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PHAGOCYTOSIS AND INTRACELLULAR KILLING OF SERUM-OPSONIZED *STAPHYLOCOCCUS AUREUS* BY MOUSE FIBROBLASTS EXPRESSING HUMAN Fcγ RECEPTOR TYPE IIa (CD32)

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ABSTRACT

Phagocytes bear more than one class of receptors for the Fc domain of IgG (FcγR). In addition the same ligand can interact with different classes of FcγR. This complexity makes it difficult to study the contribution of the various classes of FcγR to antimicrobial functions. To circumvent this difficulty, in the present study mouse 3T6 fibroblasts transfected with cDNA encoding for human FcγR type IIa (FcγRIIa-expressing cells) were used to determine the role of this receptor in phagocytosis and intracellular killing of serum-opsonized *Staphylococcus aureus*. Experiments using microbiological and fluorescent techniques to discriminate between cell-adherent and intracellular bacteria revealed that serum-opsonized bacteria are phagocytized by FcγRIIa-expressing cells, but not by parental fibroblasts. Non-opsonized bacteria were poorly internalized by FcγRIIa-expressing as well as parental fibroblasts. Furthermore, incubation of FcγRIIa-expressing cells with opsonized bacteria at 4^{o}C and incubation of FcγRIIa-expressing cells with cytochalasin E prior to addition of opsonized bacteria inhibited the phagocytosis of these bacteria almost completely. Phagocytosis of opsonized bacteria by FcγRIIa-expressing cells was partly inhibited by selective inhibition of protein tyrosine kinases (PTK). FcγRIIa cross-linking initiated transient tyrosine phosphorylation of various proteins in FcγRIIa-expressing cells. These data indicate that activation of PTK is involved in the FcγRIIa-mediated phagocytosis of opsonized *S. aureus* by transfected fibroblasts.

Human serum from normal individuals and agammaglobulinemic patients triggered the intracellular killing of *S. aureus* by FcγRIIa-expressing fibroblasts. Surprisingly, heat-inactivated human serum, IgG and incubation with anti-FcγRII antibodies followed by a bridging secondary antibody did not stimulate the killing process. The possibility that these ligands did not interact with FcγRIIa on the cells can be excluded since they induced tyrosine phosphorylation of cellular proteins. The serum factor that stimulates the intracellular killing of bacteria by FcγRIIa-expressing cells is not yet identified. Oxygen-independent mechanisms are thought to be responsible for the killing of intracellular bacteria by these cells since the NADPH oxidase inhibitor diphenylene iodonium did not affect the serum-stimulated intracellular killing of *S. aureus* and no reactive oxygen and nitrogen intermediates were produced by FcγRIIa-expressing cells after appropiate stimulation. Taken together, these data show that phagocytosis but not intracellular killing of *S. aureus* is mediated via FcγRIIa on cells expressing this receptor.

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INTRODUCTION

Phagocytes, i.e. granulocytes, monocytes and macrophages, play an important role in the resistance against infections by virtue of their ability to phagocytize and subsequently kill microorganisms. These cells express several receptors involved in the antimicrobial functions, including receptors for the Fc part of immunoglobulin G (Fcg receptors, FcγR), complement components and specific glycosylated molecules (1).

Three major classes of FcγR on human leukocytes are currently recognized; FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). These receptors can be distinguished on basis of differences in their primary sequences, molecular size, ligand affinity and specificity, cellular distribution, and reactivity with monoclonal antibodies (mAb) against FcγR (2-3). FcγRI, a 72 kDa protein that binds monomeric IgG with high affinity, is well-expressed by cells of the mononuclear phagocyte lineage and (at low levels) neutrophils. The low affinity 40 kDa FcγRII and the 50-80 kDa FcγRIII glycoprotein bind only complexed IgG. FcγRII, which is the most widely distributed IgG receptor class, is expressed by all phagocytes, B lymphocytes, and several other cell-types including platelets, specialized endothelial and epithelial cells and Langerhans' cells (4). Human FcγRII proteins can be divided into two groups, FcγRIIa which is predominantly found on phagocytes, and FcγRIIb which is preferentially expressed on B cells (5). FcγRIII is found on neutrophils, NK cells, monocytes and macrophages (5). In humans, at least two genes code for FcγRI, three genes code for FcγRII and two genes code for FcγRIII (5).

FcγR occupancy can initiate a variety of biological functions of phagocytes, e.g. phagocytosis and intracellular

killing of microorganisms, production of reactive oxygen intermediates (ROI), antibody dependent cell-mediated cytotoxicity, production and release of inflammatory mediators, and enhancement of antigen presentation (6-7). Since professional phagocytes express more than one class of FcγR on their membrane and the specificity of this receptor for ligands is relative rather than absolute (5), it is difficult to determine the relative contribution of the various classes of FcγR to initiation of biological functions. In previous studies, anti-FcγR mAb were used to investigate the relative contribution of the various classes of this receptor in the intracellular killing of bacteria by human monocytes and the signal transduction pathways involved (8-10). It was demonstrated that anti-FcγRI or FcγRII mAb as well as $F(ab')_2$ fragments of these mAb efficiently stimulated the intracellular killing of *Staphylococcus aureus* by human monocytes (8). The possibility that stimulation of the killing process after FcγR cross-linking by anti-FcγR mAb and bridging antibody differs from that after addition of serum and IgG could not be completely excluded in these studies. This possible drawback can be circumvented by determining the effects of physiological stimuli on the antimicrobial functions of FcγR-negative cells transfected with cDNA coding for human FcγR. It has been reported that such cells are able to phagocytize opsonized particles (11-15). The aim of the present study was to investigate whether mouse 3T6 mouse fibroblasts transfected with cDNA encoding for human FcγRIIa (FcγRIIa-expressing cells) are capable of phagocytosis and intracellular killing of serum-opsonized *Staphylococcus aureus*.

