followed by a signal transduction, although the physiological functions induced by Fg receptor triggering remained unknown (13). In the present study, the interaction of Fg with MO was further investigated. In contrast to our results with commercial Fg, we could not demonstrate specific binding of highly purified Fg to MO. However, a signal transduction indicated by an oxidative burst could be induced with highly purified surface-adsorbed Fg or with the carboxy-γ terminus (15 or 16 amino acids) cross-linked to bovine serum albumin (BSA). This suggests that the interaction of Fg with MO is of low affinity and may be of importance upon contact of MO with Fb

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Abbreviations: MO, monocytes; Fg, fibrinogen; Fb, fibrin; BSA, bovine serum albumin; HSA, human serum albumin; FgRP, fibrinogen-related peptide; MNC, mononuclear cells; HBSS, Hanks' balanced salt solution; CL, chemiluminescence; SOD, superoxide dismutase; Cyt. C, cytochrome C; PBS, phosphate-buffered saline.

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rather than with Fg. Moreover, a pretreatment of MO with Fg reduced their capacity to elicit an oxidative burst to another trigger, suggesting that the interaction of MO with Fb determines, in part, the defense-related functions of these cells.

MATERIALS AND METHODS

Reagents: Fg and a chromogenic Limulus amoebocyte lysate kit were from KabiVitrum (Stockholm, Sweden). Anti-Fg IgG was purchased from Cappel through Dynatech (Embrach, Switzerland); $F(ab')_2$ fragments were prepared by pepsin treatment (15). Bovine thrombin was from Hoffmann-La Roche, Basel, Switzerland; protease inhibitors were from Boehringer, Mannheim, F.R.G. Human IgG (16 %) and human serum albumin (HSA) were from the Central Laboratory of the Swiss Red Cross Blood Transfusion Service, Berne, Switzerland. Fibrinogen-related peptide (FgRP), a pentadecapeptide corresponding to the carboxy-terminus of the Fg γ chain, was either bought from Sigma (St. Louis, MO), or was kindly provided by Dr. R.F. Doolittle, San Diego. It has been synthetized and crosslinked to BSA with either bis-diazo-benzidine or glutaraldehyde (BSA-FgRP), as described (16). Anorganic and simple organic chemicals were from Merck, Darmstadt, F.R.G., all other reagents were obtained from Sigma.

Fibrinogen purification and quality control: Fg was purified from platelet-deprived plasma by affinity chromatography, using Sepharose CL-2B to which Fg (KabiVitrum) has been coupled with cyanogen bromide and then converted to monomeric Fb by thrombin activation exactly as described (17). Bound Fg was then eluted with 2 M urea/1 M NaCl/5 mM EDTA/0.05 M triethanolamine/pH 5.3, dialyzed against 0.05 M triethanolamine/0.1 M NaCl/0.02 % NaN_3/pH 7.4, and stored in aliquots at -20°C. The Fg has been radiolabeled with ^{125}I, using chloramine T (13). Quality control included FPLC on Superose 6, SDS-polyacrylamide gel electrophoresis under reducing conditions, clot disappearance within 24 hr after the addition of 200 U/ml streptokinase and 5 U/ml thrombin (plasminogen contamination test), and clot formation upon incubation of Fg with 10 mM Ca^{++} for 24 hr at 37°C (factor XIII contamination test).

Mononuclear cell isolation: Mononuclear cells (MNC) were isolated by a modified Ficoll-Hypaque procedure (18,19), avoiding the contamination with platelets and using pyrogen-free reagents. Absence of endotoxin was confirmed by a modified chromogenic Limulus amoebocyte lysate test adapted to microtiter plates and allowing the detection of $\geq$4 pg/ml lipopolysaccharide (13).

Exposure of MNC to Fg: MNC were incubated for 60 min in Hanks' balanced salt solution (HBSS) either with or without addition of Fg (3 µM) in a shaking 37°C water bath. They were washed twice with cold HBSS, re-adjusted to the required cell concentration and stored on ice in the dark until use.

Measurement of chemiluminescence: Luminol-amplified chemiluminescence (CL) was measured in uncoated or precoated (see below) polystyrene tubes (11x47mm) as described (13), using a Berthold (Wildbad, F.R.G.) Biolumat LB 950 luminometer. 10^5 MO in 250 µl HBSS were dark-adapted on ice, brought to 37°C for at least 4 min and stimulated with the following agents: phorbol 12-myristate 13-acetate (PMA; 10^{-7} M), serum-opsonized zymosan (50 µg/ml), anti-Fg IgG or $F(ab')_2$ (250 µg/ml), heat-aggregated (11 min 63°C) IgG (6.7 µM), Fg (3 µM), and BSA-FgRP. CL was recorded immediately after stimulation for 2-3 min. Similarly treated MNC were warmed up and placed in precoated tubes immediately prior to CL recording for 60-90 min. In another CL model, MNC were given to precoated microtiter strips (Removawell strips, Immulon, Dynatech), and CL was recorded by a single photon imaging system allowing the simultaneous measurement of 2 96-well plates (Hamamatsu Photonics, Herrsching, F.R.G.)(20). In both CL systems, CL was amplified by luminol which has been prepared as described (21).

