EFFECT OF THE CHEMOTACTIC PEPTIDE ON THE SUBSEQUENT SUPEROXIDE RELEASING RESPONSE IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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1. Introduction

Chemotactic factors bind to their specific receptors on the cell membrane and stimulate PMN to induce chemotaxis, degranulation, aggregation and superoxide ($O_2^{\cdot-}$) production [1,2]. Furthermore, some functions of PMN are modulated by preincubation with chemotactic factors. PMN preincubated with chemotactic factors lose their ability to directionally migrate in response to the same stimuli (chemotactic deactivation) [3]. The chemotactic deactivation of human PMN induced by preincubation with FMLP are neither accompanied by a loss of binding sites for FMLP nor by a difference in the affinity for its receptors [4]. PMN degranulation is induced by chemotactic factors in the presence of cyt B, and chemotactic factor-induced cell aggregation is enhanced by cyt B. However, the prior exposure of PMN to chemotactic factors before cyt B results in a time-dependent reduction of degranulation and cell aggregation, without altering the binding of cyt B as well as chemotactic factors (desensitization to cyt B) [5–8]. These findings suggest that the alteration of subsequent responsiveness to chemotactic factors or cyt B may arise at a step beyond the binding of these stimuli to the cell membrane.

Human PMN preincubated with FMP release $O_2^{\cdot-}$ in response to the late addition of con A, WGA or cyt B, and both the retention of FMP molecules on the cell surface membrane and the activated state of cells induced by FMP are required for maximum $O_2^{\cdot-}$ release on contact with con A [9]. Similar findings were reported [10,11]. We investigate here the kinetics of the responsiveness of FMP-pretreated PMN to the late addition of con A, WGA or cyt B, and evaluate whether Ca^{2+} actively contributes to the activation process induced by FMP or it only to the maintenance of the activated state induced by FMP.

Abbreviations: con A, concanavalin A; cyt B, cytochalasin B; FMP, *N*-formyl-methionyl-phenylalanine; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; Hepes, *N*-2-hydroxyethyl-piperazine-*N'*-2-ethane sulfonic acid; PMN, polymorphonuclear leukocytes; WGA, wheat germ agglutinin

2. Materials and methods

Cytochalasin B was purchased from Aldrich (Milwaukee WI); con A grade IV, cytochrome c type VI, FMP and superoxide dismutase from Sigma Chemicals (St Louis MO); WGA from E. Y. Labs. (San Mateo CA). Cyt B and FMP were dissolved in dimethylsulfoxide and diluted with Hepes–saline (isotonic saline solution buffered with 5 mM Hepes (pH 7.4)) immediately before use. The final concentration of dimethylsulfoxide in the reaction mixture was <5 μl/ml.

2.1. *Preparation of cells*

PMN were obtained from healthy adult donors by the dextran sedimentation and Conray-Ficoll method as in [9]. PMN preparations were suspended in Hepes–saline, and contained >98% PMN.

2.2. *Determination of PMN $O_2^{\cdot-}$ production*

$O_2^{\cdot-}$ was assayed by the reduction of ferricytochrome *c* spectrophotometrically, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double wavelength spectrophotometer; Hitachi, Tokyo), equipped with thermostatted cuvette holder as in [9,12]. The cell suspension was added to a 1 ml cuvette containing 2 mM glucose and 66 μM ferricyto-

