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Research Article

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Neutrophil Bactericidal Activity against *Staphylococcus aureus* Adherent on Biological Surfaces

Surface-bound Extracellular Matrix Proteins Activate Intracellular Killing by Oxygen-dependent and -independent Mechanisms

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Abstract

The activation patterns of surface adherent neutrophils are modulated via interaction of extracellular matrix proteins with neutrophil integrins. To evaluate neutrophil bactericidal activity, *Staphylococcus aureus* adherent to biological surfaces were incubated with neutrophils and serum, and the survival of surface bacteria was determined. When compared to albumin-coated surfaces, the bactericidal activity of neutrophils adherent to purified human extracellular matrix was markedly enhanced (mean survival: 34.2%±9.0% of albumin, $P < 0.0001$) despite similar efficient ingestion of extracellular bacteria. Enhancement of killing was observed when surfaces were coated with purified constituents of extracellular matrix, i.e., fibronectin, fibrinogen, laminin, vitronectin, or type IV collagen. In addition to matrix proteins, the tetrapeptide RGDS (the sequence recognized by integrins) crosslinked to surface bound albumin was also active (survival: 74.5%±5.5% of albumin, $P < 0.02$), and fibronectin-increased killing was inhibited by soluble RGDS. Chemiluminescence measurements and experiments with CGD neutrophils revealed that both oxygen-dependent and -independent bactericidal mechanisms are involved. In conclusion, matrix proteins enhance intracellular bactericidal activity of adherent neutrophils, presumably by integrin recognition of RGDS-containing ligands. These results indicate a role for extracellular matrix proteins in the enhancement of the host defense against pyogenic infections. (*J. Clin. Invest.* 1990. 86:942-951.) Key words: neutrophils • integrin • extracellular matrix • fibronectin • *S. aureus*

Introduction

Adherence of bacteria to cells or extracellular matrices is often the first step in colonization and subsequent infection. Both bacteria and neutrophils possess receptors or "adhesins" for extracellular matrix proteins. The adherence of neutrophils to cells and tissues is mediated by a family of cell-surface glycoproteins, the leukocyte adherence molecules Mac-1, LFA-1, and p150,95 (1, 2). A deficiency of the β_2 -subunit of these

proteins has been associated with severe bacterial or fungal infections in affected patients (3). The Mac-1 molecule on neutrophils is also the receptor for the complement fragment C3bi (4) and has recently been shown to recognize fibrinogen (5). These leukocyte adherence molecules are membrane spanning glycoproteins that mediate cell adhesion and constitute one of the three families of the integrin superfamily. In addition to the leukocyte adherence molecules, the two other families of integrins include: the receptors for fibronectin (6, 7), laminin (8, 9), type I and type IV collagen (10), and vitronectin (11-13).

The cell-binding domain of matrix proteins such as fibronectin and vitronectin recognized by integrins on a variety of cell types is made up of a short sequence of amino acids: Arg-Gly-Asp-Ser (RGDS) (for review see 14). More recently, the interaction between neutrophil integrins and fibronectin has been shown to be dependent on the recognition of RGDS (15) and the activating effect of fibronectin on ingestion of IgG-coated erythrocytes could be mimicked by RGDS containing proteins (16). Fibronectin, fibrinogen, laminin, collagen, and vitronectin enhance a variety of phagocytic functions including neutrophil oxidative metabolism (15, 17-25). However, when neutrophil-mediated killing was determined in cell suspension systems, neither enhanced uptake nor killing of bacteria could be demonstrated by solubilized matrix proteins unless additional stimuli (such as formylated peptides) were present (26-28).

Fibronectin, fibrinogen, laminin, and vitronectin may also play an important role in pathogenesis, since they possess specific binding sites for staphylococci (29-33) which serve as ligands mediating bacterial adherence to surfaces (34-37). Different matrix proteins are present in inflammatory tissue (38, 39) or are rapidly deposited on foreign material such as prosthesis and catheters (40-42). A detailed analysis of the neutrophil mediated killing of microorganisms bound to adsorbed matrix proteins rather than in suspension is therefore warranted.

Here we report that the bactericidal activity of adherent neutrophils is enhanced by extracellular matrix or by several of its purified components adsorbed to the surface. This interaction seems to be at least partially mediated via a RGDS-containing ligand interacting with neutrophil integrins. Thus, matrix proteins can be considered as important host defense modulatory factors in infections caused by pathogenic and surface adherent microorganisms.

Methods

Chemicals and materials. PBS solutions with and without 1 mM Ca^{2+} and 0.5 mM Mg^{2+} were purchased from Gibco Ltd. (Paisley, Scotland)

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and [*methyl*-³H]thymidine (54 Ci/mmol) from Amersham International (Buckinghamshire, UK). Fibronectin, fibrinogen, and pooled IgG (Sandoglobulin®), kindly provided by the Central Laboratory of the Swiss Red Cross (Bern, Switzerland), were purified as previously described (40, 43). Purified laminin was purchased from Bioreba-Diagnostica (Basel, Switzerland), and type IV collagen was obtained from Sigma Chemical Co. (St. Louis, MO). Human vitronectin (S-protein), a generous gift of Dr. Chhatwal, University of Braunschweig, FRG, was purified as described (44). No significant degradation or contaminants were seen in the preparations using SDS-PAGE (7%) electrophoresis. Human extracellular matrix, purified from human placenta by Collaborative Research (Lexington, MA), was purchased from Bioreba. RGDS, RGDSC, cysteine (C), and *N*-maleimido-6-aminocaproyl ester of 1-hydroxy-2-nitro-4-benzenesulfonic acid (mal-sac-HNSA) were purchased from Bachem (Bubendorf, Switzerland), and either RGDSC or C were crosslinked to HSA in a monitorable reaction using mal-sac-HNSA as crosslinking agent (45). After 15 min, 14 of 57 lysin groups had reacted with mal-sac-HNSA; thus, the final conjugate consisted of an estimated number of seven peptide groups per molecule of HSA. Pooled human serum was prepared from fresh blood obtained from healthy volunteers. Catalase, horseradish peroxidase, sodium azide, EDTA, acridine orange, and 5-amino-2,3-dihydro-1,4-phthalazine-dione (luminol) were purchased from Sigma Chemical Co., and FITC, ethidium bromide, and homovanillic acid from Fluka (Buchs, Switzerland).