MATERIALS AND METHODS

Culture of FcγRIIa-expressing and parental 3T6 fibroblasts:

Mouse 3T6 fibroblasts were transfected with the pPW3 FcγRIIa cDNA (in pcDX vector) and pSV_{3gpt} using a $Ca_3(PO4)_2$ precipitation method and mycophenolic acid selection, as described (11). FcγRIIa-expressing 3T6 cells and parent 3T6 cells were cultured in RPMI 1640 medium (Gibco, Irvine, UK) supplemented with 5% heat-inactivated fetal calf serum (Gibco), 0.2 µg aminopterin/ml (Sigma Chemical Co., St. Louis, MO), 10 mg $NaHCO_3$/ml, 2.3 µg deoxycytidine/ml (Sigma), 15 µg hypoxanthine/ml (Sigma), 20 µg/ml mycophenolic acid/ml (Sigma), 2 mM pyruvate (Gibco), 5 µg thymidine/ml (Sigma), 10 µg xanthine/ml (Fluka, Buchs, Switzerland) and 50 µg gentamycine/ml in 5% CO_2-incubator at 37°C. Next, the cells were harvested with 0.05% (wt/vol) trypsin (Sigma) and 0.01% (wt/vol) EDTA in phosphate-buffered saline (PBS; pH 7.4). Before being investigated in the various assays, the cells were washed with RPMI 1640 medium.

Opsonization of bacteria:

Staphylococcus aureus (type 42D) were cultured overnight at 37°C in Nutrient Broth no.2 (Oxoid Ltd., Basingstoke, UK), harvested by centrifugation at 1,500 x g for 10 min and then washed three times with PBS. For opsonization, $1x10^8$ bacteria were incubated for 30 min at 37°C under slow rotation (4 rpm) in 1 ml HBSS supplemented with 0,1% (wt/vol) gelatin (HBSS-gel) and 15% (vol/vol) heat-inactivated human serum from the blood of healthy donors with bloodgroup AB. After two washes with ice-cold HBSS-gel, the bacteria were suspended in this medium at a concentration of $5x10^7$ bacteria/ml.

Microbiological assessment of phagocytosis of S. aureus by cells:

Phagocytosis of opsonized *S. aureus* by cells was determined as described before (16). In short, equal volumes of $5x10^7$ serum-opsonized *S. aureus*/ml and $1x10^7$ cells/ml HBSS-gel were incubated at 37°C and 4 rpm. At various intervals, ranging from 0 to 90 min, a sample of this suspension was removed, centrifuged for 4 min at 110 x g, and the number of bacteria in the supernatant was determined microbiologically. Phagocytosis is expressed as the percentage decrease in the number of extracellular bacteria.

FITC-labeling of S. aureus:

S. aureus were incubated at a concentration of $1x10^9$/ml with 0.1-1.0 mg fluorescein isothiocyanate/ml (FITC, Sigma) in 50 mM $NaHCO_3$ in 100 mM NaCl (buffer, pH 9.0) for 20 min at room temperature in the dark. The bacteria were then washed twice with PBS to remove free FITC and resuspended in HBSS-gel to a concentration of $1x10^8$ bacteria/ml.

Phagocytosis of FITC-labeled serum-opsonized S. aureus by cells:

FACS analysis was used to quantitate phagocytosis of FITC-labeled opsonized *S. aureus* by cells. To determine the optimal ratio for phagocytosis, $5x10^6$cells/ml HBSS-gel were incubated with various numbers of FITC-labeled serum opsonized *S. aureus* (ratio of cells:bacteria = 1:1, 1:2, 1:5, and 1:10) under slow rotation at 37°C for 90 min. Next, the non-cell-associated bacteria were removed by centrifugation at 500 x g at 4°C for 5 min and two washes with ice-cold PBS. The cells were resuspended in 0.02 M acetate buffer pH 5.8 at a concentration of $1x10^7$/ml. To distinguish between cell-adherent and intracellular bacteria, half of the cell-suspension was centrifuged, the pellet resuspended in an equal volume of 1 mg trypan blue/ml (Merck, Darmstadt, Ger) acetate buffer (17). As control, the other half of the cell-suspension in 0.02 M acetate buffer was kept on ice. Mean fluorescence intensity (MFI) was measured on a FACScan (Becton Dickinson, Mountain View, CA) equipped with an argon-ion laser (excitation wavelength at 488 nm, laser power 300 mW) and a band pass filter of 530 nm.

Incubation of cells that had phagocytized FITC-labeled opsonized bacteria with ethidium bromide and subsequent examination of these cells by fluorescence microscopy allowed distinction of cell-adherent and intracellular bacteria (18). Briefly, after removal of extracellular bacteria, FcγRIIa-expressing cells containing bacteria were resuspended in PBS. Next, a sample of this suspension was mixed with a solution containing 25 µg ethidium bromide/ml and a cytocentrifuge preparation was made for microscopical analysis with an Orthoplan

fluorescence microscope (Leitz, Wetzlar, Germany). The numbers of cell-adherent (orange) and intracellular (green) bacteria per cell and the percentage of phagocytic cells were determined.