chrome *c* to obtain a final volume of 0.985–0.99 ml. Final cell concentration was $1-2 \times 10^6$/ml. The reaction mixture in a cuvette was preincubated for 3 min at 37°C, and the cuvette was put in a thermostatted cuvette holder (37°C) of a spectrophotometer and the reduction of cytochrome *c* was measured at 550 nm with a reference wavelength at 540 nm. Various stimulating agents (5–10 μl) were added to the reaction mixture in cuvettes to obtain final volume of 1 ml and the desired concentrations of these agents, while the time-course of cytochrome *c* reduction (the absorbance change at 550–540 nm) was followed on the recorder. The final concentrations of stimuli were 20 μM FMP, 5 μg cyt B/ml, 100 μg con A/ml and 100 μg WGA/ml [9]. Where indicated, Ca^{2+} was added as chloride salt in a final concentration of 1 mM. Cytochrome *c* reduction by human PMN stimulated by various surface active agents used here was completely abolished by superoxide dismutase (20 μg/ml), and suggested to be specific for $O_2^{\cdot-}$. The $O_2^{\cdot-}$ production was calculated from cytochrome *c* reduced for 10 min after the addition of cyt B, for 3 min after the addition of con A and for 5 min after the addition of WGA, respectively (fig.1). The values of cytochrome *c* reduced in the resting states were subtracted from those in the stimulated states. In addition, $O_2^{\cdot-}$ production induced by FMP alone was subtracted from that induced by the combination of FMP and another agent (cyt B, con A or WGA) to evaluate the net effect of cyt B, con A or WGA. As the absolute amount of $O_2^{\cdot-}$ production by PMN differed from individual to individual, the representative data of the experiment, which was done in duplicate, are shown. Each experiment was repeated 3–4 times with qualitatively similar results. In these experiments, cell viability by erythrosine B dye exclusion test was >95%. The statistical analysis employed was Student's *t*-test.

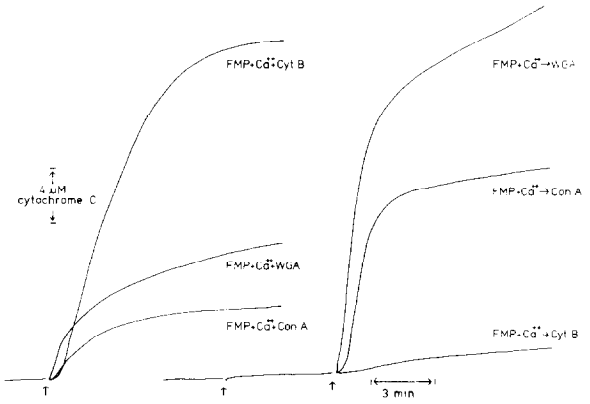
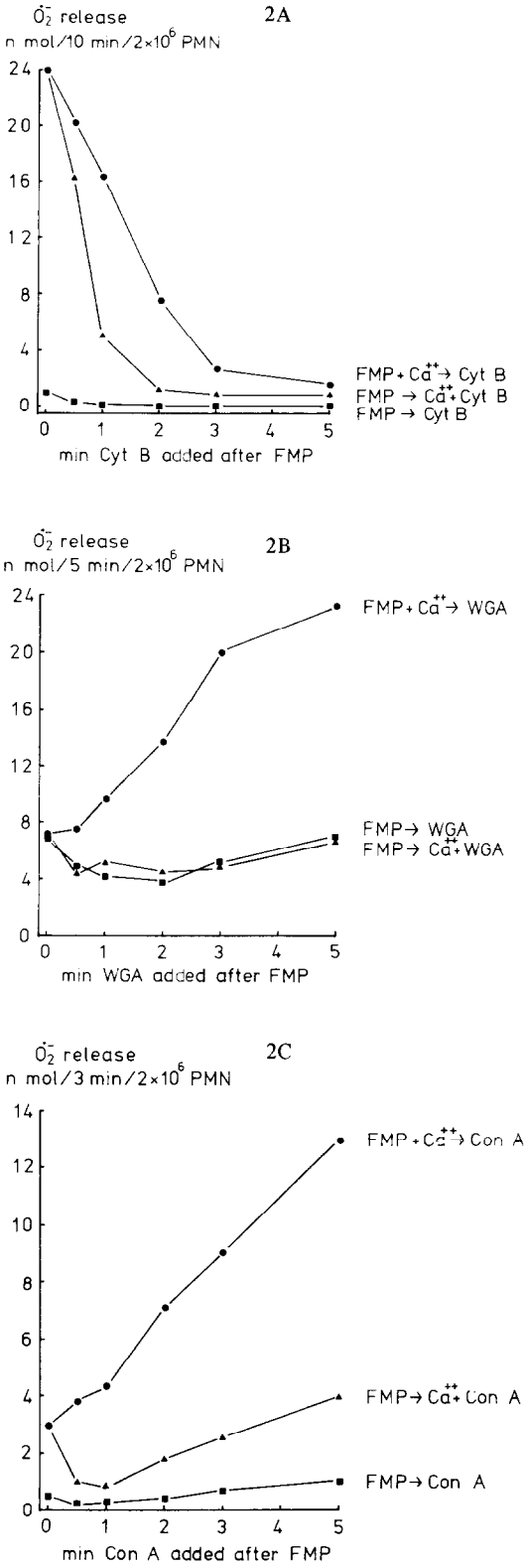