Adsorption of proteins on polymethylmethacrylate (PMMA)¹ and adherence of bacteria. Optimal protein concentrations for attachment have been described previously using radiolabeled proteins (34, 37, 46). 100 μ l of PBS containing either extracellular matrix, fibronectin, laminin, vitronectin, type IV collagen (25 μ g/ml, respectively), fibrinogen (35 μ g/ml), or IgG (100 μ g/ml) were deposited on sterilized PMMA coverslips (7 \times 7 mm) in a circle covering > 90% of the coverslips surface area. The coverslips were then incubated for 1 h at 37°C in a humid chamber and washed in PBS containing 0.5% HSA. HSA was added to block bacterial attachment to the unexposed side of the coverslips (37), and, in experiments using cysteine-albumin or RGDSC-albumin, these proteins (500 μ g/ml) were added after bacterial adherence to coat the coverslips (15 min, 37°C). In each experiment, a series of uncoated coverslips was run in parallel.

Staphylococcus aureus Wood 46, a catalase positive strain devoid of protein A, was used throughout the phagocytosis experiments. Bacteria were grown to exponential phase in Mueller Hinton broth and radiolabeled with 20 μ Ci of [³H]thymidine/ml broth as described (34), washed by centrifugation at 3,000 *g* for 10 min, and resuspended in PBS containing Ca²⁺ and Mg²⁺ to a final concentration of 1 \times 10⁹ CFU/ml. CFU and cpm counts were performed to determine the bacteria/cpm ratio. 100 μ l of the bacterial suspension were deposited on the coverslips and incubated for 1 h at 37°C. The coverslips were washed twice for 10 min in PBS. The number of adherent organisms was determined by radioactive counts and found to be slightly higher on extracellular matrix (4.0 \pm 3.0 \times 10⁶ organisms/coverslip, mean \pm SD, *n* = 6), fibronectin (4.8 \pm 5.4 \times 10⁶, *n* = 17), and fibrinogen (5.7 \pm 2.6 \times 10⁶, *n* = 12) than on laminin (1.2 \pm 0.9 \times 10⁶, *n* = 4), vitronectin (1.6 \pm 0.4 \times 10⁶, *n* = 4), type IV collagen (1.7 \pm 1.0 \times 10⁶, *n* = 4), IgG (3.6 \pm 3.5 \times 10⁶, *n* = 4), and uncoated (2.1 \pm 2.7 \times 10⁶, *n* = 11) coverslips. These results are consistent with previously published data from our laboratory (34, 37). To quantify ingestion, bacteria were grown to exponential phase without radiolabeled thymidine, washed and resuspended in carbonate buffer (pH 9.5) containing 0.01% FITC/ml and incubated for 30 min at 37°C (47). This procedure did not affect the viability of the bacteria. After washing, bacteria were resus-

ended in PBS containing Ca²⁺ and Mg²⁺ at a final concentration of 2 \times 10⁷ CFU/ml (an inoculum allowing quantification of intracellular bacteria by microscopy) and attached to the coverslips. To exclude nonspecific interactions between bacteria and FITC resulting in changes in adherence and/or phagocytosis, control experiments were performed with ethidium-bromide; identical results were found (not shown).

Phagocytic-bactericidal assay on surfaces. Human neutrophils from healthy adult volunteers or from patients with X-linked chronic granulomatous disease (CGD) were purified as previously described using a Ficoll T 500 gradient (Pharmacia LKB Biotechnology Inc, Piscataway, NJ) (48) and resuspended in PBS containing Ca²⁺ and Mg²⁺ and 10% human serum (PBS-HS) at a final concentration of 1 \times 10⁶ PMN/ml buffer solution. Coverslips with adherent bacteria were fitted horizontally into plastic tubes, a 1-ml solution of 1 \times 10⁶ PMN/ml PBS-HS was carefully overlaid, and the tubes were incubated at 37°C for 60 min without agitation (49). Albumin as the major serum component is rapidly deposited on plastic material (50, 51) and prevents nonspecific neutrophil activation (52, 53) and deposition of matrix proteins (34) on the surfaces. Therefore, for practical purposes, the uncoated and subsequently serum-treated coverslips were designated "albumin-coated" in the text. During incubation, 74% \pm 17% (mean \pm SD, *n* = 98) of the radiolabeled bacteria were detached from albumin and matrix protein-coated coverslips. Statistical analysis yielded no significant differences in detachment between the different proteins. At the end of the incubation, the fluids containing detached bacteria were drained. To recover adherent bacteria, coverslips were transferred into tubes containing 1 ml PBS-HSA, adherent bacteria were detached using a lab-sonifier (Branson Co., Danbury, CT) (10 cycles of 1 s, 50 W, 0°C), and the fluids were analyzed for CFU (using a Laser-counter; Exotech Inc., Gaithersburg, MD) and counts per minute. Sonication was effective for recovery (> 95% of the radioactivity was detached throughout all the experiments), and control experiments indicated that it was harmless to *S. aureus*. The fraction of recovered viable bacteria was calculated using the following formula: fraction (%) = CFU/radiolabeled bacteria \times 100. Survival on matrix proteins was expressed using parallel assays with albumin-coated surfaces as control (=100%). Control experiments showed that loss of tritiated thymidin from bacteria after phagocytosis was < 10% both on albumin as on matrix proteins. Thus, a possible influence on the calculated survival due to bacterial damage and subsequent loss of radioactivity could be excluded.

