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Protein Tyrosine Kinase Activity Is Essential for Fcγ Receptor-Mediated Intracellular Killing of *Staphylococcus aureus* by Human Monocytes

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Our previous study revealed that the intracellular killing of *Staphylococcus aureus* by human monocytes after cross-linking Fcγ receptor I (FcγRI) or FcγRII is a phospholipase C (PLC)-dependent process. The aim of the present study was to investigate whether protein tyrosine kinase (PTK) activity plays a role in the FcγR-mediated intracellular killing of bacteria and activation of PLC in these cells. The results showed that phagocytosis of bacteria by monocytes was not affected by the PTK inhibitors genistein and tyrphostin-47. The intracellular killing of *S. aureus* by monocytes after cross-linking FcγRI or FcγRII with anti-FcγR monoclonal antibody and a bridging antibody or with human immunoglobulin G (IgG) was inhibited by these compounds in a dose-dependent fashion. The production of O_2^- by monocytes after stimulation with IgG or IgG-opsonized *S. aureus* was almost completely blocked by the PTK inhibitor. These results indicate that inhibition of PTK impairs the oxygen-dependent bactericidal mechanisms of monocytes. Genistein and tyrphostin-47, which do not affect the enzymatic activity of purified PLC, prevented activation of PLC after cross-linking FcγRI or FcγRII, measured as an increase in the intracellular inositol 1,4,5-trisphosphate concentration. Cross-linking FcγRI or FcγRII induced rapid tyrosine phosphorylation of several proteins in monocytes, one of which was identified as PLC-γ1, and the phosphorylation could be completely blocked by PTK inhibitors, leading to the conclusion that activation of PLC after cross-linking FcγR in monocytes is regulated by PTK activity. Together, these results demonstrate that PTK activity is essential for the activation of PLC which is involved in the FcγR-mediated intracellular killing of *S. aureus* by human monocytes.

At least three distinct classes of receptors for the Fc domain of immunoglobulin G (IgG) (FcγR), i.e., a high-affinity 72-kDa FcγR (FcγRI; CD64), a low-affinity 40-kDa FcγR (FcγRII; CD32), and a low-affinity 50- to 80-kDa FcγR (FcγRIII; CD16), on human phagocytes have been recognized on the basis of their structures, primary amino acid sequences, and binding affinities for ligands (43). All three classes of FcγR on monocytes are associated with disulfide-linked γ-chains (28), which are assumed to be involved in signal transduction and functions mediated via these receptors (13, 29, 44). Interactions between FcγR and their ligands stimulate a variety of functional activities of monocytes and granulocytes, such as antibody-dependent cellular cytotoxicity (19), induction of cytokine synthesis (12), exocytosis (14), respiratory burst (34), phagocytosis (32), and intracellular killing of microorganisms (25).

One of the main intracellular signaling pathways involved in the stimulation of phagocytes via FcγR cross-linking is hydrolysis of phosphoinositol (4,5)-bisphosphate by phospholipase C (PLC), resulting in the formation of two second messengers: inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (6, 43). Ins(1,4,5)P_3 causes an increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$) whereas diacylglycerol is an endogenous activator of protein kinase C (6, 7). Experiments with agents that modulate protein kinase C activity and $[Ca^{2+}]_i$ indicate that these two messengers play an essential role in the stimulation of many functional activities of phagocytes, including the production of reactive oxygen intermediates by neutrophils and murine macrophages and intracellular killing of microorganisms by human monocytes (10, 15, 45, 46). In our previous study, we observed that stimulation of the intracellular killing of *Staphylococcus aureus* by human monocytes by cross-linking FcγRI or FcγRII is a PLC-dependent process (47).

Until now, two different mechanisms for the regulation of PLC activity have been reported. First, certain G proteins couple to PLC-β1 (40), one of the three types of PLC in human cells. Recent studies of human natural killer cells (41) and monocytes (27) suggested that cross-linking FcγR on these cells activates PLC via a G-protein-independent pathway. Second, PLC-γ is tyrosine phosphorylated and thus is activated by protein tyrosine kinases (PTKs) (27, 38). Cross-linking FcγRIII on natural killer cells or FcγRI and FcγRII on monocytic cell lines U937 and THP-1 resulted in rapid tyrosine phosphorylation of PLC-γ by PTK (27, 38, 41). Therefore, activation of PTK most likely precedes the activation of PLC in monocytes which occurs after cross-linking FcγR. The aim of the present study was to investigate whether PTK activity is essential for activation of the PLC involved in the intracellular killing of bacteria in human monocytes stimulated by cross-linking FcγRI and FcγRII.

MATERIALS AND METHODS

Chemicals, human IgG, and antibodies. All chemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.), unless indicated otherwise. Purified IgG, which contains mainly multimeric forms of IgG, was isolated from pooled normal human serum samples by ammonium sulfate precipitation and anion exchange chromatography on DEAE-Sephacel (25). The murine hybridoma cell line producing monoclonal antibody (MAb) IV-3 (anti-FcγRII; 27 μg of IgG2b per ml) was

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obtained from the American Tissue Type Collection (Rockville, Md.). The culture supernatant was dialyzed against phosphate-buffered saline (PBS) (pH 7.4) for 72 h at 4°C. Anti-FcγRI MAb 197 (1 mg of IgG2a per ml) and Fab fragments of MAb IV-3 were purchased from Medarex Inc. (West Lebanon, N.H.). MAb W6/32 (anti-HLA class I; 18 mg of IgG2a per ml) was donated by F. Koning (Department of Immunohematology and Bloodbank, University Hospital, Leiden, The Netherlands), and MAb Leu M_3 (anti-CD14; 60 μg of IgG2b per ml) was purchased from Becton & Dickinson (Mountain View, Calif.). $F(ab')_2$ fragments of goat anti-mouse IgG [$F(ab')_2$-GAM IgG] were supplied by Cappel (Durham, N.C.) and GAM κ chain was supplied by Southern Biotech. (Birmingham, Ala.).