Treatment of cells with a protein tyrosine kinase (PTK) inhibitor:

To investigate whether PTK activity was essential for FcγRIIa-mediated phagocytosis of opsonized *S. aureus*, FcγRIIa-expressing cells were incubated for 30 min at 37°C with 10 µg tyrphostin-47 (Calbiochem, La Jolla, CA), a competitive inhibitor of the binding of tyrosine to PTK (19), prior to addition of opsonized bacteria. As a control, cells were incubated with 10 µg/ml of tyrphostin-1 (19), an inactive analog of tyrphostin-47.

Assessment of tyrosine phosphorylation of cellular proteins:

Tyrosine phosphorylation of cellular proteins in FcγRIIa-expressing cells after FcγRIIa cross-linking was determined by the method of Connelly et al. (20) with minor modifications. In brief, $5x10^7$ cells/ml HBSS were stimulated at 37°C for the indicated intervals by FcγRIIa cross-linking; the reaction was terminated by mixing an 80-µl aliquot of this mixture with 100 µl of 2 x concentrated sample buffer (20% sodium dodecyl sulfate, 0.1 M dithiotreitol, 10% 2-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue in 10 mM Tris buffer pH 7.0), followed by heating at 100°C for 5 min. Cell lysates were subjected to electrophoresis in 7.5% SDS-PAGE, and proteins were electrophoretically transferred to nitrocellulose (Whatmann, Maidstone, UK). After overnight exposure to 2% BSA blots were incubated for 2 hours with 1 µg/ml antiphosphotyrosine antibody 4G10 (Upstate Biotechnology Inc, Lake Placid, NY) in Tris buffer (pH 8.0). After binding of mAb 4G10 to tyrosine phosphorylated proteins, the blot was incubated with 1 µCi/m of ^{125}I-labeled protein A (Amersham, Bucks, UK)/ ml Tris buffer. The blot was analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Intracellular killing assay:

Intracellular killing of *S. aureus* by FcγRIIa-expressing cells was determined as described (21) with minor modifications. In short, equal volumes of $1x10^7$ cells/ml and $5x10^7$ opsonized bacteria/ml of HBSS-gel were mixed and then incubated for 90 minutes at 37°C under slow rotation. Phagocytosis was stopped by shaking the tubes in crushed ice. Non-ingested bacteria were removed by differential centrifugation and two washes at 4°C. A suspension of $5x10^6$ FcγRIIa-expressing cells that had ingested serum opsonized *S. aureus* per ml of HBSS-gel was reincubated at 37°C and 4 rpm for various intervals ranging from 0 to 90 min, with one of the following stimuli: 1) 10% (vol/vol) human serum, 2) 500 µg/ml IgG, which was isolated from pooled normal human serum by ammonium sulfate precipitation and anion exchange chromatography on DEAE-Sephacel (Sigma) as described (21), 3) FcγRIIa-cross-linking by mAb IV-3 (anti-FcγRII, 27 µg/ml of murine IgG_{2b}; ATCC, Rockville, MD) followed by 25 µg/ml of $F(ab')_2$ goat anti-mouse Ig (Cappel, Durham, NC) as described (8-10), and 4) 10% (vol/vol) serum of a patient with agammaglobulinemia (Bloodbank 89/9650; less than 0.1 mg Ig/ml). As control, cells were reincubated in HBSS-gel. At various intervals, intracellular killing was terminated by transferring the tubes to crushed ice. Subsequently, the cells were disrupted to release the internalized bacteria. The number of viable bacteria was then determined microbiologically. The percentage of intracellular killing of *S. aureus* by FcγRIIa-expressing cells was calculated using the following formula:

$$\text{Intracellular killing (\%)} = (N_0-N_t)/N_0 \times 100\%$$

in which N_0 is the number of viable cell-associated bacteria at time-point 0 and N_t is the number of viable cell-associated bacteria at time t.

Incubation of cells with diphenylene iodonium:

To suppress oxygen-dependent killing mechanisms, $1x10^7$ cells/ml were incubated with 5 µM of the NADPH oxidase inhibitor, diphenylene iodonium bisulfate (DPI, 22; a generous gift from Dr. A.R. Cross, Dept. Biochemistry, University of Bristol, Bristol, UK), for 15 min at 37°C. As control, cells were incubated with 0.5% DMSO, the diluent of DPI.

Measurement of H_2O_2 production by cells:

H_2O_2 production by FcγRIIa-expressing cells without a stimulus and upon stimulation with 100 ng phorbol myristate acetate/ml was assayed by the horseradish peroxidase-mediated H_2O_2-dependent oxidation of homovanillic acid (23) and the results are expressed as nmol $H_2O_2/(1x10^6$ cells x 60 min).

Measurement of NO_2^- production by cells:

The amount of NO_2^- produced by FcγRIIa-expressing cells was determined by the Griess method (24). Briefly, 50 µl of the culture medium was mixed with 50 µl of Griess reagents consisting of 1% sulphanylamide, 0.1% naphtylethylenediamide-dihydrochloride, and 2.5% H_3PO_4. Ten min later, the absorbance by the reaction product was read at 550 nm on the Titertek Multiscan Plus. The number of cells was estimated by quantitation of the amount of cellular proteins. The results are expressed as µmol NO_2^- /mg cell protein.