Number and viability of surface adherent neutrophils. To determine the number of surface-adherent neutrophils after phagocytosis, cells were fixed on the coverslips with methanol for 1 min, stained with acridine-orange (0.01% in PBS) and observed with a fluorescence microscope. Adherent neutrophils were quantified by counting 10 microscopic observation fields, the number of adhering neutrophils per coverslip being calculated and found to be very similar on the different surfaces (2.0 \pm 0.5 \times 10⁵, 2.3 \pm 1.6 \times 10⁵, and 2.2 \pm 0.9 \times 10⁵, mean \pm SD, on albumin, fibronectin, and fibrinogen coated surfaces, respectively [*n* = 5]). Several types of control experiments were performed to check the viability of surface adherent neutrophils after 60 min: (a) > 98% of the adherent neutrophils excluded trypan blue (0.1%); (b) no differences in cytosolic free calcium levels were observed (54); (c) using more sensitive markers for membrane permeability such as ethidium bromide (55) or [³H]adenine (56), no differences in neutrophil permeability between albumin and fibronectin or fibrinogen-coated surfaces were detected.

Quantification of ingestion of *S. aureus* by neutrophils. To distinguish between ingested and cell-adherent but noningested bacteria, we modified slightly a previously described assay (47). Coverslips with FITC-labeled adherent bacteria were incubated with neutrophils (1 \times 10⁶/ml in PBS-HS) at 37°C for 60 min. At the end of the incubation period, the liquid was drained, coverslips were rinsed with ice-cold PBS, and immediately before microscopic examination, the PBS was poured off and a drop of trypan blue (0.1% in PBS) was added. The dye quenched the fluorescence of FITC-labeled *S. aureus* as confirmed by

1. **Abbreviations used in this paper:**CGD, chronic granulomatous disease; CL, chemiluminescence; NBT, nitroblue tetrazolium; PBS-HS, phosphate-buffered saline containing divalent cations and 10% human serum; PMA, phorbol myristate acetate; PMMA, polymethylmethacrylate.

experiments without neutrophils; in contrast, since the dye is excluded by neutrophils, ingested bacteria remained fluorescent. The number of bacteria in 20 cells/coverslip were assessed in triplicates in the presence and absence of trypan blue to determine the number of attached versus ingested microorganisms. After 60 min of incubation in the presence of neutrophils, a small amount of bacteria adherent to coverslips (inoculum for adherence: 1×10^9 CFU/ml) was found to be neither ingested by neutrophils nor attached: they were quantified by counting the numbers of unattached microorganisms in 10 microscope fields without trypan blue, the total number of unattached bacteria/coverslip was calculated and compared to the total number of microorganisms on the coverslip as assessed by cpm of radiolabeled bacteria.

Measurements of H_2O_2 and O_2^- production by adherent neutrophils.

We used a slightly modified method to determine H_2O_2 production based on the oxidation of homovanillic acid into a fluorescent dimer (57). Briefly, 0.5 ml of a solution containing horseradish peroxidase (4 IU/ml) and homovanillic acid (0.4 mM) were incubated with 0.25 ml of PMN (1×10^6 /ml), 0.25 ml human serum, 0.1 ml sodium azide (10 mM), coverslips with or without fibronectin (25 μ g/ml) and adherent *S. aureus*, and with 0.65 ml PBS containing Ca^{2+} and Mg^{2+} for 1 h at 37°C. At the end of the incubation period, 0.25 ml of a stop solution containing 100 mM glycine NaOH buffer and 25 mM EDTA (pH 12.0) were added, the fluids were decanted and centrifuged at 300 g for 10 min. Fluorescence of the supernatant was measured in an LS3 fluorimeter Perkin-Elmer Co. at an excitation wavelength of $\lambda = 312$ nm and an emission wavelength of $\lambda = 420$ nm. Standards were performed in parallel in the absence of coverslips and neutrophils with known amounts of H_2O_2 (between 0.1 and 10 nmol H_2O_2 /assay). O_2^- production was measured as previously described.⁴⁸

Chemiluminescence (CL) assay. We employed a previously described experimental system (58), slightly modified for surface phagocytosis. Coverslips with *S. aureus* attached as described were fitted into the bottom of 4-ml polypropylene tubes containing luminol (2×10^{-5} M) and neutrophils (1×10^6 /ml) in 1 ml PBS-HS. CL was measured continuously for 60 min with a 6-channel Biolumat LB 9505 (Berthold Co., Wildbad, FRG) and integral and peak cpm values were calculated. Controls performed without bacteria yielded 9.6%, 6.0%, and 22.6% of integral cpm values obtained with bacteria on fibronectin, fibrinogen, and albumin coverslips, respectively. Thus, a high proportion of the measured CL response was a result of the interaction of neutrophils with bacteria.

Statistical analysis. Two-way analysis of variance, paired and unpaired *t* tests and Wilcoxon's test for related rankable scores were performed using the BMDP statistical software package (59). All statistical analysis was performed using two-tailed significance tests.

Results

Inhibition of growth of *S. aureus* on albumin coated surfaces by adherent neutrophils. *S. aureus* Wood 46 were attached to coverslips and incubated with neutrophils and HS for 60 min. After phagocytosis and recovery of adherent bacteria from the albumin-coated coverslips, the fraction of recovered viable over radiolabeled bacteria was found to be $180.2 \pm 17.5\%$ ($n = 32$). To determine growth of bacteria in the absence of neutrophils, adherent *S. aureus* were incubated with HS alone; the growth was $421.8 \pm 70.6\%$ ($n = 12$). Thus, in the presence of neutrophils, growth was significantly inhibited ($P < 0.02$, paired *t* test). 60 min incubation time were found to be optimal, since after prolonged incubation in the presence of neutrophils, replication of bacteria continued: after 2 h incubation, $386.7 \pm 49.3\%$ (mean \pm SEM, $n = 3$), and after 3 h, $730.2 \pm 241.8\%$ of the radiolabeled bacteria were viable. These experiments demonstrated that neutrophils effectively inhibit growth of *S. aureus* bound to albumin coated surfaces

for a limited time period. In further experiments with matrix proteins, parallel assays were performed on albumin as control.

