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Different sensitivities of the responses of human neutrophils stimulated with immune complex and C5a anaphylatoxin to pertussis toxin

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When stimulated with immune complex or C5a anaphylatoxin, human neutrophils undergo an increase in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) that precedes the onset of superoxide (O_2^-) production. The extracellular Ca^{2+} is required for the O_2^- production of neutrophils stimulated by C5a, but is only partially required for those by immune complex. The addition of pertussis toxin to neutrophils does not inhibit the rise in $[Ca^{2+}]_i$ and O_2^- production induced by immune complex but does inhibit those induced by C5a. These results suggest that a different sequence of reaction is involved by different stimulants, the anaphylatoxin and immune complex.

Neutrophil; Pertussis toxin; Immune complex; C5a

1. INTRODUCTION

When exposed to appropriate stimuli, human neutrophils undergo phagocytosis, O_2^- production, chemotaxis and degranulation [1]. Other responses of neutrophils to stimulation include phosphatidylinositol metabolism, protein phosphorylation and an increase in Ca^{2+} influx and release from cell membranes or intracellular stores with a rapid rise in $[Ca^{2+}]_i$ [2,3]. These latter responses have received much attention because of their casual involvement in the activation of neutrophils. Recent studies indicate that GTP-binding proteins are also involved in the regulation of neutrophil responses elicited by a chemotactic peptide, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) [4,5]. Thus, the stimulus response of human neutrophils has been studied extensively, using fMLP as a stimulant [6]. While fMLP is a synthetic analogue of the natural chemoattractants derived from some bacteria, its in vivo function must await verification.

Human neutrophils possess the surface membrane receptors for Fc regions of IgG molecules (Fc receptors) [7] and the receptor for C5a anaphylatoxin (C5a receptor) [8], a low-molecular-mass fragment released from C5 when the complement cascade is activated. The stimulations of neutrophils by immune complex or C5a, naturally occurring immune stimulants, are also reported to result in the O_2^- production and degranulation [8–10]. At present, much less information is available on the signal transduction mechanisms of neutrophils through Fc receptors or C5a receptor, in spite of their physiological significance.

The present paper shows that the pertussis toxin-sensitive G-protein system is not involved in the reactions responsible for the rise in $[Ca^{2+}]_i$ and O_2^- production of neutrophils stimulated by immune complex, but involved in those stimulated by C5a.

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Abbreviations: $[Ca^{2+}]_i$, concentration of intracellular Ca^{2+}; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; O_2^-, superoxide; PBS, phosphate-buffered saline

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2. MATERIALS AND METHODS

Immune complex was prepared by incubating tetanus toxin (Behring Diagnostics, La Jolla, CA, 200 U/ml) with human anti-tetanus toxin IgG (Tetanobulin® , Green Cross, Osaka, 200 U/ml) for 1 h at 37°C, as described previously [9,10]. C5a was prepared by incubating highly purified human C5 with C5 convertase (produced from cobra venom factor, factor B and factor D). Details for preparation of C5 and C5a will be published elsewhere.

Human peripheral blood was collected in acid-citrate-dextrose and neutrophils were isolated utilizing Dextran T-500 and Ficoll-Paque (Pharmacia, Uppsala) sedimentation followed by hypotonic lysis of contaminating erythrocytes as previously described [11]. Neutrophils thus obtained were finally suspended in phosphate-buffered saline (PBS; 135 mM NaCl, 5 mM KCl, 10 mM sodium phosphate buffer, pH 7.4) at 5×10^7 cells/ml and kept on ice. Where indicated, the cells (2×10^7/ml) were incubated with or without 100 ng of pertussis toxin (islet-activating protein, generously provided by Dr Toshiaki Katada, Faculty of Science, Tokyo Institute of Technology) per ml for 2 h at 37°C, washed and resuspended in PBS.

O_2^- production was monitored continuously as the superoxide dismutase-inhibitable reduction of cytochrome *c*, as described in [11,12]. All assay mixtures were incubated with cytochalasin B (5 μg/ml) for 2 min before stimulation. $[Ca^{2+}]_i$ was determined with a fluorescent probe, Fura-2 (molecular Probes, Junction City, OR) by the procedure described by Scanlon et al. [13].