Statistical analysis:

All data represent means ± SEM of at least three experiments. The significance of differences was analyzed by Mann-Whitney U test.

RESULTS

Binding and phagocytosis of *S. aureus* by FcγRIIa-expressing fibroblasts:

Microbiological assessments revealed that heat-inactivated serum-opsonized *S. aureus*, but not non-opsonized *S. aureus*, were phagocytized by FcγRIIa-expressing cells (Figure 1)

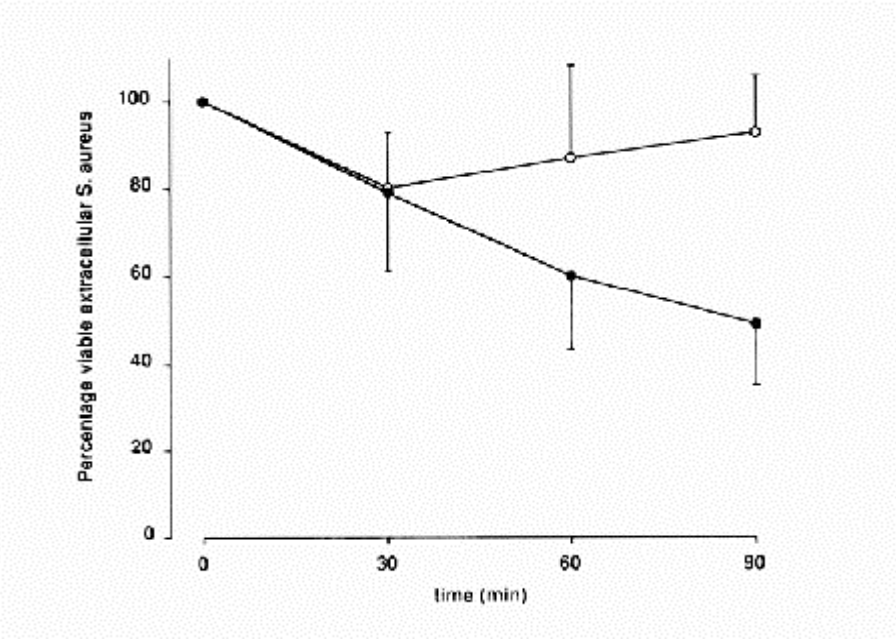

Figure 1. Phagocytosis of opsonized but not non-opsonized *S. aureus* by FcγRIIa-expressing fibroblasts.
Equal volumes of $5x10^7$/ml of heat-inactivated serum-opsonized *S.aureus* (●-●) or non-opsonized *S. aureus* (○-○) and $1x10^7$/ml of FcγRIIa-expressing fibroblasts HBSS-gel were incubated at 37°C under slow rotation. At various intervals thereafter, samples of this suspension were removed and centrifuged to separate extracellular from cell-associated bacteria. Subsequently, the number of extracellular viable bacteria was determined microbiologically and the decrease in the number of extracellular bacteria, i.e. phagocytosis, was calculated.

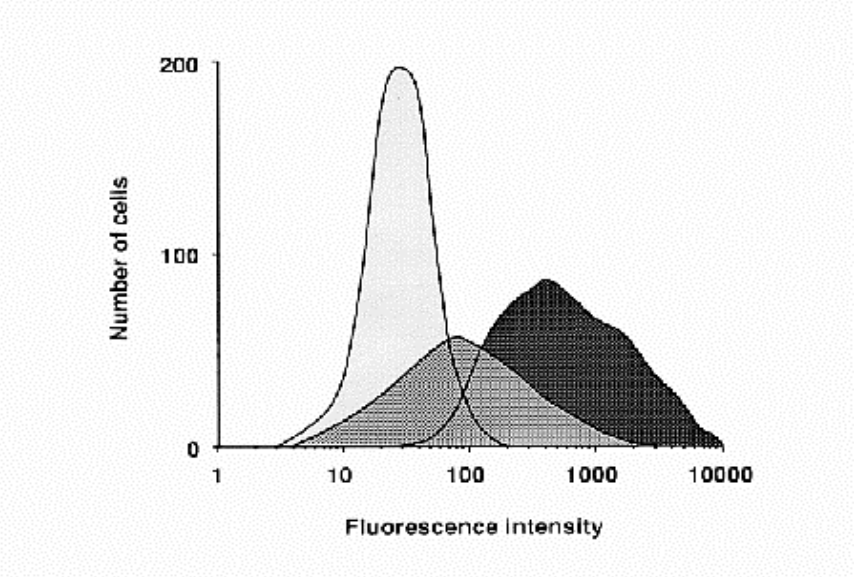

Figure 2. Binding and phagocytosis of serum-opsonized *S.aureus* by FcγRIIa-expressing cells.
FcγRIIa-expressing cells were incubated for 90 min at 37°C with FITC-labeled heat-inactivated serum-opsonized *S.aureus* at a cell-to-bacterium ratio of 1:5. Free bacteria were removed and the cells resuspended in 0.02 M acetate buffer. Half of this cell-suspension was centrifuged and then resuspended in acetate buffer and the other half resuspended in acetate buffer containing 1 mg trypan blue/ml to quench the fluorescence of cell-adherent bacteria. Subsequently, the fluorescence of cells was measured on FACScan. Dark grey curves represent fluorescence from both cell-adherent and intracellular bacteria, light grey curves reveal fluorescence from intracellular bacteria and white curves indicate non-specific fluorescence of cells. Results are representative for three individual experiments.

