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IN HUMAN NEUTROPHILS THE BINDING TO IMMUNOCOMPLEXES INDUCES THE TYROSINE PHOSPHORYLATION OF FcγRII BUT THIS PHOSPHORYLATION IS NOT AN ESSENTIAL SIGNAL FOR Fc-MEDIATED PHAGOCYTOSIS

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Summary. It has been recently suggested that protein tyrosine phosphorylation is involved in FcγRs-mediated signalling. In this paper we have investigated if in human neutrophils a tyrosine phosphorylation of FcγRII takes places after the binding with immunocomplexes and if this phosphorylation plays a role in phagocytic signal. The immunoprecipitation with mAb anti-FcγRII of lysates of neutrophils challenged in suspension with insoluble immunocomplexes (IIC) or sheep erythrocytes opsonized with IgG (E-IgG), followed by immunoblotting with anti-phosphotyrosine antibody, demonstrated that FcγRII was tyrosine phosphorylated. When neutrophils were pretreated with different doses of tyrosine kinase inhibitors, genistein or erbstatin, the phosphorylation of FcγRII induced by IIC or E-IgG was dose dependently inhibited. In these conditions both genistein and erbstatin failed to inhibit the phagocytosis of E-IgG. These results demonstrated that in human neutrophils in suspension the binding to Fc of IgG induces a tyrosine phosphorylation of FcγRII but this phosphorylation is not an essential signal for phagocytosis of IgG-opsonized particles.

In human neutrophils the receptors for IgG (FcγRs) are costitutively represented by the transmembrane FcγRIIA and FcγRIIC, and by the GPI-anchored FcγRIIIB (1,2). Many studies have been performed on the signalling function of these two receptors (1-12).

In this paper we have investigated if in human neutrophils a tyrosine phosphorylation of FcγRII takes place after the binding with immunocomplexes and whether this phosphorylation is essential for the phagocytic signal. The rationale of this investigation is based on the following findings: 1) In neutrophils the binding of FcγRs causes a rapid stimulation of tyrosine phosphorylation of several proteins (13,14); 2) The cytoplasmic tail of human FcγRII contains (15-19) a single highly conserved motif, which is closely related to the motifs (TAM, tyrosine-containing activation motif) found in T- and B-lymphocyte

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antigen receptor, β and γ chains of FcεRI of mastcells and basophils, γ and ζ chains of FcγRIII of macrophages and NK cells (15-22). This motif contains tyrosine residues that are phosphorylated by src-family tyrosine kinases during receptor-mediated cell activation. This phosphorylation has been shown in FcγRI and FcγRII of a human monocytic leukemia cell line (23,24), FcγRII of platelets, HEL or COS-1 transfected cells (17), FcγRII of B-lymphocytes (25), FcγRIII of NK cells (26,27), FcεRI of mast cells and basophils (28,29); 3) FcγRII of human neutrophils is associated with src-like tyrosine kinase Fgr, which becomes activated after cross-linking of FcγRII (30); 4) The tyrosine phosphorylation of FcγRIIA and FcγRIIIA of monocytes is required for a phagocytic signal (19,21,24); 5) Inhibitors of tyrosine kinases inhibit the Fc-mediated phagocytosis in COS-1 cells transfected with FcγRIII of macrophages (31), in P338 transfected with FcγRIIA of neutrophils and macrophages (16), and in adherent mouse macrophages (32).

The results presented in this paper show that in human neutrophils the binding with insoluble IIC or E-IgG induces a tyrosine phosphorylation of FcγRII, and that this phosphorylation is not essential for phagocytosis.

MATERIALS AND METHODS

Reagents. BSA, DFP and SDS were from Sigma. Rabbit anti-BSA serum, genistein, erbstatin were from Calbiochem. Acrylamide, N'N'-methylenebisacrylamide, tetramethylenediamine, Coomassie Blue R-250 and blotting nitrocellulose membrane were from Bio-Rad. Rabbit anti-sheep red blood cell IgG was from USB (Cleveland, OH). IgG2bk anti-phosphotyrosine mouse monoclonal antibody from hybridoma 4G10 was from UBI, N.Y. The anti FcγRII was mAb IV.3 purified from ascitic fluid of mice injected with hybridoma cell lines obtained from American Type Culture Collection (ATCC), Bethesda, MA.

Preparation of neutrophil suspensions. Human neutrophils (95% pure) were prepared from healthy donors as previously described (12).