Isolation of monocytes. Monocytes were isolated from buffy coats of blood samples from healthy donors by differential centrifugation on Ficoll-amidotrizoate gradients (ρ = 1.077 g/ml; Pharmacia, Uppsala, Sweden) (9). The layer containing mononuclear cells was washed four times with PBS plus 0.5 U of heparin per ml and then suspended to a concentration of 10^7 monocytes per ml of Hanks' balanced salt solution (HBSS) containing 0.1% gelatin (HBSS-GEL). This preparation consisted of about 30% monocytes, 67% lymphocytes, and less than 3% granulocytes. For measurement of the intracellular biochemical changes, monocytes were purified from the mononuclear cell suspensions by elutriation centrifugation (47). These suspensions consisted of about 85% monocytes and 15% lymphocytes. The cell viability in the various suspensions exceeded 98%, as determined by trypan blue exclusion.

Cross-linking FcγR on monocytes. Cross-linking FcγR was achieved by incubation of monocytes or monocytes containing bacteria with the optimal concentration of anti-FcγRI or anti-FcγRII MAb at 4°C for 15 min when performing the killing assay and otherwise at 37°C for 3 min, followed by the addition of 25 μg of $F(ab')_2$-GAM IgG per ml. In some experiments, IgG was used to cross-link FcγR on monocytes.

Opsonization of bacteria. *S. aureus* (type 42D) and *Streptococcus pyogenes* (group A) were cultured overnight at 37°C, washed twice with PBS, and opsonized with 10% (vol/vol) serum, prepared from the blood samples of healthy donors with blood group AB, or 500 μg of IgG per ml as previously described (25, 47). After removal of excess serum or IgG, the bacteria were suspended in HBSS-GEL at a concentration of 10^7 bacteria per ml.

Intracellular killing assay. Intracellular killing of bacteria by monocytes was determined as previously described (17, 45). In short, equal volumes of 10^7 monocytes per ml of HBSS-GEL and 10^7 opsonized bacteria per ml of HBSS-GEL were incubated at 37°C under slow rotation for 3 min. Phagocytosis was stopped by shaking the tubes in crushed ice, and the free bacteria were removed by differential centrifugation and washing. Next, 5×10^6 monocytes containing ingested bacteria per ml were reincubated in HBSS with or without a stimulus at 37°C under slow rotation for various intervals. Intracellular killing was terminated by spinning down the cells at 4°C; after the addition of distilled water containing 0.01% bovine serum albumin (BSA) (fraction V), the monocytes were disrupted by vigorously vortexing. The number of viable intracellular bacteria was determined microbiologically; intracellular killing is expressed as the percentage decrease in the number of viable intracellular bacteria (17, 25).

Measurement of O_2^- production. The O_2^- production by monocytes at rest and after stimulation with IgG or IgG-opsonized bacteria was assessed by reduction of ferricytochrome *c* (type IV) as previously described (5). Results are expressed as nanomoles per 2×10^6 monocytes per 60 min.

Treatment of monocytes with PTK inhibitors. To investigate whether PTK activity is essential for FcγR-mediated intracellular killing and signaling, monocytes were incubated with various concentrations of genistein (Calbiochem Corp., La Jolla, Calif.), which inhibits PTK by competing with ATP for binding to PTK (2), at 37°C for 10 min or with various concentrations of tyrphostin-47, a competitive inhibitor of the binding of tyrosine to PTK (26), at 37°C for 30 min before phagocytosis. As controls, cells were incubated with 0.1% dimethyl sulfoxide, the diluent of genistein, or with tyrphostin-1, the inactive analog of tyrphostin-47.

Assay for PLC activity. The method of Kurioka and Matsuda (23) with minor modifications was used to determine the effects of PTK inhibitors on PLC. Briefly, 0.3 U of purified PLC (EC 3.1.4.3.) was added to 1 ml of 125 mM Tris-HCl (pH 7.2) supplemented with 40% sorbitol, 5 mM *p*-nitrophenylphosphoryl-choline (NPPC), and the PTK inhibitors. Next, this mixture was incubated at 37°C for a 60-min period during which time the rate of hydrolysis of NPPC was monitored by measuring A_{410}.

Competition binding assay for Ins(1,4,5)P_3. The assay to determine the intracellular Ins(1,4,5)P_3 concentration has been described elsewhere (31). In short, monocytes (5×10^7 per ml of PBS) were stimulated at 37°C for selected intervals. The reaction was terminated by mixing a 50-μl aliquot of the cell suspension with 50 μl of ice-cold 3.5% perchloric acid; after centrifugation, the supernatants were neutralized with 25 μl of saturated $KHCO_3$. The Ins(1,4,5)P_3 content in the cell extract was allowed to compete with [2-^{3}H]Ins(1,4,5)P_3 (specific activity, 20 to 60 Ci/mmol; Amersham, Bucks, United Kingdom) for binding to components of bovine adrenal cortex microsomal preparation. The Ins(1,4,5)P_3 content of these cell lysates was quantified by comparison with a standard curve, using unlabelled Ins(1,4,5)P_3 (Amersham). The mean intracellular Ins(1,4,5)P_3 concentrations were calculated from the amount of Ins(1,4,5)P_3 and the mean cell volume of monocytes (31, 47).

Measurement of changes in the $[Ca^{2+}]_i$. For measurement of the $[Ca^{2+}]_i$, purified monocytes were loaded with 1.5 μM acetoxymethyl ester of 1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methyl-phenoxy)-ethane-*N*,*N*,*N'*,*N'*-tetraacetic acid (FURA-2) (FURA-2/AM) in Ca^{2+}-containing medium at 37°C for 30 min in the dark (45). $[Ca^{2+}]_i$ was measured by recording the fluorescence intensities at 340-, 360-, and 380-nm excitation wavelengths and at a 500-nm emission wavelength on a RF-5001PC Shimadzu spectrofluorometer (Shimadzu Co., Kyoto, Japan) equipped with a magnetic stirrer at 37°C. The $[Ca^{2+}]_i$ was calculated from the ratios of the fluorescence at 340 and 380 nm as previously described (21, 45). Calibration of the FURA-2 fluorescence was performed by lysing the cells with 0.1% Triton X-100 in the presence of 1 mM extracellular Ca^{2+} and then adding ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid (EGTA) to a final concentration of 10 mM.