Table 1. Fluorescence microscopic examination of the number of intracellular and cell-adherent FITC-labeled heat-inactivated, serum-opsonized *S. aureus*.

Phagocytosis	Cells with bacteria (%)	Intracellular bacteria (number of bacteria/cell)	Cell-adherent bacteria (number ofbacteria/cell)
at 37°C	90±2	5.6±1.8	3.8±2
at 4°C	78±8	0.1±0.1	6.9±2
Cytochalasin E pre-incubated cells*	85±6	0.5±0.2	5.9±2

FcγRIIa-expressing cells were incubated for 90 min at 37°C with FITC-labeled heat-inactivated serum-opsonized *S.aureus* at a cell-to-bacterium ratio of 1:10. Subsequently, free bacteria were removed and a sample of the cell-suspension was mixed with ethidium bromide to discriminate between intracellular and cell-adherent bacteria. In each preparation, 50 cells were examined and the percentage of cells with bacteria as well as the numbers of cell-adherent and intracellular bacteria was determined by fluorescence microscopy. Results are means ± SEM of four experiments.
* FcγRIIa-expressing cells were pre-incubated with 10 μg/ml of cytochalasin E for 5 min at 37°C.

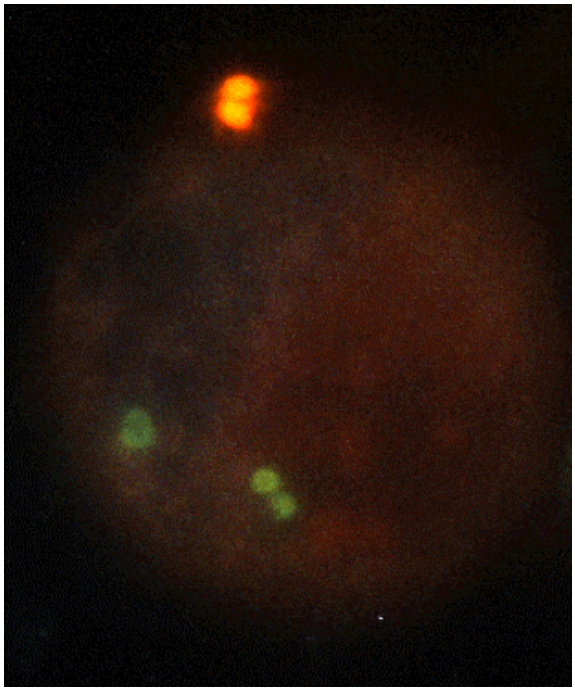

Figure 3. Identification of cell-adherent and intracellular FITC-labeled *S.aureus*.
FcγRIIa-expressing cells were incubated for 90 min at 37°C with FITC-labeled heat-inactivated, serum-opsonized *S.aureus*. Subsequently, free bacteria were removed and a sample of the cell-suspension was mixed with ethidium bromide to stain the extracellular bacteria. Microscopical examination allowed identification of cell-adherent (orange) and intracellular (green) *S. aureus*.

Because this microbiological assay does not allow discrimination between cell-adherent and intracellular bacteria, phagocytosis was assessed by using FITC-labeled bacteria in combination with agents that modify the fluorescence of cell-adherent bacteria without affecting the fluorescence of intracellular bacteria. Incubation of FcγRIIa-expressing cells with different concentrations of FITC-labeled heat-inactivated serum-opsonized *S. aureus* for various intervals revealed that a cell-to-bacterium ratio of 1:5 and an incubation period of 90 min were optimal for quantitation of phagocytosis by FACS analysis (results not shown). All further experiments were performed under these conditions unless specified otherwise. Using trypan blue to quench the fluorescence of cell-adherent bacteria (17) it was found that FcγRIIa-expressing cells efficiently phagocytized opsonized S.aureus (Figure 2).

Phagocytosis but not binding of bacteria to FcγRIIa-expressing cells was blocked when incubations were performed at 4°C; preincubation of $1x10^7$ FcγRIIa-expressing cells with 10 μg/ml of the cytoskeleton inhibitor cytochalasin E for 5 min at 37°C led to a largely decreased phagocytosis of bacteria, although binding was not affected (results not shown).

For accurate determination of the percentage of intracellular bacteria, FcγRIIa-expressing cells that had been incubated with FITC-labeled serum-opsonized bacteria were examined by fluorescence microscopy using ethidium bromide to discriminate between intracellular and cell-adherent bacteria (Figure 3).

In agreement with the results obtained by FACS analysis, binding of bacteria to FcγRIIa-expressing cells was observed after incubation at 37°C and 4°C and after preincubation of cells with cytochalasin E. Phagocytosis of bacteria was only observed when FcγRIIa-expressing cells were incubated with opsonized bacteria at 37°C (Table 1).

The percentage of phagocytizing cells was 90±2% (n=4), which is similar to the percentage cells expressing the human FcγRIIa (89±3%), as determined by FACS analysis. Together, these data indicate that FcγRIIa-expressing fibroblasts are able to phagocytize heat-inactivated, serum-opsonized bacteria.