Preparation of IIC and opsonized erythrocytes. 200 μl of BSA (0.5 mg/ml) were mixed with 100 μl of rabbit anti-BSA serum (6 mg/ml) and incubated at 25°C for 60 min. The IIC formed were washed and resuspended in 300 μl of HBSS. Sheep erythrocytes were prepared and opsonized with subagglutinating concentration of rabbit anti-sheep red blood cell IgG as previously described (33).

Stimulation of neutrophils with IIC or E-IgG. Neutrophils suspended in HBSS ($1.5x10^7$/ml) were treated with IIC (90 μg protein /ml) or E-IgG (ratio, neutrophil : erythrocytes, 1:10) at 37°C under continuous shaking. When required, various doses of genistein in DMSO (3μl DMSO/ml) were added to the neutrophil suspension 10 min before the addition of IIC or E-IgG. In these cases control neutrophils were treated with the same amount of DMSO (3μl DMSO/ml). In the case of erbstatin, neutrophils ($3-5x10^6$/ml) were pretreated for 60 min at 37°C with the inhibitor, washed twice, and resuspended in HBSS at $1.5x10^7$/ml. Samples of stimulated and unstimulated neutrophils were drawn out and analysed for phagocytosis and processed for immunoprecipitation and determination of tyrosine phosphorylation of FcγRII. The phagocytosis of E-IgG was evaluated by microscopic examination at x 1500 magnification as previously described (12).

Immunoprecipitation. After stimulation, cell samples were diluted with a 10-fold excess of ice-cold HBSS containing 1 mM PMSF, 2 mM di-isopropylfluorophosphate, 2mM Na_3VO_4 and 10 μM phenylarsine oxide, and rapidly centrifuged (4°C) at 500xg for 7 min. The cell pellet was resuspended in 500 μl of solubilization buffer (25 mM Tris, pH-7.5, 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS containing 2 mM Na_3VO_4, 10 μM phenylarsine oxide, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 1mM EDTA, 1mM PMSF), extracted for 30 min on ice and then centrifuged at 15000xg for 10 min to remove insoluble material. The supernatants were incubated for 60 min at 4°C under rotation with 4

μg of IV.3 mAb conjugated to trisacryl-protein A (Pierce), then washed three times with 700 μl of ice-cold solubilization buffer. The washed beads were then boiled for 5 min in Laemmli sample buffer, sedimented and the supernatant was subjected to SDS/10% PAGE (34). The western blot was performed as described in (35). The blots were incubated overnight with anti-phosphotyrosine 4G10 antibodies (1 μg/ml) or with anti-FcγRII receptor IV.3 mAb (2 μg/ml) diluted in TBS buffer (50 mM Tris, pH-7.5, 170 mM NaCl) containing 3% (w/v) BSA and 0.2% (v/v) NP40. After various washings with TBS, and incubation for 60 min in HRP-labelled anti-mouse IgG (Sigma, St Louis) diluted 1:2000 in TBS containing 3% (w/v) BSA and 0.2% (v/v) NP40, bound antibodies were revealed by enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham U.K.) using Kodak X-Omat AR film. The "stripping" protocol has been already reported in (35).

RESULTS

The results reported in Fig. 1 show that IV.3 mAb precipitated a 40 kDa protein from lysates of control and IIC-treated neutrophils and that only the protein from IIC-treated neutrophils was tyrosine phosphorylated. Moreover, an irrelevant mouse antibody was not able to precipitate the 40 kDa protein (Fig. 1 lines 4).

As shown in Fig. 2A the stimulation of tyrosine phosphorylation of FcγRII was dose