Assessment of tyrosine phosphorylation of proteins. The tyrosine-phosphorylated proteins in monocytes after cross-linking FcγR was determined by the method of Connelly et al. (11) with minor modifications. In brief, 5×10^7 purified monocytes per ml of HBSS were stimulated at 37°C for the indicated intervals with various ligands of FcγR; the reaction was stopped by mixing an 80-μl aliquot of the cell suspension with 100 μl of 2× sodium dodecyl sulfate (SDS) sample buffer (2× SDS sample buffer consists of 20% SDS, 0.1 M dithioerythritol, 10% β-mercaptoethanol, 10% glycerol, and 0.005% bromophenol blue in 10 mM Tris buffer [pH 7.0] at 100°C), followed by heating at 100°C for 5 min. After the cell lysates

were run on an SDS–7.5% polyacrylamide gel, the proteins were electrophoretically transferred to nitrocellulose paper (Whatmann International Ltd., Maidstone, United Kingdom). After being blocked with 2% BSA overnight, the blot was incubated for 2 h with 1 μg of anti-phosphotyrosine MAb 4G10 (Upstate Biotechnology Inc., Lake Placid, N.Y.) per ml of Tris buffer (pH 8.0). The binding of the antibody to tyrosine-phosphorylated proteins on the blot was assessed with 1 μCi ^{125}I-labelled protein A (Amersham) per ml of Tris buffer analyzed with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Assessment of tyrosine phosphorylation of PLC-γ1. Purified monocytes (3×10^7) in 400 μl of HBSS-GEL were stimulated by cross-linking FcγRII at 37°C for various intervals. The reaction was stopped by adding 200 μl of ice-cold lysis buffer (3% Triton X-100, 15% glycerol, 150 mM NaF, 3 mM Na_3VO_4, 15 μg of leupeptin, 3 mM phenylmethylsulfonyl fluoride, 15 μg of aprotinin, 60 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid]; pH 7.2), and the samples were incubated for 30 min on ice (33). After removal of nuclear and cellular debris by centrifugation at 12,000 × *g* for 15 min at 4°C, 500 μl of the supernatant were mixed with 10 μl of polyclonal PLC-γ1-specific antiserum (Upstate Biotechnology Inc.) and maintained for 2 h before the addition of 50 μl of protein A-Sepharose 6MB (Pharmacia). After overnight incubation at 4°C, the beads containing the immunoprecipitates were first washed four times with a buffer containing 1% Triton X-100, 0.1% SDS, 150 mM NaCl, and 50 mM Tris (pH 7.5) and then washed once with PBS, and thereafter the proteins were eluted from the beads with 50 μl of SDS sample buffer and boiled for 5 min. Tyrosine phosphorylation of proteins was assessed by Western blotting as described above.

Statistical analysis. All results are presented as means ± standard deviations (SD), unless specified. The significance of the differences was analyzed by Student's *t* tests.

RESULTS

Effects of PTK inhibitors on the intracellular killing of *S. aureus* by monocytes. Inhibitors of PTK were used to find out whether PTK activity is essential for the FcγR-mediated intracellular killing of bacteria by monocytes. Monocytes were incubated with various concentrations of genistein at 37°C for 10 min before the addition of opsonized *S. aureus*. After phagocytosis and removal of non-cell-associated bacteria, these monocytes were incubated at 37°C for various intervals with purified IgG in the presence of genistein. The results showed that genistein inhibited the IgG-stimulated intracellular killing of *S. aureus* by monocytes in a dose-dependent fashion (Fig. 1A). The genistein concentration yielding half-maximum inhibition was 110 μM. To determine which class of FcγR mediated the PTK-dependent killing process, monocytes that had been exposed to genistein and then to phagocytosed opsonized *S. aureus* were subsequently incubated with anti-FcγRI or anti-FcγRII MAb and the bridging antibody F(ab')$_2$-GAM IgG. The results revealed that cross-linking FcγRI and FcγRII stimulated intracellular killing, as described earlier (47), which in turn was inhibited by about 60% by 110 μM genistein (Table 1). Cross-linking of antigens on monocytes by MAbs Leu M_3 and W6/32, that served as IgG isotype-matched controls for the anti-FcγR MAb, and F(ab')$_2$-GAM IgG did not stimulate the killing process (Table 1).

Since genistein interferes with the binding of ATP to protein kinases and, therefore, might inhibit protein kinases other than PTK as well, we also investigated whether tyrphostin-47, a competitive inhibitor of the binding of tyrosine to PTK,