Increase of neutrophil bactericidal activity on surface-bound extracellular matrix proteins. We coated coverslips with extracellular matrix purified from human placenta and subsequently determined the survival of adherent *S. aureus*. As shown in Fig. 1, the bactericidal activity of adherent neutrophils on human extracellular matrix was significantly increased, since only $34.2 \pm 9.0\%$ (mean \pm SEM) of the bacteria survived when compared to albumin surfaces. In order to analyze in detail the influence of individual components of human extracellular matrix on neutrophil bactericidal activity, we coated coverslips with purified proteins known to be constituents of the extracellular matrix. Surface bound fibronectin, fibrinogen, laminin, vitronectin, and type IV collagen all increased neutrophil bactericidal activity (Fig. 1). The effect was present to a different extent and most pronounced on fibronectin (mean survival \pm SEM: $47.4 \pm 4.6\%$ of control) and least pronounced on fibrinogen ($76.0 \pm 12.8\%$ of control). In contrast, surface-bound IgG failed to enhance killing of *S. aureus*: survival on IgG was even slightly higher compared to albumin (difference not significant).

The influence of surface bound fibronectin on neutrophil bactericidal activity was present at all neutrophil concentrations tested. Using different neutrophil concentrations, survival was smaller on fibronectin when compared to albumin over the whole range of neutrophil concentrations tested (Fig. 2).

To determine the effect of matrix proteins in suspension on killing of surface bound *S. aureus*, we added 300 μ g/ml of soluble fibronectin to the phagocytic assay containing albumin coated coverslips and adherent bacteria under conditions described in Fig. 1. No enhanced killing was observed in the presence of solubilized fibronectin, i.e., $115 \pm 20\%$ (mean \pm SEM, $n = 3$) of the number of control *S. aureus* were

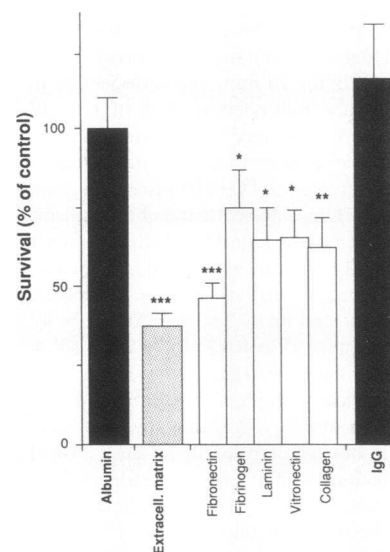


Figure 1. Survival of opsonized *S. aureus* adherent to various surface-bound matrix proteins in the presence of neutrophils. Coverslips were incubated with human extracellular matrix ($n = 6$), fibronectin ($n = 17$), laminin ($n = 7$), vitronectin ($n = 8$), type IV collagen ($n = 10$) (25 μ g/ml, respectively), fibrinogen (35 μ g/ml) ($n = 10$), IgG (100 μ g/ml) ($n = 8$), or were left uncoated ($n = 32$). Radiolabeled *S. aureus* were attached, and the coverslips were subsequently

exposed to PBS containing 10% human serum and 1×10^6 PMN/ml. After phagocytosis, bacteria were detached by sonication and the fraction of recovered viable bacteria compared to albumin as control. Results are means of n experiments \pm SEM performed in triplicate. *P* values calculated with the paired *t* test (*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$).

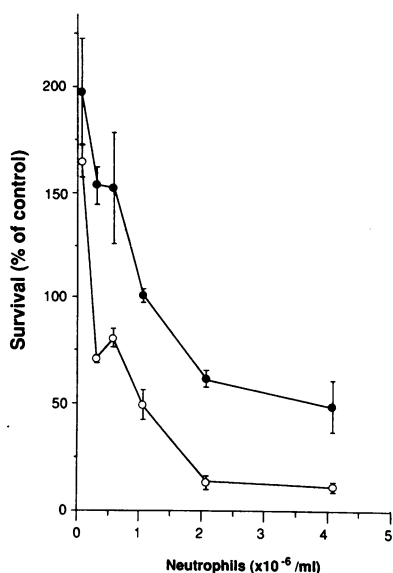


Figure 2. Influence of different neutrophil concentrations on survival. Experimental conditions as in Fig. 1. Results are means of triplicate determinations \pm SEM on fibronectin (open circles) and albumin (closed circles).

viable. Thus, the matrix proteins have to be bound to the surface to increase neutrophil bactericidal activity.

Effect of the tetrapeptide RGDS on neutrophil bactericidal activity on surfaces. A variety of extracellular matrix proteins contain the RGDS-sequence, which has been shown to mediate the proteins' interaction with integrins (14). To investigate the effect of RGDS on bactericidal activity in a surface phagocytosis system, we coated surfaces with RGDS crosslinked to albumin. We found that survival of bacteria was significantly decreased when compared to albumin or C-albumin ($74.0\% \pm 5.5\%$ vs. $100.0\% \pm 9.7\%$ and $97.8\% \pm 7.2\%$, mean survival \pm SEM, on RGDS-albumin, albumin, and C-albumin, respectively, $P < 0.02$, paired *t* test), suggesting activation of the neutrophils by the surface-bound and albumin-coupled tetrapeptide (Fig. 3). As another approach, we added RGDS (230 μ M) in suspension to the assay containing fibronectin-coated coverslips and neutrophils in order to inhibit competitively the protein-integrin interaction. We found, that the increase in neutrophil bactericidal activity on fibronectin was significantly diminished in the presence of the tetrapeptide in suspension ($46.8\% \pm 5.0\%$ vs. $76.2\% \pm 3.9\%$, mean survival in the absence versus presence of soluble RGDS, $P < 0.02$) (Fig. 3).

Ingestion of surface-adherent *S. aureus* by neutrophils. To explain the mechanisms of increased bactericidal activity, we first investigated the ingestion of *S. aureus* by neutrophils on protein-coated surfaces. After 30 s, bacteria were already associated with neutrophils (Fig. 4 A), and after 15 min almost all the microorganisms interacted with the cells (Fig. 4 B). Only $2.5\% \pm 0.8\%$, $0.4\% \pm 0.2\%$ and $0.6\% \pm 0.3\%$ (means \pm SEM, $n = 3$) of the total number of microorganisms bound to the surfaces were respectively found to be non-cell-associated on albumin, fibronectin, and fibrinogen (Fig. 4 B, arrows). Upon addition of trypan blue to fluorescence-labeled bacteria to distinguish between neutrophil adherent and ingested microorganisms, ingested compared to total neutrophil-associated bacteria was similar on the three surfaces: $85\% \pm 19\%$, $91\% \pm 26\%$, and $95\% \pm 25\%$ (mean \pm SEM, $n = 5$) on albumin, fibronectin, and fibrinogen, respectively (Fig. 5). Thus, the majority of the neutrophil associated *S. aureus* were ingested independently of the extracellular matrix protein and only a small fraction of the microorganisms was neither cell associated nor ingested after

phagocytosis. Therefore, our system was optimal for assessing the role of extracellular ligands on intracellular killing.