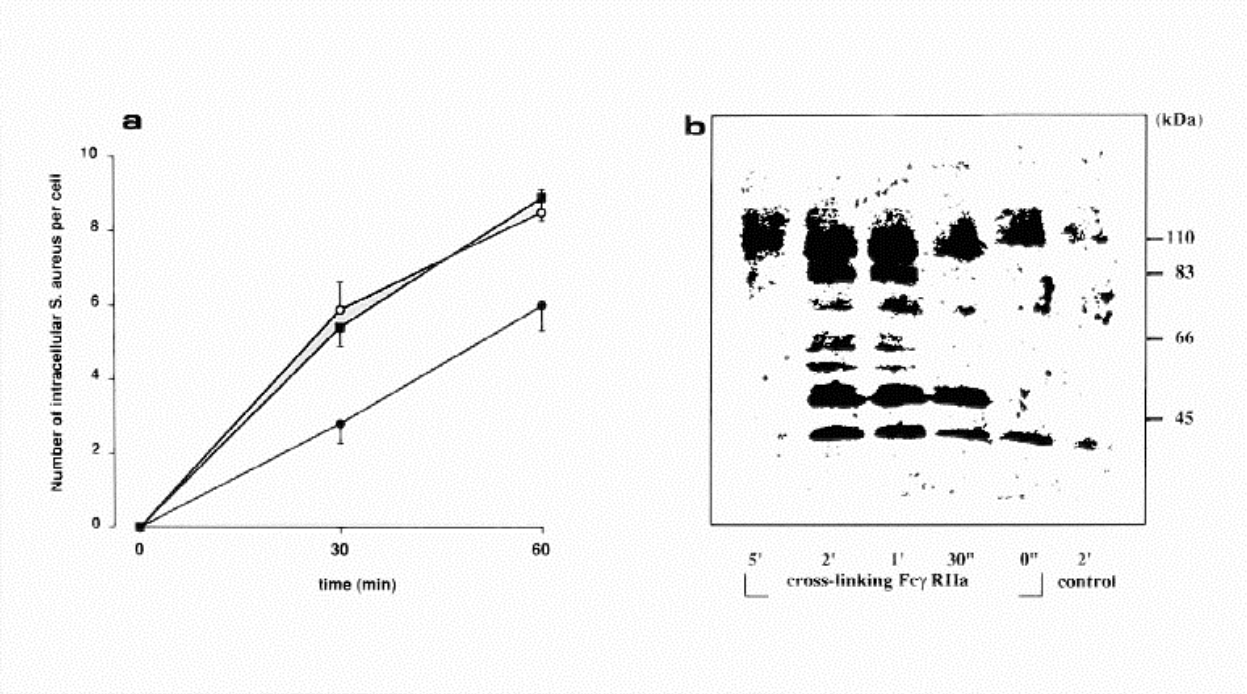

Figure 4. Protein tyrosine kinase activation and phagocytosis of FITC-labeled opsonized *S. aureus* to FcγRIIa-expressing cells.
a. Effect of tyrphostin-47 on phagocytosis of *S. aureus* by FcγRIIa-expressing cells.
FcγRIIa-expressing cells were pre-incubated for 30 min at 37°C with 10 μM tyrphostin-47 (●-●), tyrphostin-1 (■-■) or PBS (○-○) and then incubated with FITC-labeled heat-inactivated, serum-opsonized *S. aureus*. Next, free bacteria were removed and a sample of the cell-suspension was mixed with ethidium bromide and the number of intracellular (green) and cell-adherent (orange) bacteria was determined in 50 cells by microscopic examination. Results are mean number of intracellular bacteria/FcγRIIa-expressing cell ± SEM.
b. Effects of FcγRIIa cross-linking on tyrosine phosphorylation of proteins in cells.
FcγRIIa-expressing cells were incubated for 3 min with PBS or with 2mg/ml of anti-FcγRIIa antibody IV-3 and then 25mg bridging secondary antibody was added to achieve cross-linking of FcγRIIa. At indicated intervals, the reaction was stopped by addition of 2 x concentrated SDS sample buffer at 100°C, and the the lysates were subjected to 7.5% SDS-PAGE, followed by Western blot analysis with using-phosphotyrosine mAb 4G10 and ^{125}I-labeled protein A. The results of one experiment quantified on a PhosphorImager are respresentative of three individual experiments are given.

Role of FcγRIIa in the phagocytosis of opsonized *S. aureus*:

To obtain more insight into the role of FcγRIIa in phagocytosis of opsonized bacteria by FcγRIIa-expressing cells, two sets of experiments were performed. First, phagocytosis of FITC-labeled, non-opsonized *S. aureus* by FcγRIIa-expressing cells was determined. The results revealed that about twenty-fold less non-opsonized than serum-opsonized bacteria were phagocytized by FcγRIIa-expressing cells, i.e. the mean number of intracellular non-opsonized bacteria amounted to 0.3±0.1/cell and serum-opsonized bacteria to 5.6±1.8/cell (n=4). Second, we compared phagocytosis of FITC-labeled serum-opsonized bacteria by FcγRIIa-expressing and parental fibroblasts. The results revealed that, in contrast to FcγRIIa-expressing cells, parental fibroblasts hardly phagocytized opsonized *S. aureus*, i.e. $5\pm1x10^4$ bacteria/$5x10^6$ cells (n=4). Together, these data indicate the FcγRIIa mediates the phagocytosis of opsonized bacteria by FcγRIIa-expressing cells.