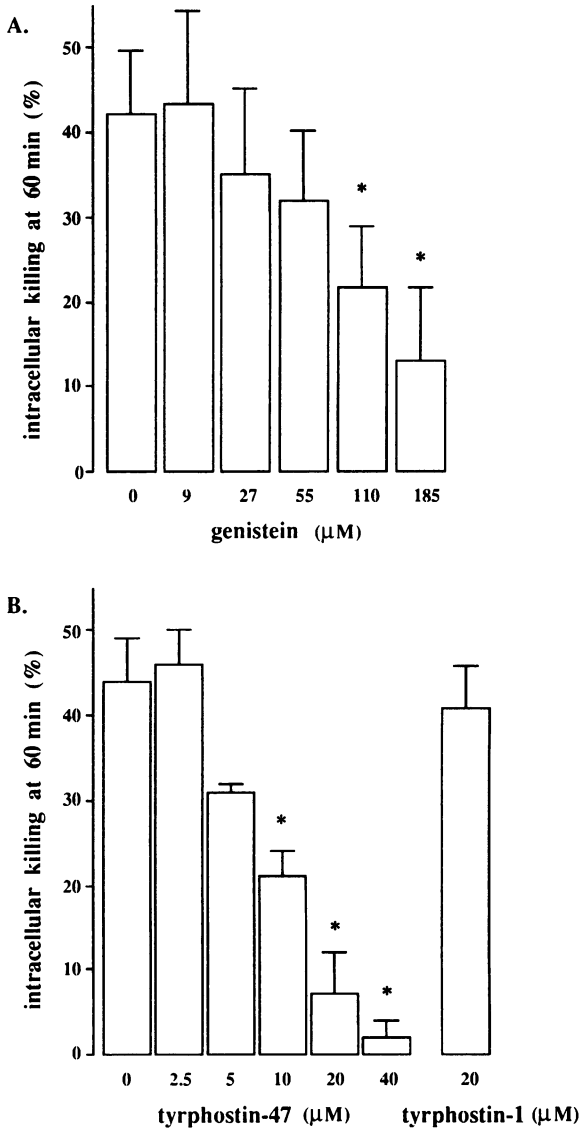

FIG. 1. Effects of genistein and tyrphostin-47 on the intracellular killing of *S. aureus* by human monocytes stimulated with IgG. Monocytes were preincubated with various concentrations of genistein at 37°C for 10 min (A) or tyrphostin-47 at 37°C for 30 min (B). The cells were then allowed to ingest serum-opsonized *S. aureus* for 3 min. After removal of noningested bacteria, the monocytes were reincubated with 500 μg of IgG per ml at 37°C in the presence of these inhibitors. The intracellular killing was determined at 60 min; the results are means ± SD of three paired experiments. Asterisks indicate those values that are significantly different from the values obtained with the control monocytes ($P < 0.01$).

affected the FcγR-mediated killing process in monocytes. The results revealed that tyrphostin-47 inhibited the IgG-stimulated intracellular killing of *S. aureus* by these cells in a dose-dependent fashion (Fig. 1B). The tyrphostin-47 concentration yielding half-maximum inhibition was 10 μM. The inactive compound tyrphostin-1 did not affect ($P > 0.1$) the killing process.

The phagocytosis of serum-opsonized bacteria was not affected by these PTK inhibitors, the number of cell-associated bacteria at the beginning of killing assay amounted to (1.3 ±

TABLE 1. Effect of genistein on the FcγRI or FcγRII-mediated intracellular killing of *S. aureus* by human monocytes[a]

Stimulus	% Intracellular killing of *S. aureus* by monocytes pretreated with:		% Inhibition[b]
	Buffer	Genistein	
Cross-linking FcγRI	30 ± 7	13 ± 5[c]	57
Cross-linking FcγRII	29 ± 9	11 ± 7[d]	64
Cross-linking HLA class I	10 ± 4	ND[e]	ND
Cross-linking CD14	8 ± 6	ND	ND
HBSS	5 ± 7	3 ± 8	

[a] Monocytes were preincubated with 110 μM genistein or buffer at 37°C for 10 min. After phagocytosis and three washes, monocytes containing *S. aureus* were incubated with 5 μg of anti-FcγRI MAb 197, 2 μg of anti-FcγRII MAb IV-3, or 5 μg of anti-HLA class I MAb W6/32 or anti-CD14 MAb Leu M_3 per ml and then 25 μg of $F(ab')_2$-GAM IgG per ml or buffer was added. The intracellular killing of *S. aureus* was determined at 60 min; the results are the means ± SD of four paired experiments. Statistical differences between the values for genistein-preincubated monocytes and control cells were evaluated by the paired Students' *t* test.

[b] Inhibition = [1 − (killing in genistein-preincubated cells/killing in control cells)] × 100%.

[c] Significantly different from value obtained with buffer ($P < 0.01$).

[d] Significantly different from value obtained with buffer ($P < 0.001$).

[e] ND, not done.

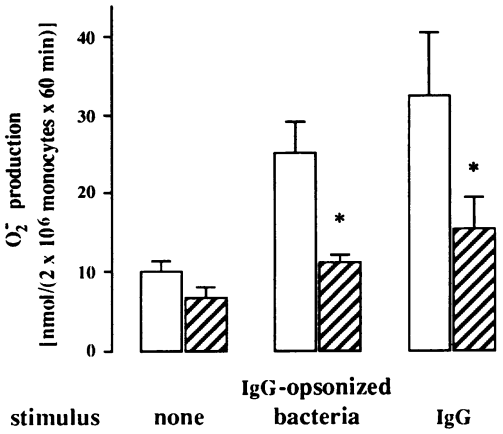

FIG. 2. Effect of genistein on the O_2^- production by monocytes. Monocytes were incubated with 110 μM genistein (hatched bars) or buffer (open bars) at 37°C for 10 min and then stimulated with 500 μg of IgG per ml, 10^7 IgG-opsonized *S. aureus* cells per ml, or buffer in the presence of 100 μM ferricytochrome *c*. The results are means ± SD of three paired experiments, each in duplicate. Asterisks indicate those values that are significantly different from the values obtained with the control monocytes ($P < 0.01$).

0.3) × 10^6/ml and (1.3 ± 0.2) × 10^6/ml for cells incubated with 110 μM genistein or 40 μM tyrphostin-47 and (1.4 ± 0.3) × 10^6 bacteria per ml for control monocytes ($n = 3$).

Genistein and tyrphostins at the concentrations used in present study did not affect the expression of FcγRI and FcγRII on either monocytes or monocytes that had ingested *S. aureus*, as assessed by fluorescence-activated cell sorting analysis, the proliferation of bacteria, and the viability of monocytes (data not shown).