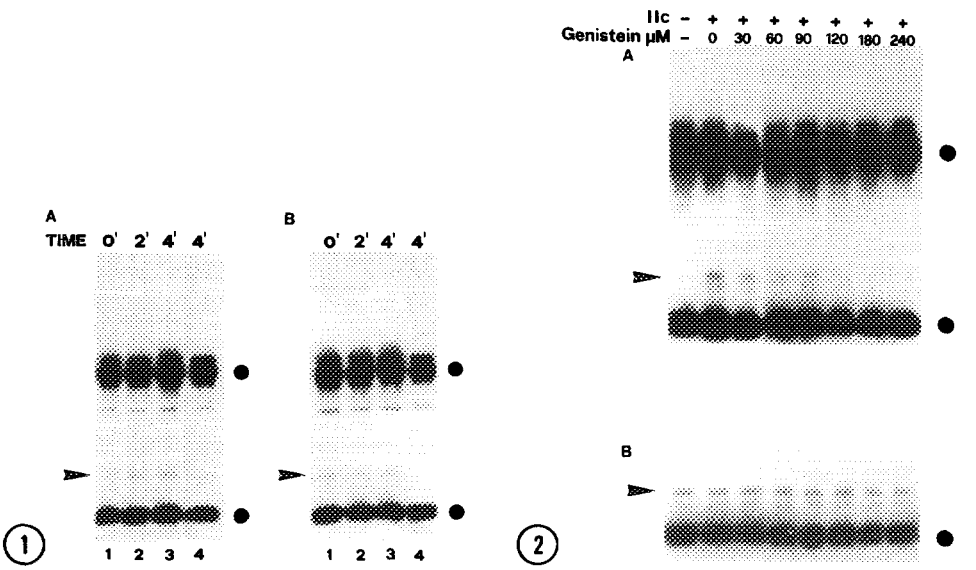

Fig. 1. Tyrosine phosphorylation of FcγRII in human neutrophils.
Neutrophils ($1.5x10^7$/ml) were stimulated with 90 μg/ml IIC for the time indicated. The cells were then sedimented and extracted in ice-cold lysis buffer as described in Materials and Methods.The extracts were immunoprecipitated with specific anti FcγRII receptor mAb IV.3 (lines 1,2,3) or mouse irrelevant antibodies (lines 4) and analyzed on an immunoblot probed with anti-phosphotyrosine mAb 4G10 (A) or IV.3 mAb (B). The arrows indicate the position of FcγRII receptor. The immunoglobulin molecules are designated with dots. Data are from one experiment representative of four.

Fig. 2. Effect of genistein on tyrosine phosphorylation of FcγRII receptor induced by IIC.
Neutrophils were incubated for 10 min in the presence or absence of the indicated doses of genistein, then stimulated with 90 μg/ml IIC for 4 minutes, blocked, extracted and immunoprecipitated with anti FcγRII mAb IV.3. After SDS/10% PAGE, the blots were probed with anti-phosphotyrosine mAb 4G10 (A), then stripped and reprobed with anti FcγRII IV.3 (B). The arrows indicate the position of FcγRII. Dots indicate the immunoglobulin chains. Data are from one experiment representative of three.

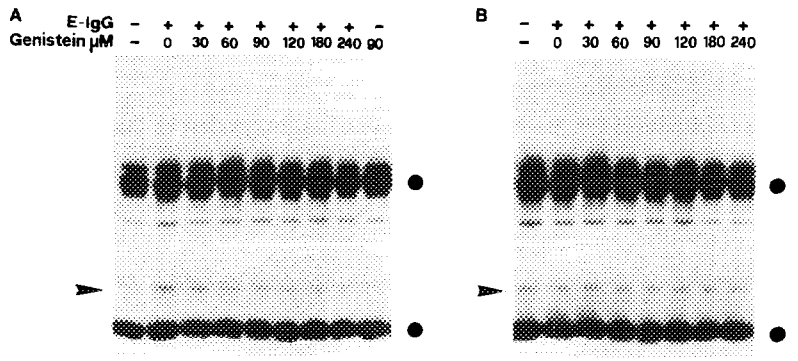

Fig. 3. Effect of genistein on FcγRII tyrosine phosphorylation induced by E-IgG.
The protocol was as in Fig. 2, except that neutrophils were stimulated with E-IgG for 10 minutes in the presence or in the absence of the indicated doses of genistein. Extracts were immunoprecipitated with mAb IV.3, subjected to SDS/10% PAGE, immunoblotted with anti-phosphotyrosine 4G10 (A) and then stripped and reprobed with mAb IV.3 (B). The arrows indicate the position of FcγRII. Dots indicate the positions of immunoglobulin chains. Data are from one experiment representative of three.

dependently inhibited by the tyrosine kinase inhibitor genistein. The same blot was then "stripped" and reprobed with IV.3 mAb (Fig. 2B), to ensure that comparable amounts of proteins had been immunoprecipitated and transferred to nitrocellulose membrane.

To understand the role of this tyrosine phosphorylation in phagocytosis we have investigated the tyrosine phosphorylation of FcγRII during Fc-mediated phagocytosis. The results reported in Fig. 3A show that phagocytosis of E-IgG was associated with tyrosine phosphorylation of FcγRII. The time point at which the lysates of neutrophils were analyzed was that corresponding to the maximal rate of phagocytosis as shown in Fig. 4A. When neutrophils were pretreated with different doses of genistein the tyrosine phosphorylation of