Involvement of PTK in the phagocytosis of serum-opsonized *S. aureus* by FcγRIIa-expressing cells:

Since activation of protein tyrosine kinases (PTK) is implicated in FcγRIIa-mediated phagocytosis by phagocytic cells (9, 25-27), the effect of inhibitors of PTK on the phagocytosis of serum-opsonized *S. aureus* by FcγRIIa-expressing cells was investigated. The results revealed that the PTK-inhibitor tyrphostin-47, but not its inactive analog tyrphostin-1, inhibited ($p<0.05$) phagocytosis of *S. aureus* by FcγRIIa-expressing cells (Figure 4a). Neither of tyrphostins ($p>0.1$) affected binding of the bacteria to these cells (data not shown).

To find out whether FcγRIIa cross-linking results in tyrosine phosphorylation of cellular proteins, the pattern of tyrosine phosphorylated proteins after stimulation of cross-linking of FcγRIIa on cells was determined. The

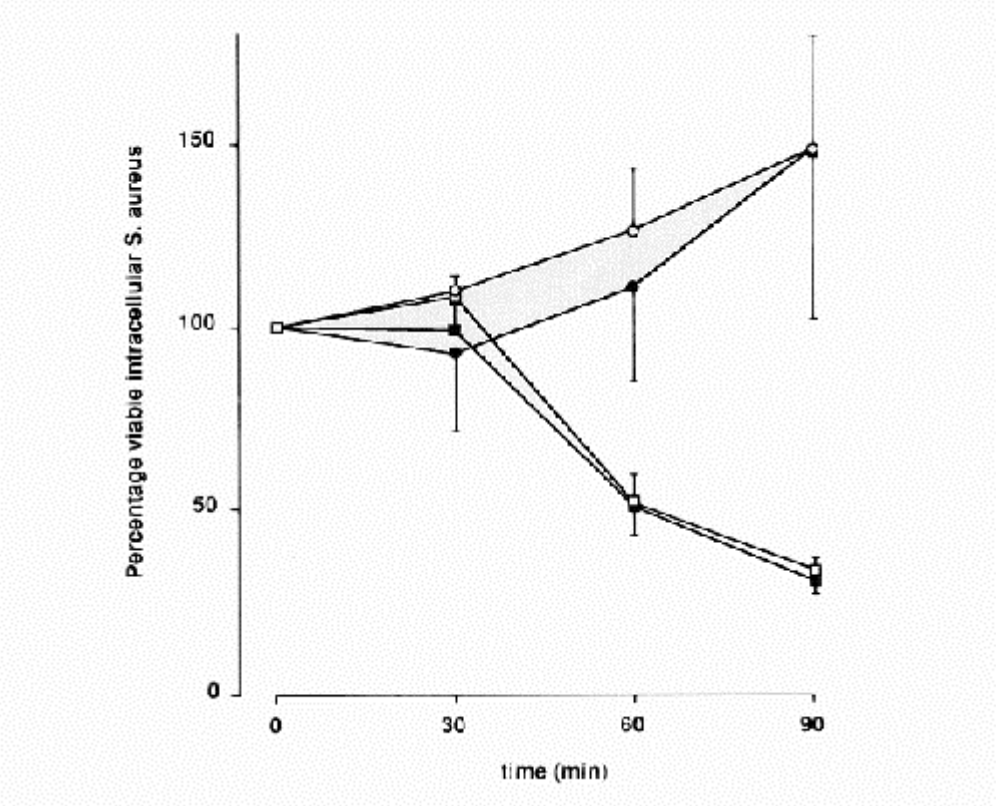

Figure 5. Intracellular killing of *S. aureus* by FcγRIIa-expressing fibroblasts.
FcγRIIa-expressing fibroblasts were incubated with serum-opsonized *S. aureus* for 90 min. Then, extracellular bacteria were removed by washing and killing of the internalized bacteria was initiated by the following stimuli: normal human serum (■-■), serum from patients with agammaglobulinemia (□-□), heat-inactivated serum (●-●) or, as control HBSS (○-○). At indicated intervals, a sample was taken and the number of viable bacteria was determined. Results are means ± SEM of 5-8 experiments.

results showed that FcγRIIa cross-linking induced within 30 sec an increase in the tyrosine phosphorylation of muliple proteins, which became dephosphorylated after 2 min of stimulation (Figure 4b). Incubation of cells with tyrphostin-47 reduced the tyrosine phosphorylation of the various proteins by FcγRIIa cross-linking (results not shown), as reported previously (9). Furthermore, incubation of cells with mAb LeuM3 (anti-human CD14 antibody, Becton Dickinson (San Jose, CA), serving as an isotype-matched control) followed by bridging antibody did not induce an increase in tyrosine phophorylation of proteins (Figure 4b, control).

Intracellular killing of *S. aureus* by FcγRIIa-expressing cells:

To find out whether FcγRIIa-expressing cells were capable of intracellular killing of *S. aureus*, cells that had ingested opsonized *S. aureus* were incubated with serum. It is known that serum is obligatory for obtaining maximal killing of ingested *S. aureus* by human monocytes and mouse macrophages (8, 21). The results revealed that serum efficiently stimulated the intracellular killing of *S. aureus* by FcγRIIa-expressing cells (Figure 5).