Oxidative metabolism of surface-adherent neutrophils in the presence of *S. aureus*. A similar basal release of H_2O_2 by neutrophils was detected on albumin and fibronectin coated surfaces in the absence of bacteria (3.2 ± 1.9 and 3.4 ± 1.7 nmol $H_2O_2/10^6$ PMN, respectively, mean \pm SEM, $n = 4$). In the presence of *S. aureus* adherent to coverslips, significantly higher amounts of H_2O_2 were released during 60 min incubation on albumin and fibronectin (7.9 ± 1.8 and 6.9 ± 1.6 nmol $H_2O_2/10^6$ PMN, mean \pm SEM, $P < 0.02$ and < 0.05 , respectively, $n = 4$). No difference in H_2O_2 release was seen on the two surface conditions. Additionally, we analyzed the neutrophil oxidative metabolism by continuous measurement of neutrophil chemiluminescence in our surface phagocytosis assay. Fig. 6 A shows a representative curve of CL on surfaces coated with albumin or different matrix proteins; the largest CL response was found on fibrinogen, whereas on albumin and fibronectin the response was smaller. This observation is confirmed by analysis of data obtained in 10 independent experiments (Fig. 6 B); significantly higher integral cpm values were found on fibrinogen when compared to fibronectin and albumin. Additionally, on albumin surfaces CL peaks were transient and decreased rapidly, whereas on fibronectin and fibrinogen the response was more sustained. Analysis of the data calculating integral over peak counts per minute values yielded significantly higher values on fibronectin and fibrinogen surfaces when compared with albumin (Fig. 6 C). This indicates a more sustained CL response, and thus a presumably more prolonged production of oxygen radicals in neutrophils on the two matrix proteins.

Characteristics of the surface bactericidal activity of neutrophils from patients with CGD. To evaluate the role of oxygen metabolites on antistaphylococcal activity we investigated surface phagocytosis with neutrophils from three patients with CGD. Neutrophils from these patients are unable to produce reactive oxygen intermediates (as shown by negative nitroblue tetrazodium slide tests and lack of detectable superoxide pro-