Fig.1. $O_2^{\cdot-}$ production by human PMN stimulated by the simultaneous or sequential addition of stimuli. Cell concentrations used were 2×10^6/ml. Left: 20 μM FMP, 1 mM Ca^{2+} and 5 μg cyt B/ml (100 μg con A/ml or 100 μg WGA/ml) were added simultaneously. Right: Cells were challenged with 20 μM FMP plus 1 mM Ca^{2+}, and 5 min thereafter exposed to 5 μg cyt B/ml (100 μg con A/ml or 100 μg WGA/ml). Arrows (↑) indicate the points of addition.

3. Results and discussion

As shown in fig.1, when PMN were challenged with FMP and 5 min thereafter exposed to cyt B, con A or WGA, remarkable $O_2^{\cdot-}$ release was seen after the addition of cyt B, con A or WGA, and the increase of $O_2^{\cdot-}$ release was significantly larger than the sum of the two increments induced by the two agents separately as in [9]. The enhancement of $O_2^{\cdot-}$ release was also seen even when FMP and another agent (cyt B, con A or WGA) were added simultaneously. However, the time interval between the addition of FMP and another agent was critical to induce the maximum $O_2^{\cdot-}$ production according to the agents used. $O_2^{\cdot-}$ production induced by the sequential addition of FMP and cyt B was much less than that induced by the simultaneous addition of FMP and cyt B, whereas $O_2^{\cdot-}$ production induced by the sequential addition of FMP and con A (or WGA) was much larger than that induced by the simultaneous addition of FMP and con A (or WGA) (fig.1).

To study the kinetics of the responsiveness to the late addition of cyt B, con A or WGA, PMN were preincubated with FMP for various times from 0–5 min, and thereafter exposed to cyt B, con A or WGA. Extracellular Ca^{2+} was added with FMP or added later with another agent (cyt B, con A or WGA) to evaluate the role of Ca^{2+}. As shown in fig.2A, cyt B-induced $O_2^{\cdot-}$ production by PMN preincubated with FMP and Ca^{2+} decreased rapidly in a time-dependent fashion. The reduction of $O_2^{\cdot-}$ releasing response to the late addition of cyt B was more rapid when PMN were preincubated with FMP in the absence of extracellular Ca^{2+}. These findings are similar to the previous works in lysosomal enzyme secretion and cell aggregation induced by the chemotactic factors (FMLP and C5a) and cyt B (time-dependent desensitization to cyt B) [5–8]. When extracellular Ca^{2+} was omitted through-