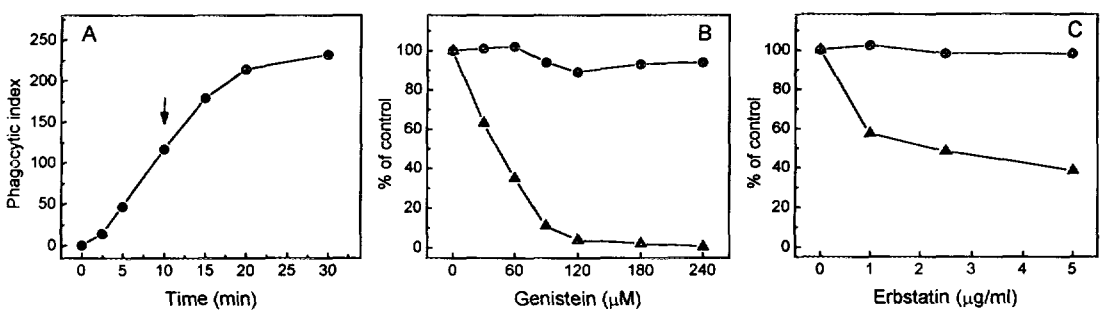

Fig. 4. Effect of genistein and erbstatin on phagocytosis and tyrosine phosphorylation of FcγRII in human neutrophils.
Time-course of phagocytosis of E-IgG (A). The arrow indicates the time point at which phagocytosis and tyrosine phosphorylation of FcγRII were investigated. Phagocytic index=number of E-IgG ingested/100 neutrophils.
Comparison between the effect of genistein (B) or erbstatin (C) on phagocytosis of E-IgG (●) and on tyrosine phosphorylation of FcγRII (▲) measured densitometrically. Data in panel B are of the same experiment reported in Fig. 3A. The densitometric values, calculated as height of the densitometric peaks, are presented as arbitrary units. Data are from one experiment representative of three.

FcγRII induced by E-IgG was dose-dependently inhibited (Fig. 3A). Fig. 3B shows the same blot "stripped" and reprobed with IV.3 mAb. Similar results were obtained using another tyrosine kinase inhibitor, erbstatin (Fig. 4C).

In order to understand the function of tyrosine phosphorylation of this receptor we have compared the phagocytosis of E-IgG in the presence or absence of genistein or erbstatin. The results reported in Fig. 4B and 4C show that when tyrosine phosphorylation of FcγRII was inhibited the ingestion of E-IgG was unchanged.

While this finding demonstrates that the tyrosine phosphorylation of this receptor is not essential for phagocytosis, it is likely that the tyrosine phosphorylation of other proteins is involved in the phagocytic process in neutrophils and that in our experimental conditions the phosphorylation of these proteins was not inhibited by the two drugs. In order to clarify this problem we have compared the effect of genistein and erbstatin on the pattern of protein tyrosine phosphorylation induced by E-IgG and on phagocytosis. The results presented in Fig. 5 demonstrate that in conditions where the two drugs almost completely suppressed protein tyrosine phosphorylation (Fig. 5A) the phagocytic index was unchanged (Fig. 5B).

DISCUSSION

The results presented in this paper demonstrate that in human neutrophils in suspension the FcγRII was tyrosine phosphorylated after the binding to IIC or E-IgG and that the suppression of the phosphorylation by a tyrosine kinase inhibitor was not accompanied by a depression of phagocytosis of E-IgG.

The first result was expected on the basis of the results presented by others that 1) the cytoplasmic tail of FcγRII possesses the highly conserved motif containing tyrosine residues

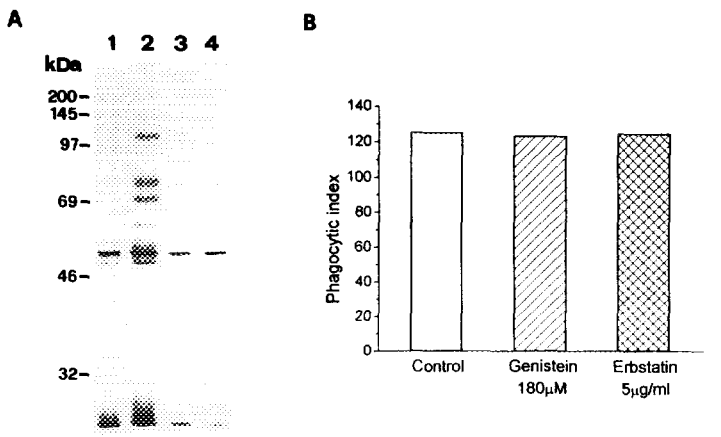

Fig. 5. Effect of genistein and erbstatin on protein tyrosine phosphorylation (A) and on phagocytosis (B). Neutrophils were incubated in the absence (lane 2) or presence of 180 μM genistein (lane 3) or 5μg/ml erbstatin (lane 4) at 37°C under agitation as described in Materials and Methods. Cells were then stimulated with E-IgG. Reactions were stopped after 10 min and samples were boiled in electrophoresis sample buffer, subjected to SDS/10% PAGE, and immunoblotted with anti-phosphotyrosine mAb 4G10. Lane 1= resting neutrophils. The phagocytosis of E-IgG was measured in the same experiment. Data are from one experiment representative of three.

that are phosphorylated by scr-family of tyrosine kinases upon cell activation (16-24), and that 2) FcγRII of neutrophils is associated with the src-like tyrosine kinase Fgr, whose activity rapidly increases after cross-linking of the receptor (30).