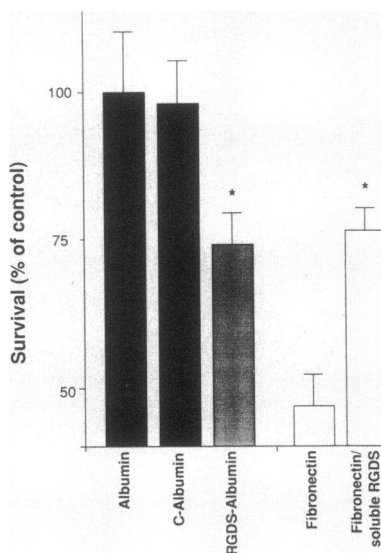
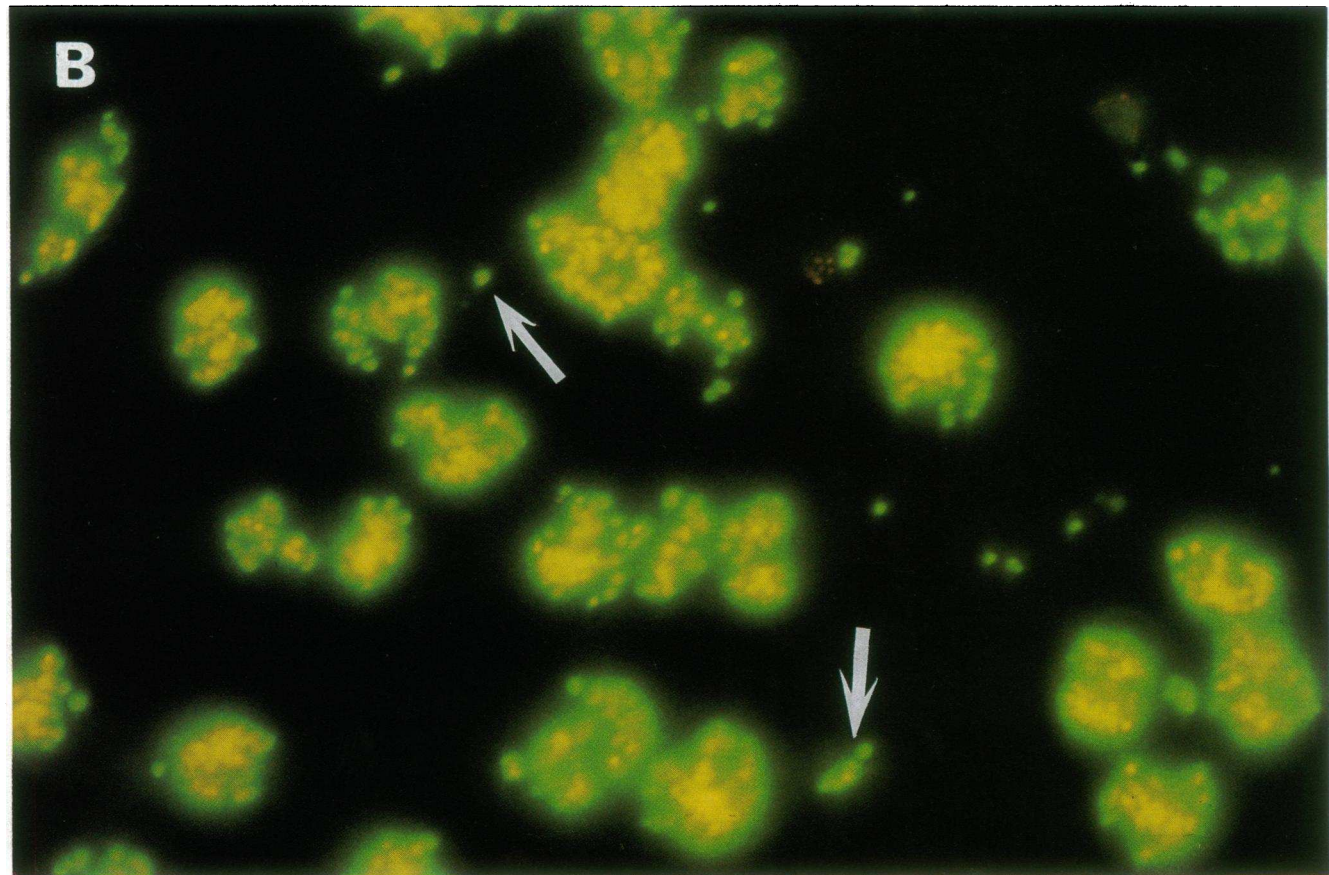
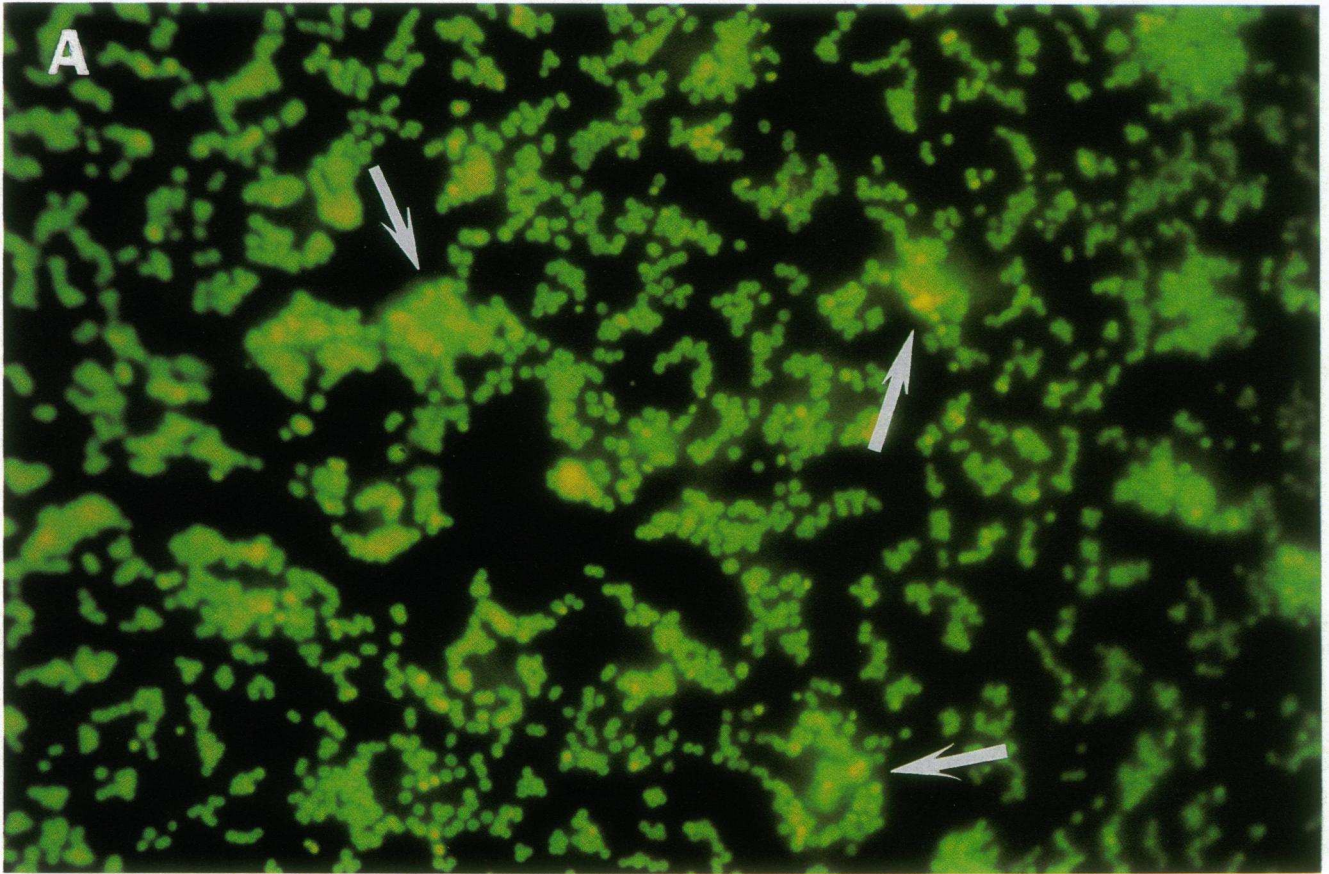


Figure 3. Effect of surface-bound RGDS-albumin on neutrophil bactericidal activity and of RGDS in suspension on fibronectin-increased bactericidal activity. Surfaces were coated either with HSA or fibronectin as described in Fig. 1, or with C-albumin or RGDS-albumin (500 μ g/ml). Neutrophils were added with or without RGDS in suspension (230 μ M). Experiments on albumin, C-albumin and RGDS-albumin were performed in the presence of catalase (5,000 IU/ml) to minimize any

oxidative damage of the integrins (16, 75). Results are means of four experiments \pm SEM performed in triplicate. * $P < 0.02$ (paired *t* test).



duction both in suspension and on surfaces). Thus, these cells are ideally suited to test oxygen independent neutrophil bactericidal activity.

As expected, increased survival of *S. aureus* was found when bacteria were exposed to neutrophils from CGD patients compared to cells from controls (Fig. 7). However, even in the presence of CGD neutrophils, killing was significantly increased on fibronectin-coated surfaces compared to albumin surfaces. Interestingly, in the presence of fibrinogen, a more potent stimulator of the respiratory burst in our experimental system, all the bactericidal enhancing activity was abrogated. Control experiments using the trypan blue quenching technique demonstrated that the ingestion of microorganisms by the neutrophils was similar in these two conditions.