Measurement of O_2^- production: The production of O_2^- was determined by measurement of superoxide dismutase (SOD) inhibitable cytochrome C (Cyt. C) reduction (22). The latter was recorded in precoated microtiter plates, using a thermostated (37°C) ELISA reader (Molecular Devices, Palo Alto, CA.) equipped with a 550 nm narrow

band filter and a 540 nm filter (reference wave length). Reagents and cells were sequentially dispensed into a 96-well plate in a heating block thermostatet at 37°C, the last addition being the prewarmed cells (time 0). Wells received catalase (5 U/ml, Sigma No. C40), Cyt. C (50 μM; Sigma, No. C2506), and MNC ($2x10^5$ MO/well) in a total volume of 200 μl. Control wells also contained SOD (5 U/ml; Sigma, No. S2515). After a lag phase of 10 min, the plate was transferred to the reader for kinetic recording over 60 to 90 min.

Coating of polystyrene with proteins: Polystyrene tubes, Nunclon plates and Removawell strips were treated for 3 hr with proteins at varying concentrations, rinsed 3 times with phosphate-buffered (10 mM, pH 7.4) saline (PBS) and used for MO stimulation on the same day.

RESULTS AND DISCUSSION

Composition of commercial and purified Fg: Fig. 1 shows the elution profile of Fg subjected to FPLC on Superose 6. Whereas the commercial Fg contained molecular species of a higher molecular weight than monomeric Fg, possibly Fg aggregates (23), purified Fg was devoid of such material. Commercial Fg is known to contain factor XIII and plasminogen (8), but purified Fg was devoid of these activities.

Failure to demonstrate binding of monomeric Fg to MO: We could not demonstrate a specific binding of radiolabeled Fg to MNC or purified MO. Likewise, MNC pretreated with undiluted heparinized plasma did not show Fg binding when tested by an indirect immunofluorescence method, using goat anti-Fg IgG as the first and fluorescein isothiocyanate-coupled rabbit anti-goat IgG as the second antibody. This raised the possibility that the binding to MO reported for commercial Fg (13) was due to Fg aggregates, which have been shown to form even in the absence of thrombin (23).

Fg-treated MO respond by an oxidative burst when treated with anti-Fg: Our group reported that MNC preincubated with commercial Fg respond with an oxidative burst, as measured by luminol-amplified CL, when treated with specific anti-Fg antibodies (13). Divalent antibodies were required, but the Fc portion was not. This was

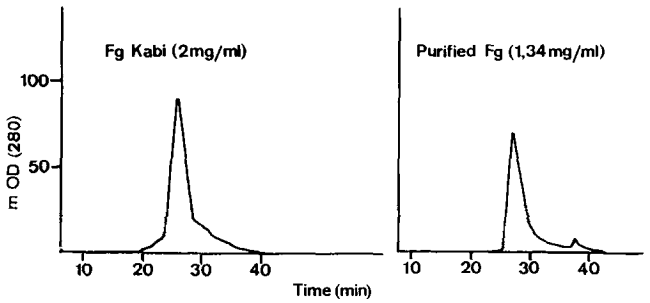

Figure 1. FPLC chromatogram of commercial Fg (Kabi) and of highly purified Fg. Both preparations showed a peak after 27 min; the small peak in purified Fg. (low molecular weight range) has not been identified. Note the absence of high-molecular-weight contaminants in purified Fg.

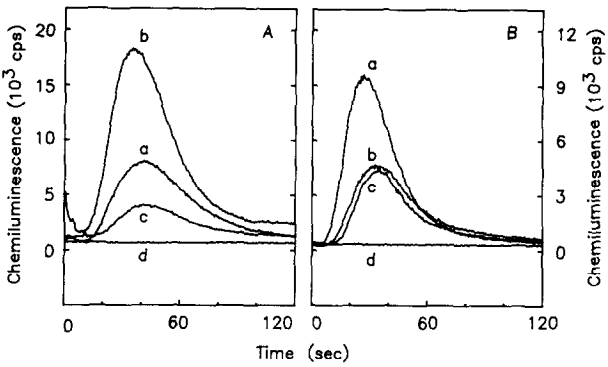

Figure 2. Temporal traces of CL recordings from Fg-pretreated, then washed MNC stimulated with aggregated IgG (a), with anti-Fg IgG (b), with anti-Fg $F(ab')_2$ or with HBSS (d). While the kinetics was similar in all experiments, the strength of the signal and the ratio of signals emitted by different stimuli varied between donors.In the experiments shown in Fig. A and B, preincubation was performed in the presence (2 m<u>M</u>) or absence, respectively, of Ca^{++}.