The finding that the phagocytosis of E-IgG occurred normally when tyrosine phosphorylation of FcγRII was inhibited, demonstrates that, in the experimental conditions employed here, this phosphorylation is not an essential process for the phagocytic activity in human neutrophils. This finding was unexpected on the basis of data presented by others on mononuclear phagocytes or other transfected cells. In fact, it has been shown that inhibitors of protein tyrosine kinases depressed the Fc-mediated phagocytosis in COS-1 cells transfected with FcγRIII of macrophages (31), in P338 transfected with FcγRII of neutrophils and macrophages (16), in monocytic cell line THP-1 (24), and in thioglycollate elicited mouse macrophages (32). Moreover, the presence of tyrosine residues in the cytoplasmic domains of FcγRIIA and FcγRIIIA transfected in COS-1 cells is required for phagocytic signal (19,21) as shown by the finding that these cells transfected with truncated forms of the receptor lacking the tyrosine-containing motif have no phagocytic activity. Similar requirement in TAM sequence in the γ subunit of cytoplasmic tail of FcγRIII has been recently shown in rat basophilic leukemia cells (36).

The discrepancy between our data on neutrophils and those of others on mononuclear phagocytes can be explained as follows; 1) It is likely that the tyrosine phosphorylation of FcγRs is a signal for phagocytosis in mononuclear phagocytes but not in neutrophils; 2) The results showing that the tyrosine-containing motif of FcγRs is necessary for phagocytosis does not imply that the phosphorylation of these residues is the required signal. It cannot be ruled out that the presence, and not the phosphorylation, of these residues is the requisite for the phagocytic signal; 3) The finding obtained by others using mononuclear phagocytes that the inhibition of tyrosine kinases depressed the phagocytosis (16,24,31,32) could be due to the inhibition of the tyrosine phosphorylation of other proteins required for phagocytosis; 4) Our finding that genistein and erbstatin inhibited tyrosine phosphorylation of FcγRII in neutrophils without affecting the phagocytosis of E-IgG did not rule out the possibility that the activation of other tyrosine kinases and the phosphorylation of other proteins are required for phagocytosis also in neutrophils and that, in our conditions, these tyrosine kinases were not inhibited by genistein or erbstatin, thus allowing a normal phagocytic activity also in the presence of these inhibitors and in the absence of phosphorylation of FcγRII. However, we have shown that this was not the case. In fact, the comparison between the effect of genistein and erbstatin on the general pattern of protein tyrosine phosphorylation and on phagocytosis has clearly demonstrated that the phagocytosis of E-IgG was unaffected also when the increment of tyrosine phosphorylation of all the proteins was almost completely suppressed. Before to conclude that tyrosine phosphorylation of the proteins is not involved in Fc-mediated phagocytosis we have to further clarify whether tyrosine phosphorylation of some proteins minimally represented in neutrophils and therefore difficult to detect could be involved. Whichever is the case, the results presented here clearly demonstrate that in neutrophils in suspension the tyrosine phosphorylation of FcγRII is not essential for phagocytosis.

It is known (1-14) that the activation of Fc receptors in phagocytes and also in non phagocytic cells is followed by the stimulation of several processes and functions such as turnover of phosphoinositides and hydrolysis of other phospholipids, increase in $[Ca^{2+}]i$, production of oxygen metabolites, enzymes secretion, aggregation, cytotoxicity, production of eicosanoids, PAF, cytokines and other inflammatory mediators. Thus it is likely that in neutrophils the phosphorylation of FcγRII is a process functional not to phagocytosis but to other cell responses associated with the phagocytosis. One of these could be the activation of phosphoinositide turnover. In fact, we have recently shown that IIC induced a genistein-sensitive activation of phosphoinositide hydrolysis mediated by tyrosine phosphorylation of phospholipase Cγ2 (manuscript in preparation). The phosphorylation of tyrosine residues of FcγRII could be the mechanism by which phospholipase Cγ2, that contains SH2 domains (37,38), is recruited on the plasmamembrane and subsequently tyrosine phosphorylated and activated.

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