Effect of genistein on the O_2^- production by monocytes stimulated via FcγR. Since genistein inhibits the intracellular killing of *S. aureus* by monocytes and this killing process involves oxygen-dependent bactericidal mechanisms (45), we investigated the effect of genistein on the O_2^- production by these cells after stimulation with purified IgG or IgG-opsonized bacteria. The results showed that genistein markedly ($P < 0.01$) inhibited the FcγR-mediated O_2^- production by monocytes (Fig. 2).

Effect of genistein on the intracellular killing of *Streptococcus pyogenes* by monocytes stimulated via FcγR. Since *Streptococcus pyogenes*, a catalase-negative bacterium, can be intracellularly killed in the absence of reactive oxygen intermediates formed by monocytes (16, 17), we determined whether inhibition of PTK impairs the intracellular killing of this bacterium. IgG-stimulated intracellular killing of *Streptococcus pyogenes* at 30 and 60 min amounted to 38% ± 6% and 51% ± 5%, respectively, for monocytes preincubated with 110 μM genistein and 42% ± 3% and 54% ± 3%, respectively, for control cells ($n = 3$). These data demonstrated that genistein does not affect the IgG-stimulated intracellular killing of *Streptococcus pyogenes* by monocytes.

Effects of PTK inhibitors on changes in the intracellular $Ins(1,4,5)P_3$ and $[Ca^{2+}]_i$ in monocytes induced by cross-linking FcγR. To find out whether PTK activity is involved in the activation of PLC after the cross-linking FcγR, we determined the effects of PTK inhibitors on changes in the intracellular $Ins(1,4,5)P_3$ concentration and $[Ca^{2+}]_i$ in monocytes. Cross-linking FcγRII induced a threefold increase in the intracellular $Ins(1,4,5)P_3$ concentration (Fig. 3) and $[Ca^{2+}]_i$ (Table 2) in monocytes with lag times of 5 s and 15 to 20 s, respectively. Incubation of these cells with genistein or tyrphostin-47 almost completely abolished the increase in the intracellular $Ins(1,4,5)P_3$ concentration stimulated by cross-linking FcγRII (Fig. 3). Genistein only slightly affected the maximum increase in the $[Ca^{2+}]_i$ in monocytes stimulated by cross-linking FcγRII, although it did cause a delay in the onset of the increase in the $[Ca^{2+}]_i$. Similarly, cross-linking FcγRI induced a twofold increase in the intracellular $Ins(1,4,5)P_3$ concentration in monocytes, which was almost completely inhibited by genistein (Table 2). This inhibitor delayed the

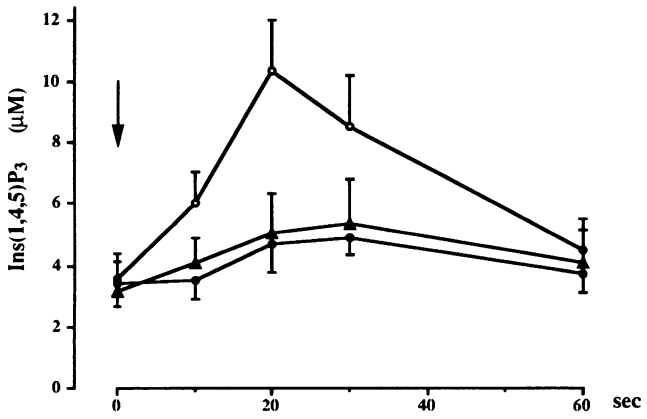

FIG. 3. Effects of genistein and tyrphostin-47 on the FcγR-stimulated intracellular $Ins(1,4,5)P_3$ formation in monocytes. Monocytes were incubated with 2 μg of anti-FcγRII MAb IV-3 per ml at 37°C for 3 min, 25 μg of $F(ab')_2$-GAM IgG per ml was added for the indicated intervals, and the reaction was stopped by the addition of perchloric acid. The $Ins(1,4,5)P_3$ content of the cell extracts was determined by a competition binding assay (31). The monocytes were incubated with 110 μM genistein (closed circles) or 10 μM tyrphostin-47 (closed triangles) or buffer (open circles) before stimulation, as described in Materials and Methods. The results are means ± SD of three paired experiments, each done in triplicate. The arrow indicates the addition of $F(ab')_2$-GAM IgG to cross-linking FcγR.

TABLE 2. Effects of genistein on the maximum intracellular Ins(1,4,5)P_3 concentration and $[Ca^{2+}]_i$ and the lag time for the increase in the $[Ca^{2+}]_i$ in monocytes stimulated by cross-linking FcγRI or FcγRII[a]

Stimulus	Ins(1,4,5)P_3 concn (μM)		$[Ca^{2+}]_i$ (nM)		Lag time(s) for $[Ca^{2+}]_i$ increase	
	Vehicle	Genistein	Vehicle	Genistein	Vehicle	Genistein
None (control)		3.4 ± 0.5	82 ± 15	91 ± 20		
Cross-linking FcγRI	7.0 ± 1.0	4.5 ± 0.9[b]	325 ± 51	280 ± 42	18 ± 4	27 ± 3[b]
Cross-linking FcγRII	10.3 ± 1.4	4.7 ± 0.9[b]	367 ± 39	329 ± 70	16 ± 2	26 ± 2[b]

[a] Purified monocytes were treated with 110 μM genistein or vehicle (0.1% dimethyl sulfoxide) at 37°C for 10 min before stimulation. The maximum intracellular Ins(1,4,5)P_3 concentrations in monocytes which had been incubated with anti-FcγRI MAb 197 or anti-FcγRII MAb IV-3 for 3 min were determined 20 s after the addition of $F(ab')_2$-GAM IgG. The changes in the $[Ca^{2+}]_i$ in FURA-2/AM-loaded monocytes were monitored as previously described (45), expressed as maximum $[Ca^{2+}]_i$ and the lag time of the increase in the $[Ca^{2+}]_i$. The results are means ± SD of at least three paired experiments.