3. RESULTS

Fig.1A (upper tracing) shows the time course of O_2^- production of neutrophils stimulated by immune complex in the presence or absence of extracellular Ca^{2+}. The presence of extracellular Ca^{2+} in the reaction medium enhanced the O_2^- production. Fig.1A (lower tracing) shows that Fura-2-loaded neurophils responded to immune complex with an increase in $[Ca^{2+}]_i$ from 100 to 340 nM in the presence of extracellular Ca^{2+}. The onset of the increment in $[Ca^{2+}]_i$ occurred within 15 s and preceded the onset of O_2^- production by 40–50 s. After the peak has been reached in 1 min, there is a subsequent decline in $[Ca^{2+}]_i$. Without extracellular Ca^{2+}, a smaller fluorescent increase and its rapid decline were observed, correlating with less O_2^- production. The immune complex caused dose-dependent increases in $[Ca^{2+}]_i$ and O_2^- production (fig.2). The correlation of $[Ca^{2+}]_i$ change with O_2^- production was observed (fig.2, inset) and the coefficient of correlation was 1.0. These results suggest that the rise in $[Ca^{2+}]_i$ is a requirement for the Fc receptor-mediated activation of neutrophils.

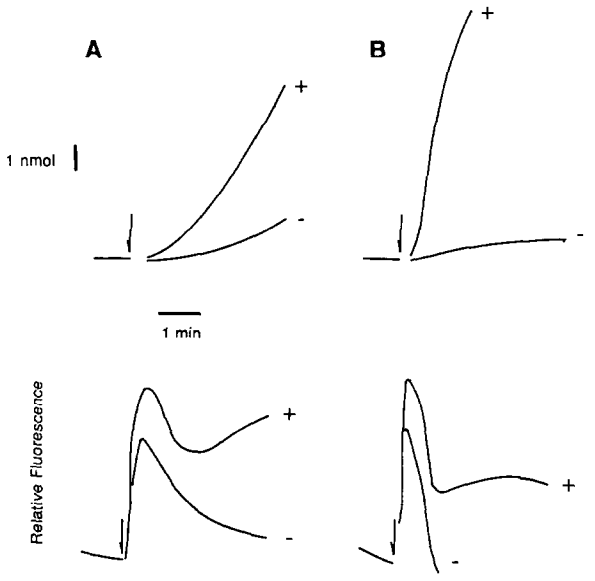

Fig.1. Effects of extracellular Ca^{2+} on O_2^--production and an increase in $[Ca^{2+}]_i$. Continuous measurements of O_2^- production (upper tracings) and fluorescence change (lower tracings) of 1×10^6 neutrophils per ml were performed as described in section 2. The addition of (A) immune complex (80 μg/ml) and (B) C5a (80 ng/ml) is shown by the arrow in the presence (+) or absence (−) of 1 mM extracellular Ca^{2+}, respectively (+, PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$; −, PBS containing 5 mM EGTA and 1 mM $MgCl_2$). Comparable results were obtained in each three separate neutrophil preparations.

When neutrophils were treated with C5a, the absence of extracellular Ca^{2+} reduced the extent of rise in $[Ca^{2+}]_i$ (fig.1B). The recovery phase of fluorescence tracing was accelerated in the absence

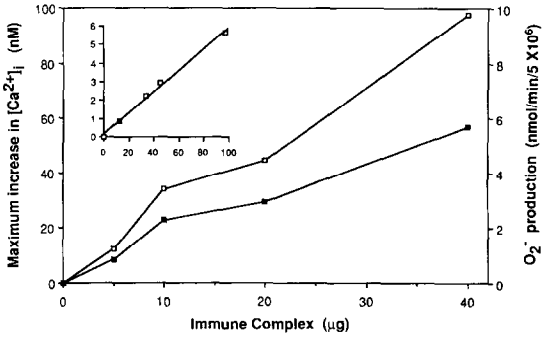

Fig.2. Dose response curves for effect of immune complex on O_2^- production (■) and maximum increase in $[Ca^{2+}]_i$ (□). All assays were done in the presence of 1 mM extracellular Ca^{2+}, as described in fig.1. (Inset) Relationship between O_2^- production (ordinate) and maximum increase in $[Ca^{2+}]_i$ (abscissa).