Discussion

Circulating neutrophils adhere to endothelial cells, leave the vascular compartment (60) and move into tissues along chemotactic gradients (61, 62). Several lines of evidence suggest that a variety of functions of neutrophils adherent to surfaces differ in many aspects from neutrophils in suspension. (a) Superoxide production of neutrophils adhering to polystyrene surfaces is enhanced in response to FMLP and C5a (53). (b) Adherence primes neutrophils and renders them susceptible to the activating effect of cytokines (52). (c) Ingestion of surface-bound bacteria by neutrophils is observed even in the absence of opsonins (63).

Whereas an assessment of viable microorganisms in suspension after phagocytosis by neutrophils is easy to perform, so far this has not been possible with bacteria adherent to solid surfaces. The use of acridine orange in combination with a crystal violet quenching technique has been used to differentiate between viable and dead organisms as assessed by the green or red fluorescence of the stained bacteria (17). However, a major disadvantage of the acridine orange technique is the indirect determination of viability by assessment of changes in fluorescence: determinations of CFU counts reflect a more reliable estimation of the survival of ingested bacteria. Therefore, we employed an assay using viable and radiolabeled *S. aureus* adherent to surface-adsorbed matrix proteins and used sonication as an effective and harmless method to detach the bacteria after exposure to neutrophils. This experimental system allowed us to assess carefully the survival of bacteria bound to artificial surfaces by viable counts. As a first important observation, we found that large amounts of surface bound bacteria remained viable on albumin-coated surfaces despite adequate numbers of neutrophils and opsonic activity. This observation confirmed previously published results showing a greater survival of adherent *S. aureus* when compared with *S. aureus* in suspension after exposure to neutrophils (49).

Natural or artificial surfaces are readily coated with a variety of extracellular matrix proteins, e.g., fibronectin or fibrinogen (40, 41). These proteins, which are ubiquitously present

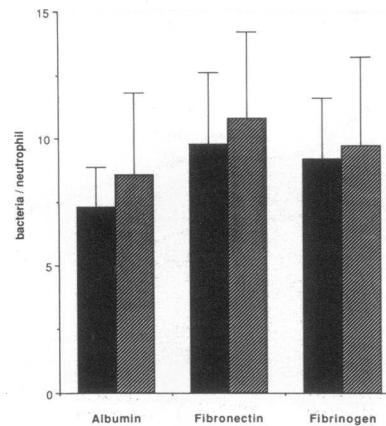


Figure 5. Ingestion of *S. aureus* bound to matrix proteins by neutrophils. *S. aureus* were charged with FITC and surface phagocytosis was performed in the presence of serum as described in Methods. Coverslips were observed by fluorescence microscopy in parallel with phase contrast microscopy in the presence (black columns, intracellular microorganisms) or absence (shaded columns,

total number of microorganisms) of trypan blue (0.1%), and the number of neutrophil associated bacteria was determined in 20 neutrophils/coverslip. Results are means \pm SEM of five experiments performed in duplicate.

as part of connective tissues, are suggested to trigger a variety of different events associated with phagocytosis. (a) Many bacterial strains are known to possess specific binding sites for these proteins (30–33) and adherence and subsequent colonization of surfaces are considered to be important steps in early infection. (b) Extracellular matrix proteins can mediate close contact between bacteria and neutrophils (27, 28), since they are dimeric or multimeric molecules (35, 64, 65) and bind specifically to receptor proteins of the integrin family on neutrophils (66). (c) The interaction of solubilized matrix proteins with neutrophils has been reported to modulate a variety of different functions in neutrophils such as chemotaxis and the triggering of the respiratory burst (17, 21) with subsequent superoxide production (23, 24). In our assay, coating with human extracellular matrix as well as with a variety of its purified components such as fibronectin, fibrinogen, laminin, type IV collagen and vitronectin to surfaces significantly enhanced killing of *S. aureus*. Furthermore, we found that the deposition of the matrix proteins on the surface appears to be the required step to elicit enhanced killing, since fibronectin in suspension did not trigger this effect as shown by others in different conditions (17, 21, 26). It is interesting that in addition to albumin, the only protein tested not belonging to the matrix protein family and recognized by a receptor not belonging to the integrin family, namely IgG, did not enhance staphylococcal killing.

A variety of matrix proteins such as fibronectin (67) and vitronectin (68) contain the tetrapeptide sequence RGDS in their respective cell binding sites, which has been shown to mediate the recognition of the ligand by the receptor. Integrins interacting with RGDS have been described on a variety of phagocytic cells (15, 69) and, more recently, a novel neutrophil integrin recognizing this sequence has been characterized (16, 70). The activation of bactericidal activity on RGDS-al-

Figure 4. Interaction of adherent *S. aureus* with neutrophils. *S. aureus* were grown to exponential phase, charged during 30 min with FITC (0.01%) and attached to PMMA. Coverslips with adherent bacteria were incubated with neutrophils (1×10^6 /ml in PBS-HS) as described in Methods and examined by fluorescence microscopy. After 30 s, bacteria started to become associated with neutrophils (arrows) (A) and after 15 min most bacteria interacted with the cells (B, arrows: non-cell-associated microorganisms).