[b] Significantly different from values obtained with monocytes treated with vehicle ($P < 0.01$).

onset of the increase in the $[Ca^{2+}]_i$ in monocytes stimulated by cross-linking FcγRI, but the maximum $[Ca^{2+}]_i$ was only slightly lower (Table 2). Since tyrphostin-47 interfered with the fluorescence of FURA-2, this inhibitor cannot be used to study the involvement of PTK in the FcγR-mediated changes in the $[Ca^{2+}]_i$. Genistein and tyrphostin-47 at the concentrations used in the present study did not inhibit the enzymatic activity of purified PLC, as indicated by the hydrolytic rate for NPPC (data not shown).

Tyrosine phosphorylation of proteins in monocytes after cross-linking FcγR. To find out whether FcγR cross-linking activates PTK, we determined the pattern of tyrosine-phosphorylated proteins after cross-linking FcγRI or FcγRII on monocytes. Purified IgG induced a rapid increase in the tyrosine phosphorylation of cytoplasmic proteins with apparent molecular masses of about 110, 85, 66, and 50 to 40 kDa (Fig. 4A). The 85- and 66-kDa proteins were the most prominent. The 50- to 40-kDa band most likely contains an artifact

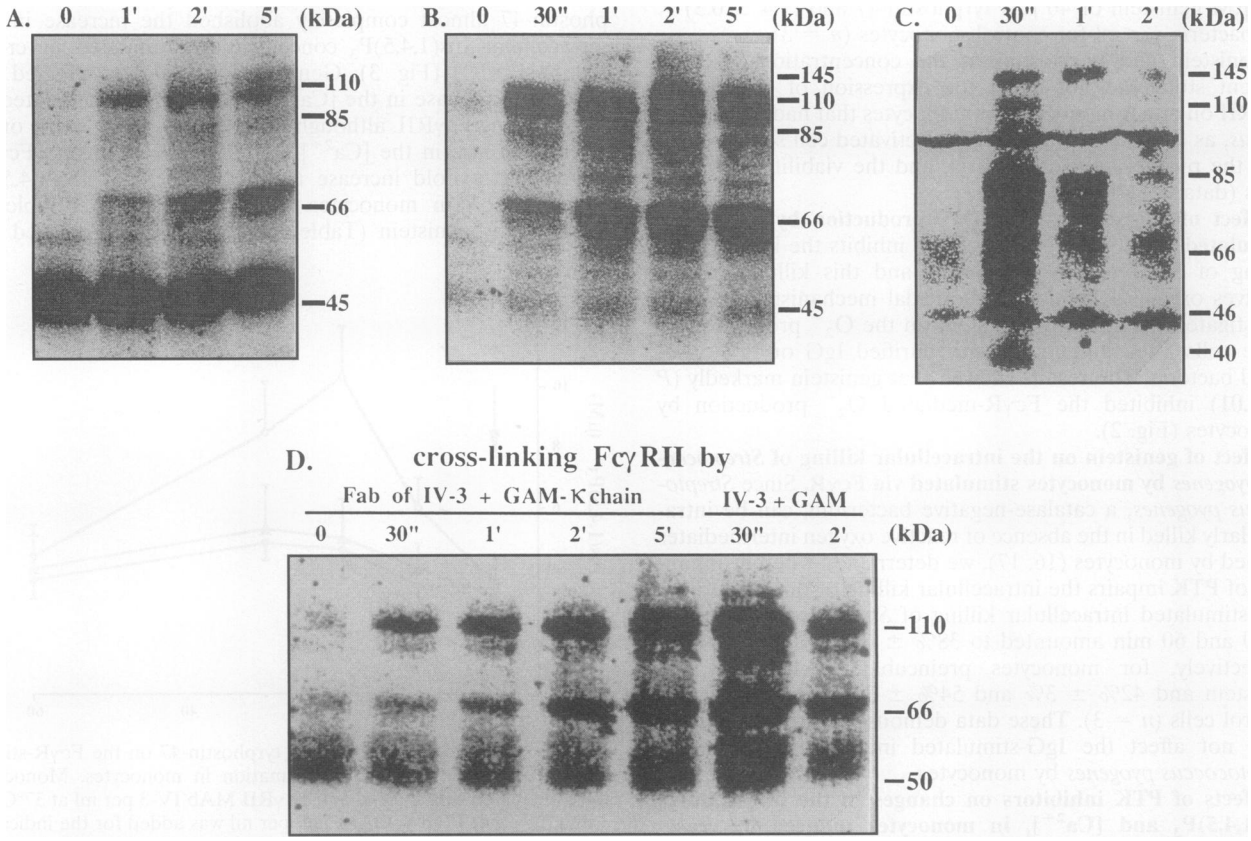

FIG. 4. Effects of cross-linking FcγR on tyrosine phosphorylation of proteins in monocytes. Purified monocytes (5×10^7/ml of HBSS) were stimulated with 250 μg of human IgG per ml (A), 5 μg of anti-FcγRI MAb 197 per ml (B), 2 μg of anti-FcγRII MAb IV-3 per ml (C and D), or 5 μg of Fab fragments of MAb IV-3 (D) at 37°C. Cross-linking of FcγRI and FcγRII was obtained by the addition of 25 μg of $F(ab')_2$-GAM IgG (B and C) or GAM κ chain (D) per ml to the cells which had been incubated with one of the anti-FcγR MAbs for 3 min. At the indicated times (in seconds [″] or minutes [′]) the reaction was terminated by the addition of 2× SDS sample buffer at 100°C, and then the lysates were subjected to SDS–7.5% polyacrylamide gel electrophoresis, followed by Western blot analysis with an anti-phosphotyrosine MAb 4G10 and ^{125}I-labelled protein A. The results were quantified with a PhosphorImager. Results are representative of at least three individual experiments.