To determine whether oxygen-dependent microbicidal mechanisms are involved in the intracellular killing of opsonized *S. aureus* by FcγRIIa-expressing fibroblasts, cells containing *S. aureus* were incubated with the NADPH oxidase-inhibitor DPI before stimulation with normal human serum. The results showed that DPI did not affect ($p>0.1$) the killing process, i.e. intracellular killing by DPI-treated cells and control cells at 90 min was respectively 94±2% and 96±2%, (n=3). In agreement with these observations, FcγRIIa-expressing cells did not ($p>0.1$) produce H_2O_2 upon PMA stimulation or after addition of 100-fold excess of opsonized *S. aureus*. Upon stimulation of the cells with 100-fold excess serum opsonized-bacteria, no NO_2^- -production was observed (n=3). The combination of 10 mg/ml of lipopolysaccharide, 100 units/ml of recombinant rat interferon-gamma and 100 units/ml of recombinant mouse tumor necrosis factor-alpha stimulated NO_2^- production by FcγRIIa-expressing cells. This amount was 1±1 mmol NO_2^-/mg cell protein for non-stimulated cells and 34±4 mmol NO_2^-/mg cell protein for stimulated cells (n=3).

The possible role of FcγRIIa in this killing process was investigated by incubating FcγRIIa-expressing

cells that had ingested bacteria with heat-inactivated serum, purified IgG, anti-FcγRII mAb and bridging secondary antibody. The results revealed that heat-inactivated serum (Fig. 5) as well as the other FcγR-specific stimuli (results not shown) did not trigger intracellular killing of *S. aureus* by these cells. Contrary, serum from patients with agammaglobulinemia stimulated the killing process (Figure 5). These data indicate that heat-labile serum factor(s) stimulate the intracellular killing of *S. aureus* by FcγRIIa-expressing cells.

DISCUSSION

The present results indicate that phagocytosis but not intracellular killing of serum-opsonized *S. aureus* is mediated by FcγRIIa fibroblasts expressing this receptor. The conclusion that human FcγRIIa mediates phagocytosis of heat-inactivated serum-opsonized, *S. aureus* by mouse 3T6 fibroblasts stably transfected with this receptor is based on the following lines of evidence. First, by means of two different techniques to discriminate between cell-adherent and intracellular bacteria, we have demonstrated that bacteria are truly phagocytized by FcγRIIa-expressing fibroblasts. Second, incubation of FcγRIIa-expressing fibroblasts and opsonized bacteria at 4^{o}C or pre-incubation of the fibroblasts with cytochalasin E inhibited phagocytosis of opsonized *S. aureus*, as has been found for professional phagocytes (16). Third, FcγRIIa-expressing, but not parental, fibroblasts efficiently phagocytized opsonized *S. aureus*. Fourth, non-opsonized bacteria were poorly phagocytized by these cells. These observations are in agreement with reports that human FcγRIIa introduced into murine 3T6 fibroblasts (13), II A 1.6 B cells (14-15) and P388D1 macrophage-like cells (26) mediated phagocytosis of opsonized particles. Interestingly, transfection of the same receptor into CHO cells resulted in enhanced binding but not internalization of opsonized erythrocytes (12). Clearly, many cell types (but not all) are equipped to phagocytize particles including opsonized bacteria. Another important finding is that selective inhibition of PTK reduced the phagocytosis of opsonized *S. aureus* by FcγRIIa-expressing fibroblasts, as reported earlier for professional phagocytes (9, 25-27). Reduction of the phagocytosis of opsonized bacteria by the PTK inhibitor tyrphostin-47 was not due to a cytotoxic effect of the inhibitor or suppression of expression of human FcγRIIa by the compound (unpublished observations), as has been reported earlier for human monocytes (8).

The second conclusion pertains to the ability of FcγRIIa-expressing fibroblasts to intracellularly kill bacteria. Our observation that serum, both from healthy controls and from patients suffering from agammaglobulinemia, induced intracellular killing of opsonized *S. aureus* indicates that 3T6 fibroblasts exhibit antimicrobial activity. The identity of the serum factor that stimulates the killing process is not clear, since FcγR-specific stimuli, such as heat-inactivated serum, IgG and cross-linking FcγRIIa with a specific mAb and bridging second antibody, did not trigger the killing process. The possibility that these FcγR-specific stimuli do not result in intracellular signaling can be excluded since FcγRIIa cross-linking caused rapid tyrosine phosphorylation of cellular proteins in fibroblasts expressing this receptor. Of course, it could be that signaling down-stream of tyrosine phosphorylation of proteins after FcγRIIa cross-linking on the cells is impaired. In this connection, it can be speculated that cross-linking of FcγRIIa on fibroblasts does not lead to the formation of complexes of FcγRIIa and g-chains. These complexes may be essential to the stimulation of the killing process, as has recently been reported for interleukin-2 release, immune complex internalization and antigen presentation by FcγRIIa-expressing murine B cells IIA.1.6. (14-15). The possible explanation that the antimicrobial effector mechanisms in fibroblasts are rather limited is very attractive. We have found that the NADPH oxidase-inhibitor DPI did not affect the killing process in FcγRIIa-expressing fibroblasts. Upon stimulation with opsonized bacteria and phorbol ester reactive oxygen and nitrogen intermediates were not produced by FcγRIIa-expressing fibroblasts. In contrast to human monocytes (8,28) the mechanism(s) underlying the intracellular killing of *S. aureus* by FcγRIIa-expressing fibroblasts is most probably oxygen-independent. It has been reported that in these fibroblasts, opsonized erythrocytes end up in phagolysosomes where they are believed to be degraded by the contents of lysosomes (13). It could be that phagocytized *S. aureus* are degraded by the action of lysosomal proteins in FcγRIIa-expressing fibroblasts as well.

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