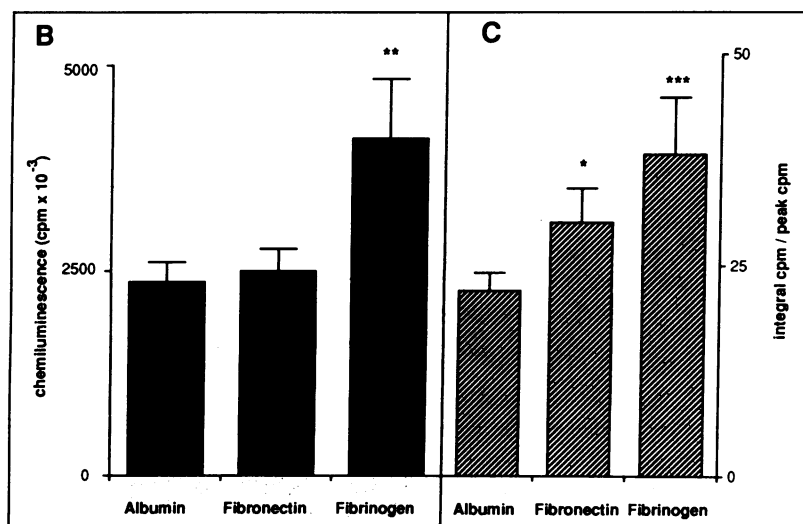
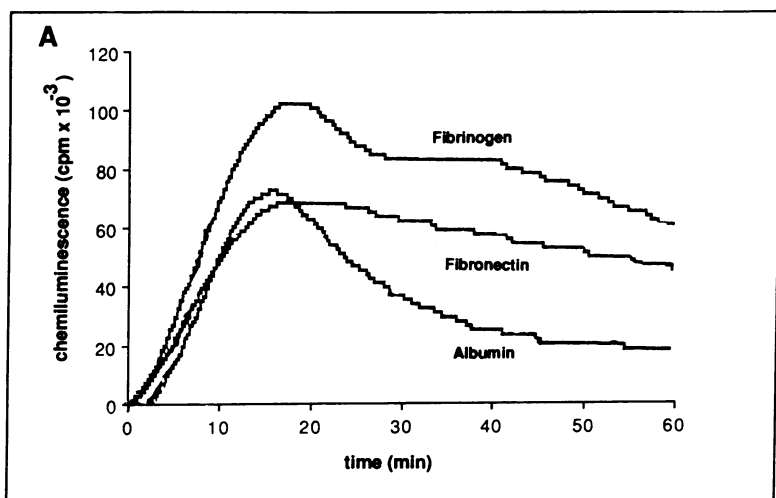


Figure 6. CL response of neutrophils to surface-adherent *S. aureus*. Reaction mixtures included: coverslips with *S. aureus* Wood 46 adherent to various extracellular matrix protein, 1×10^6 neutrophils in 1 ml PBS-HS and luminol (2×10^{-5} M). CL response was measured continuously over 60 min at 37°C and the curves shown are representative of 10 similar experiments. (A) Coverslips were coated either with fibronectin, fibrinogen, or albumin (see Methods). (B and C) Identical conditions as in A. Integral cpm (B) and integral over peak cpm (C) (means \pm SEM, $n = 10$) on matrix proteins are compared to results on albumin with the unpaired *t* test (*** $P < 0.002$; ** $P < 0.02$; * $P < 0.05$).

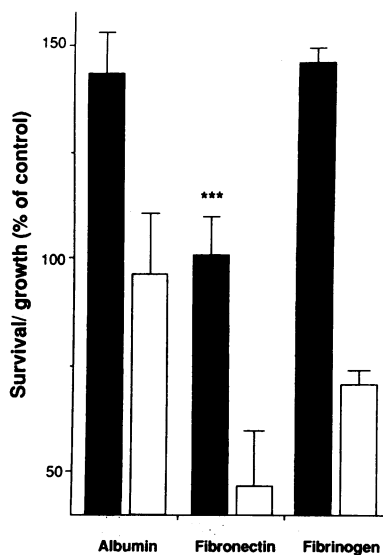


Figure 7. Bactericidal activity of neutrophils obtained from patients with CGD against *S. aureus*. Experimental conditions as in Fig. 2. (Black columns) CGD-PMN. (White columns) Control PMN. Results are means of four experiments \pm SEM performed in three CGD patients (average of five determinations per experiment). Parallel NBT slide tests of CGD cells were entirely negative under identical experimental conditions as in Fig. 1 or upon the addition of PMA (50 nM).

*** $P < 0.001$, (comparing matrix protein versus albumin by two-way analysis of variance).

bumin in our surface phagocytosis assay suggests that this neutrophil function is at least partly modulated via RGDS as ligand.

We initially thought that enhanced neutrophil oxidative metabolism could entirely explain the increased killing on matrix proteins since the respiratory burst of neutrophils is increased by solubilized and surface-adsorbed matrix proteins (17, 19, 21, 71). However, when we assessed the hydrogen peroxide production of surface-adherent neutrophils in the presence of *S. aureus*, we could not find a difference in the peroxide release on fibronectin as compared with albumin. The amount of released peroxide in our system was even smaller than that reported previously (52), possibly due to the described lag phase before onset of peroxide production. We assessed oxygen radical production continuously by using CL, which is a more sensitive reaction and detects also intracellular oxygen radical production (72) and was found to be convenient for the assessment on coverslips under conditions identical to the bactericidal experiments. The significantly larger and more sustained CL response on matrix proteins, particularly on fibrinogen when compared with albumin suggested a

higher and more prolonged exposure of bacteria to bactericidal reactive oxygen species. The pronounced CL response on fibrinogen surfaces may be due to a direct interaction between fibrinogen and the CR3 (C3bi) receptor on the neutrophil (5).

If oxidative metabolism is the major mechanism by which intracellular killing on matrix surfaces is increased, this effect should be abrogated in neutrophils from a patient with CGD (73). Survival on all surfaces in the presence of CGD cells was indeed found to be significantly increased when compared with control neutrophils. However, fibronectin enhanced the intracellular killing of *S. aureus* in CGD cells when compared to albumin and fibrinogen. Thus, even in the absence of oxygen radicals, surface bound fibronectin triggers a reduction of viable microorganisms, suggesting possible activation of non-oxidative killing mechanisms. The nature of the antistaphylococcal bactericidal mechanisms involved and how they may be enhanced by activation of the fibronectin receptor remains to be determined. This is however the first example of enhancement of nonoxidative killing in neutrophils.

The presence of matrix proteins on surfaces and the subsequent adherence of bacteria has been considered to be a virulence factor (26, 34, 37, 40, 74) in staphylococcal infection since they enhance bacterial adherence and subsequent colonization. Our present results show enhanced intracellular killing of surface-bound bacteria due to the presence of immobilized matrix proteins. It is therefore suggested that these proteins support the host cellular defense via an increase of oxygen-dependent and independent bactericidal activity by neutrophils. The nature of the deposited matrix proteins on biological surfaces could determine the levels of the neutrophil bactericidal activity. The exact role of these proteins in the interaction between the host and the invading microorganism remains to be defined in complex persistent pyogenic infections such as endocarditis, osteomyelitis, or in the presence of foreign materials.

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