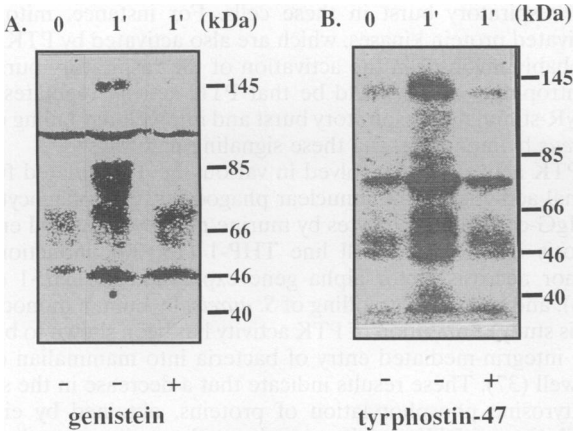

FIG. 5. Effects of genistein and tyrphostin-47 on tyrosine phosphorylation of proteins in monocytes after cross-linking FcγRII. Purified monocytes were incubated with (+) or without (−) 110 μM genistein (A) for 10 min or with 10 μM tyrphostin-47 (B) for 30 min before stimulation. Stimulation of the cells by cross-linking FcγRII and assessment of the patterns of tyrosine-phosphorylated proteins were performed as described in the legend to Fig. 4. Results are representative of at least three individual experiments.

resulting from the binding of ^{125}I-labelled protein A to degraded human IgG, which was added as a stimulus. Cross-linking FcγRI or FcγRII on monocytes stimulated within 30 s tyrosine phosphorylation of multiple proteins. The patterns of tyrosine-phosphorylated proteins, e.g., proteins with apparent molecular masses of 145, 110, 85, 66, and 45 kDa, in monocytes elicited by cross-linking either FcγRI or FcγRII were similar (Fig. 4B and C). Cross-linking FcγRII with Fab fragments of MAb IV-3 followed by GAM κ chain stimulated tyrosine phosphorylation of proteins similar to that with intact MAb IV-3 and F(ab′)$_2$-GAM IgG (Fig. 4D). Interaction between monocytes and either anti-FcγR MAb or F(ab′)$_2$-GAM IgG alone failed to stimulate tyrosine phosphorylation of proteins (data not shown).

Cross-linking of antigens on monocytes by MAb Leu M$_3$ and MAb W6/32, which served as isotype-matched controls for the anti-FcγR MAb, and F(ab′)$_2$-GAM-IgG did not induce tyrosine phosphorylation in monocytes (data not shown), which confirmed the specificity of the immunoblot assay. Furthermore, incubation of monocytes with genistein or tyrphostin-47 prevented the tyrosine phosphorylation of proteins induced by cross-linking FcγR (Fig. 5).

Tyrosine phosphorylation of PLC-γ1 in monocytes after cross-linking FcγRII. Next, we investigated whether PLC-γ1 is tyrosine phosphorylated after cross-linking FcγR on monocytes. The results revealed that cross-linking FcγRII on monocytes induced tyrosine phosphorylation of a protein with a molecular mass of 145 kDa in the immunoprecipitates prepared with polyclonal PLC-γ1-specific antiserum. Tyrosine phosphorylation of this protein was detected within 30 s after cross-linking FcγRII, with a maximum at 1 min and a gradual decline thereafter (Fig. 6).

DISCUSSION

The main conclusion to be drawn from the present results is that activation of PTKs is essential for stimulation of intracellular killing of *S. aureus* by human monocytes by cross-linking FcγRI or FcγRII and for the activation of PLC involved in this process. This conclusion is based on two lines of evidence. First, two selective PTK inhibitors, which act through entirely different mechanisms (2, 26), inhibited the FcγR-mediated intracellular killing of *S. aureus* by monocytes and the increase in the intracellular Ins(1,4,5)P$_3$ concentration in these cells. Second, cross-linking FcγRI or FcγRII on monocytes induced the rapid tyrosine phosphorylation of several proteins, one of which was identified as PLC-γ1. This tyrosine phosphorylation of proteins could be completely blocked by PTK inhibitors.

The inhibitory effects of genistein and tyrphostin-47 on the intracellular killing of *S. aureus* and the PLC activation stimulated by FcγR were not due to cytotoxic effects of these PTK