interpreted as evidence for signal transduction occurring upon crosslinking of receptor-bound Fg. Fig. 2 shows that anti-Fg induced CL in MNC pretreated with Fg devoid of aggregates. This response was obtained regardless of whether cells had been preincubated with Fg in the presence (2 m<u>M</u>) or absence of Ca^{++} (Fig. 2), or in the presence of EDTA (5 m<u>M</u>; not shown). Relatively high amounts of Fg (3 μ<u>M</u>) were required for obtaining a strong response. This suggested that MO bind Fg with relatively low affinity in a Ca^{++}-independent manner, and crosslinking of the bound ligand mediates signal transduction.

<u>Triggering of an oxidative burst by surface-bound Fg</u>: We then tested whether surface-bound Fg could duplicate in untreated MNC the signal transduction inducible in Fg-pretreated cells with antibody. MNC placed in either IgG-coated or Fg-coated tubes elicited luminol-amplified CL (Fig. 3A,B). Likewise, MNC placed in IgG- or Fg-pretreated microtiter plate wells, displayed CL as evidenced by the single photon imaging record of these plates (Fig. 3D,E). HSA-coated and uncoated vessels induced a weak response only. A significant, although a weak response, was also seen when plates were precoated with FgRP. Similar results were obtained when O_2^- production was determined instead of photon emission (Fig. 3C). These results support the notion that crosslinking of MO Fg receptors by Fg induces a signal transduction and that MO isolated in the absence of stimulating or activating conditions, express receptors for Fg (13).

<u>Fg interacts with MO by the carboxy-γ terminus</u>: Fg was reported to interact with certain bacteria (16), with thrombocytes (24) and with polymorphonuclear leukocytes (12) by a site represented by FgRP. This pentadecapeptide has been tested between 10^{-3} and 10^{-6} <u>M</u> and failed to induce CL in MNC (not shown). If this peptide was presented to cells as a multimer, crosslinked to BSA (16), luminol-amplified CL was induced, and this was inhibitable by an excess of monomeric Fg (Table 1). Since the