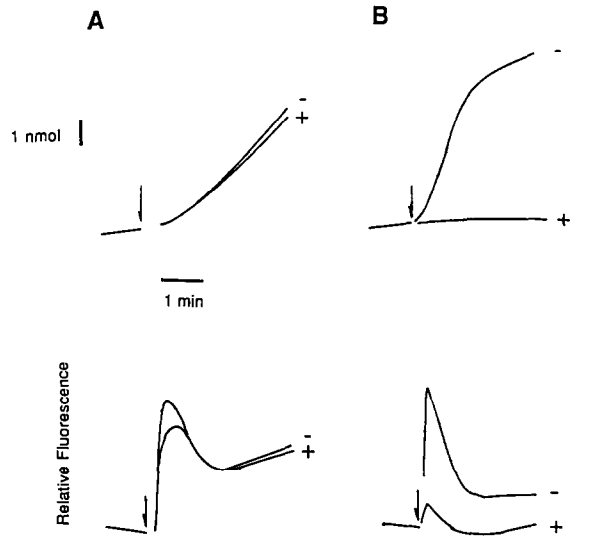

Fig.3. Effects of pertussis toxin pretreatment on O_2^- production and an increase in $[Ca^{2+}]_i$. Neutrophils were incubated in the presence (+) or absence (−) of 100 ng/ml pertussis toxin for 2 h at 37°C. Continuous measurement of O_2^- production (upper tracings) and fluorescence change (lower tracings) of 1×10^6 neutrophils per ml were performed as described in section 2. The addition of (A) immune complex (80 μg/ml) and (B) C5a (80 ng/ml) is shown by the arrow in the presence of 1 mM extracellular Ca^{2+}, respectively. Comparable results were obtained in each three separate neutrophil preparations.

of extracellular Ca^{2+}. No O_2^- production was detected in this condition (fig.1B, upper tracing), showing that the rise in $[Ca^{2+}]_i$ is not sufficient to produce O_2^-.

Next, we used pertussis toxin to determine whether any G-proteins are involved in the coupling of Fc or C5a receptor occupancy to the rise in $[Ca^{2+}]_i$ and subsequent O_2^- production. This toxin exerted little discernable inhibition on the increase of Fura-2 fluorescence of neutrophils stimulated with immune complex (fig.3A, lower tracing). Furthermore, the O_2^- production of Fc receptor-stimulated neutrophils was completely refractory to inhibition by pertussis toxin (fig.3A, upper tracing). In the response to C5a, the cells pretreated with pertussis toxin exhibited a significant reduction in a rise in $[Ca^{2+}]_i$ and subsequent O_2^- production, as shown in fig.3B.

4. DISCUSSION

As shown in fig.1, the maximum Fura-2 fluorescence increase following either immune complex or C5a became greater when the extracellular Ca^{2+} was present. The Ca^{2+} influx from the extracellular medium is added to the Ca^{2+} release from intracellular stores. This influx from the extracellular buffer seems to be a requirement for the C5a receptor-mediated cell activation, as no O_2^- production was detected in the absence of extracellular Ca^{2+}. The extracellular calcium is also known to be required for the maximum responses (such as O_2^- production, increase in $[Ca^{2+}]_i$) of neutrophils stimulated with fMLP [14,15]. In contrast, the Ca^{2+} release from intracellular stores is able to induce the O_2^- production through Fc receptors, while the Ca^{2+} influx enhances this response. Although the exact role of Ca^{2+} is unclear, the rise in $[Ca^{2+}]_i$ appears to be an essential intermediatory step in the activation of neutrophils via Fc receptors, as shown in fig.2.

The G-protein inhibitor, pertussis toxin, is reported to strongly inhibit the fMLP-induced O_2^- production and an increase in $[Ca^{2+}]_i$ [4,5]. In agreement with these studies [4,5], the O_2^- production and change in Fura-2 fluorescence of fMLP-stimulated neutrophils were nearly completely inhibited by pertussis toxin (not shown). The inhibitory effect of pertussis toxin on the C5a receptor-mediated change in $[Ca^{2+}]_i$ was found to correlate with the inhibition of O_2^- production. Thus, the C5a receptor is suggested to be coupled to a guanine nucleotide regulating protein, as already known in the case of fMLP receptor. On the other hand, pertussis toxin did not inhibit the immune complex-induced O_2^- production and the rise in $[Ca^{2+}]_i$, suggesting that the IgG Fc receptors are not linked to the pertussis toxin-sensitive G-protein. It is also reported that complete inhibition of the responses to certain chemoattractants (fMLP and platelet activating factor) by pertussis toxin could be observed under the conditions where only slight inhibition of the responses to a lectin, concanavalin A, occurred [16]. Binding of multi-valent concanavalin A or the IgG immune complex, but not their mono-valent derivatives (monovalent concanavalin A nor monomer IgG), to the respective binding sites on the neutrophil surface results in the crosslinking of the sites, which initiates several functional responses of the cells [10,17]. On the other hand, C5a and fMLP, which are monovalent, do not cause the crosslink-

ing of sites. In a variety of neutrophil ligand-receptor systems, it seems likely that the cell responses triggered by multi-valent ligand binding to receptors is not associated with the G-protein system.

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