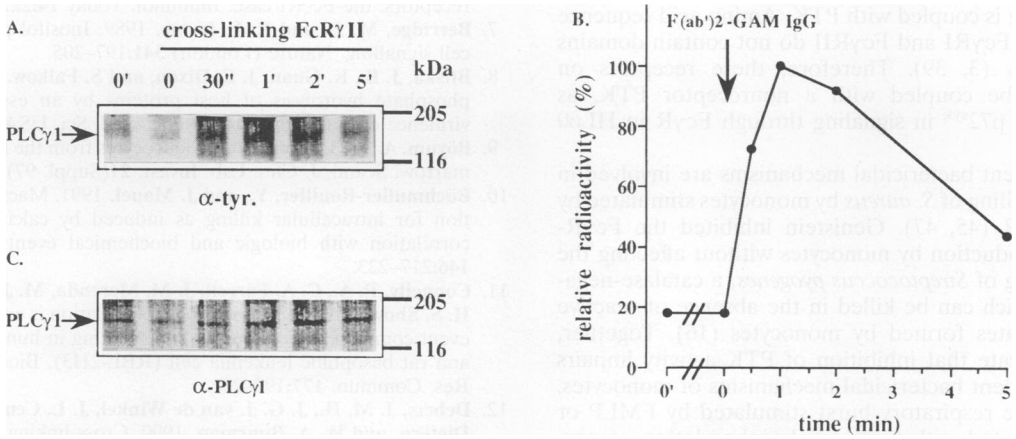

FIG. 6. Kinetics of tyrosine phosphorylation of PLC-γ1 in monocytes after cross-linking FcγRII. (A) Purified monocytes (3×10^7 per sample) were incubated at 37°C with buffer (lane 1) or with 2 μg of anti-FcγRII MAb IV-3 per ml for 3 min, and then 25 μg of F(ab′)$_2$-GAM IgG per ml was added to achieve cross-linking FcγRII. At the indicated intervals, the reaction was terminated and PLC-γ1 in monocytes was immunoprecipitated with PLC-γ1-specific antiserum. The immunoprecipitates were then eluted, subsequently resolved by SDS-polyacrylamide gel electrophoresis, followed by Western blot analysis with an anti-phosphotyrosine MAb as described in the legend to Fig. 4. The arrow indicates the bands for PLC-γ1. (B) The relative radioactivities in the PLC-γ1 bands shown in panel A were determined with a PhosphoImager and expressed as a percentage of the value obtained 1 min after cross-linking FcγRII. (C) To ascertain that each lane received similar amounts of PLC-γ1, after the blot was stripped, it was reprobed with the PLC-γ1 antiserum. Results are representative of three individual experiments.

inhibitors, since the phagocytosis of serum-opsonized *S. aureus* and the intracellular killing of *Streptococcus pyogenes* by monocytes were not affected by these drugs. Our observation that the expression of both FcγRI and FcγRII on monocytes and monocytes containing bacteria was not affected by genistein and tyrphostin-47 excludes the possibility that the PTK inhibitors induced loss of FcγR from the cell surface.

The conclusion that activation of PLC in monocytes after cross-linking FcγR is regulated by PTK is consistent with observations by others in monocytic cell lines U937 and THP-1 (27, 35, 38). Our observation that genistein only slightly affected the maximum increase in the $[Ca^{2+}]_i$ in monocytes contradicts the results of Rankin et al. (35). However, the concentration of genistein used by these researchers, which is 3.6 times higher than that used in the present study, can inhibit protein kinases other than PTK as well (2). It is of interest to note that in Rankin's study, the FcγR-mediated increase in $[Ca^{2+}]_i$ was not affected by 1 μM herbimycin-A, although others have shown that this concentration of herbimycin-A almost completely abolished the FcγR-mediated tyrosine phosphorylation of PLC-γ1 and hydrolysis of phosphoinositol (4,5)-bisphosphate in the monocytic cell line U937 (27). Furthermore, our data are in agreement with the observation that genistein did not inhibit the FcγR-mediated increase in the $[Ca^{2+}]_i$ in a murine macrophage cell line transfected with FcγRIIA (32). The identity of the intracellular activator(s) of the increase in the $[Ca^{2+}]_i$ in monocytes after cross-linking FcγR is not known. Rearrangements of cytoskeleton or activation of phospholipase A_2 might contribute to the increase in the $[Ca^{2+}]_i$ after cross-linking FcγR in monocytes, as shown in neutrophils and platelets after ligation of FcγR (4, 36).

Our observation that cross-linking FcγRI or FcγRII on monocytes induced similar patterns of tyrosine-phosphorylated proteins is consistent with reports that occupancy of these receptors induces similar biochemical and functional changes in monocytes (6, 43). Intact MAbs might induce the simultaneous cross-linking of FcγRI and FcγRII, since intact anti-FcγR MAbs could bind to FcγR via their Fc regions as well as via their antigen-binding regions (43). However, this possibility is very unlikely, since we observed similar patterns of tyrosine-phosphorylated proteins after cross-linking FcγRII by intact MAb IV-3 and its Fab fragments. At present, it is not clear how FcγR cross-linking is coupled with PTK. Amino acid sequence data indicate that FcγRI and FcγRII do not contain domains with PTK activity (3, 39). Therefore, these receptors on monocytes must be coupled with a nonreceptor PTK, as reported for PTK $p72^{syk}$ in signaling through FcγR in HL60 cells (1).

Oxygen-dependent bactericidal mechanisms are involved in the intracellular killing of *S. aureus* by monocytes stimulated by cross-linking FcγR (45, 47). Genistein inhibited the FcγR-mediated O_2^- production by monocytes without affecting the intracellular killing of *Streptococcus pyogenes*, a catalase-negative bacterium which can be killed in the absence of reactive oxygen intermediates formed by monocytes (16). Together, these results indicate that inhibition of PTK activity impairs the oxygen-dependent bactericidal mechanisms of monocytes. In neutrophils, the respiratory burst stimulated by FMLP or IgG is also associated with tyrosine phosphorylation of proteins (24). The role of PTK in the respiratory burst in monocytes after cross-linking FcγR is not clear. It could be that PTK activity regulates activation of PLC-γ and thus Ca^{2+} phospholipid-dependent protein kinase C, which is essential for activation of the NADPH oxidase in monocytes (30). It should be realized that signal transduction pathways other than the PLC-dependent pathway are involved in the stimulation of the respiratory burst in these cells. For instance, mitogen-activated protein kinases, which are also activated by PTK, are probably involved in the activation of the respiratory burst in neutrophils (42). It could be that PTK activity regulates the FcγR-stimulated respiratory burst and intracellular killing of *S. aureus* by monocytes via these signaling pathways.

PTK activation is involved in various FcγR-mediated functional activities of mononuclear phagocytes, e.g., phagocytosis of IgG-coated erythrocytes by murine macrophages and endocytosis in monocytic cell line THP-1 (18, 20), induction of tumor necrosis factor alpha gene expression in THP-1 cells (38), and intracellular killing of *S. aureus* by human monocytes (this study). Inhibition of PTK activity has been shown to block the integrin-mediated entry of bacteria into mammalian cells as well (37). These results indicate that a decrease in the state of tyrosine phosphorylation of proteins, obtained by either inhibition of PTK activity or enhanced tyrosine phosphatase activity, e.g., by the *Yersinia* outer membrane protein (YopH) (8, 22), is an efficient mechanism of impairment of the antimicrobial activities of phagocytes, thus favoring the survival of certain microorganisms.

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