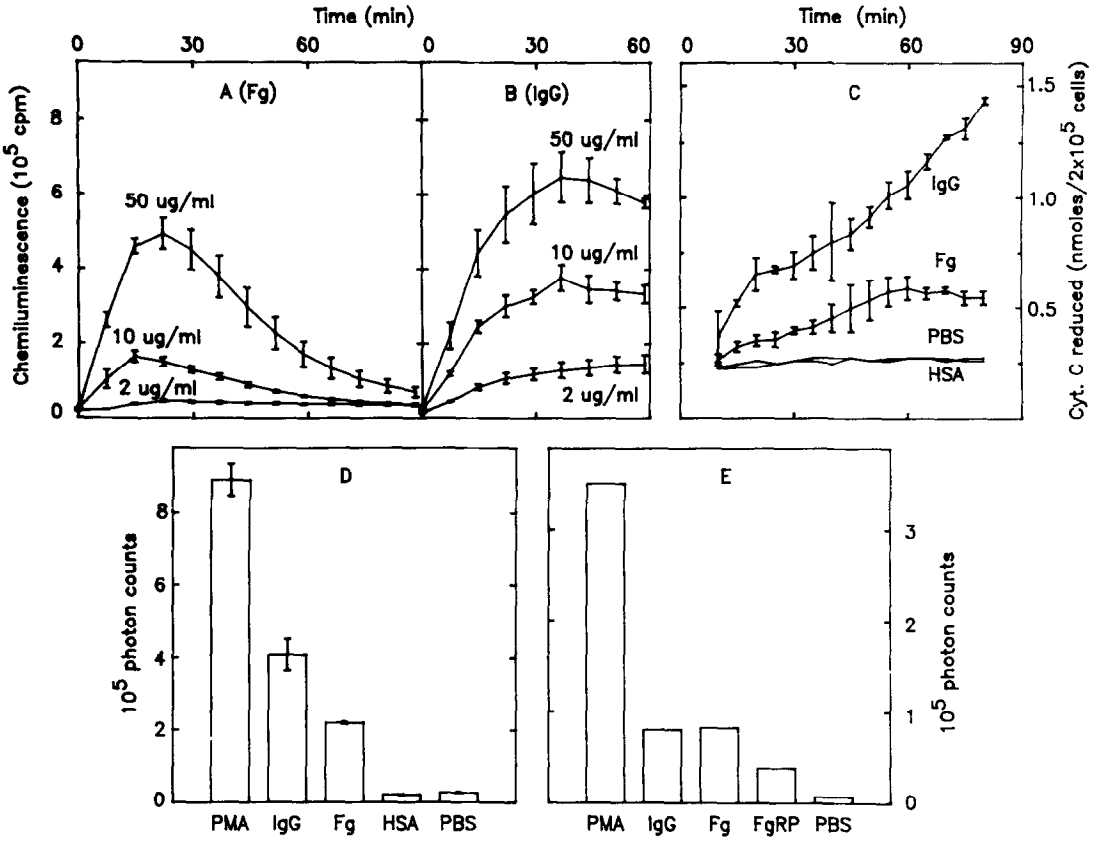

Figure 3. Evidence that surface-bound purified Fg induces an oxidative burst. In the experiment of Fig. A and B, CL was recorded with untreated MNC placed in tubes precoated with Fg or IgG, respectively, at the indicated concentrations. Means of 5 ± S.D. are shown. In Fig. C,D and E, MO were placed in microtiter plates pretreated either with IgG, Fg, HSA (all at 50 µg/ml), or FgRP (100 µM). Fig. C shows O_2^- production as indicated by SOD-inhibitable Cyt. C reduction. Fig. D and E show single photon emission within 45 min in the presence of luminol. Controls were unstimulated and PMA-stimulated cells placed in PBS-pretreated wells. Error bars indicate S.D. of triplicates.

FgRP site is also expressed on Fb, it suggests that MO not only bind to Fb, but are stimulated to elicit an oxidative burst, a notion of potential importance for understanding pathophysiology of inflammation associated with Fb deposits (1,25). Apart from a significance for coagulation-related and fibrinolysis-related events, the

Table 1: Ability of multimeric FgRP to induce in MO an oxidative burst

Stimulus	Concentration	CL response (Peak cps)
BSA-FgRP (Glu)[a]	30.0 µM	1'577
BSA-FgRP (BDB)[b]	0.6 µM	2'266
BSA-FgRP (BDB) / Fg	0.6 µM / 3 µM	577
aggreg. IgG	6.7 µM	11'581
HBSS		344

[a]BSA-FgRP crosslinked with glutaraldehyde.
[b]BSA-FgRP crosslinked with bis-diazobenzidine.

Table 2: MO pretreated with Fg (60 min 3 μM), then washed, display reduced CL upon stimulation with fluid-phase or surface-bound trigger molecules

CL stimulus	10^3 photon counts over 45 min	
	Fg-pretreated cells	Control cells
Controls: PBS	12.0	11.5
HSA	15.2	12.6
Stimuli: Surface-bound IgG	142.2 (50 %)[a]	273.9
" - " Fg	120.1 (47 %)	216.7
PMA	311.7 (54 %)	657.7

[a]Figures in background are percent activity of control cells (background-corrected).

capacity of MO to mount an oxidative burst upon triggering by multimeric Fg may be of advantage for defense against Fg-coated bacteria. Whether other effector functions are induced by this interaction, is under study.

Modulation of MO response by interaction with Fg: The interaction of MO with Fg not only induces effector functions such as the generation of reactive oxygen, it also modulates the responsiveness of MO to other stimuli. Thus, MO pretreated with Fg in suspension tend to show a diminished CL response to a variety of CL stimuli when compared with similarly incubated control cells. This is seen most clearly when CL stimulation is performed in microtiter plates (Table 2). Moreover, MO exposed to surface Fg display diminished phagocytosis of IgG-coated erythrocytes (to be reported elsewhere). Such findings raise the possibility that Fg and/or Fb not only induce certain effector functions in MO, but modulate(s) their responsiveness to other defense-related functional triggers. The differentiation of MO into macrophages has been shown to be accelerated by plasma proteins, including Fg (26), and the migratory properties of MO within and on Fb gels were found to be critically dependent on the Fb gel composition (27). Moreover, Mac-1, the putative Fg receptor on MO (12-14), appears as an important control element for a variety of effector functions. Thus, interaction of Leishmania parasites with MO via C3b/iC3b receptors, dampens the oxidative burst elicited by antibodies carried by the same parasite (28). Evidence for interaction of Leishmania parasites with CR3 has recently been provided (29). Certain anti-Mac-1 antibodies impair the capacity of MO to ingest via FcR (30). Unraveling the hierarchy of MO response patterns upon stimulation by different plasma constituents, including Fg, will further the understanding of pathophysiological processes with a mononuclear phagocyte involvement. The use of Fg-covered surfaces, and of FgRP-carrying particles or molecules, may be one approach towards this goal.

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