out the reaction, cyt B-induced O_2^- production by FMP-pretreated PMN was markedly diminished and a rapid loss of responsiveness to cyt B was seen (fig.2A). On the other hand, con A- or WGA-induced O_2^- production by PMN preincubated with FMP in the presence of extracellular Ca^{2+} increased in a time-dependent fashion as the time interval between the addition of FMP and con A (or WGA) was lengthened, whereas PMN preincubated with FMP in the absence of extracellular Ca^{2+} showed the initial reduction of responsiveness to the late addition of con A (or WGA) plus Ca^{2+}, which was followed by the time-dependent increase (or recovery) of the responsiveness (fig.2B,C). When extracellular Ca^{2+} was omitted throughout the reaction, the responsiveness to the late addition of con A was markedly diminished, whereas statistically no significant difference was seen in the responsiveness to WGA whether or not Ca^{2+} was added with WGA. It is unlikely that the time-dependent increase of O_2^- production in response to the late addition of con A or WGA may reflect the number of FMP molecules on the cell membrane, since the binding of chemotactic peptides to their receptors on human PMN is rapid with a $t_{1/2} < 2$ min at 37°C [1]. In addition, the binding of chemotactic peptides to their receptors is not affected by the presence or absence of extracellular Ca^{2+} [13]. FMLP had been shown to transiently increase the steady-state level of cell-associated $^{45}Ca^{2+}$ in the presence of extracellular Ca^{2+}, whereas it transiently decreased the steady-state level of cell-associated $^{45}Ca^{2+}$ in the absence of extracellular Ca^{2+} [14]. These findings and the fact that the initial reduction was reversed by adding extracellular Ca^{2+}, suggest that the initial reduction of O_2^- production may be associated with the initial decrease of exchangeable Ca^{2+} of

Fig.2. Effect of preincubation time with FMP on (A) Cyt B-, (B) Con A- or (C) WGA-induced O_2^- production by human PMN. (●) FMP + Ca^{2+} → cyt B (con A or WGA); PMN were challenged with 20 μM FMP plus 1 mM Ca^{2+}, and at the indicated times thereafter exposed to 5 μg cyt B/ml (100 μg con A/ml or 100 μg WGA/ml). (▲) FMP → Ca^{2+} + cyt B (con A or WGA); PMN were challenged with 20 μM FMP in the absence of extracellular Ca^{2+}, and at the indicated times thereafter exposed to 1 mM Ca^{2+} plus 5 μg cyt B/ml (100 μg con A/ml or 100 μg WGA/ml). (■) FMP → cyt B (con A or WGA); PMN were challenged with 20 μM FMP, and at the indicated times thereafter exposed to 5 μg cyt B/ml (100 μg con A/ml or 100 μg WGA/ml). Ca^{2+} was omitted throughout the reaction.

cells preincubated with FMP in the absence of extracellular Ca^{2+} and that Ca^{2+} may actively contribute to the activation process by FMP rather than the maintenance of the activated state induced by FMP [9].

As described in [9], the enhancing effect of FMP is almost, but not completely, abolished when FMP molecules are washed out from the cell surface membrane after preincubation with FMP for 5 min at 37°C, suggesting that the enhancement of $O_2^{\cdot-}$ production may result from the interaction between FMP–receptor complexes and another ligand–receptor complexes. Chemotactic factors activate many metabolisms of PMN including the metabolism of membrane phospholipids [15,16]. It is conceivable that FMP-induced changes of cell membrane may affect the interaction between FMP–receptor complexes and another ligand (cyt B, con A or WGA)–receptor complexes on the cell membrane.

These experiments have shown that the subsequent $O_2^{\cdot-}$ releasing response of human PMN to the late addition of cyt B, con A or WGA is modulated by the prior exposure to a chemotactic peptide (FMP) and that the modulating effect is rapid and influenced by extracellular Ca^{2+}. The time-dependent loss of responsiveness to cyt B (desensitization to cyt B) do not imply a general functional hypo-responsiveness of FMP-pretreated PMN, since the same cells release remarkable $O_2^{\cdot-}$ in response to con A or WGA. Therefore, these cells are rather activated [9,17]. It is possible that the same metabolic events induced by FMP may be responsible not only for the increased responsiveness to con A or WGA but also for the decreased responsiveness to cyt B.

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References

[1] Williams, L. T., Snyderman, R., Pike, M. C. and Lefkowitz, R. J. (1977) Proc. Natl. Acad. Sci. USA 74, 1204–1208.
[2] Aswanikumar, S., Corcoran, B. A., Schiffmann, E., Day, A. L., Freer, R. J., Showell, H. J., Becker, E. L. and Pert, C. B. (1977) Biochem. Biophys. Res. Commun. 74, 210–217.
[3] Ward, P. A. and Becker, E. L. (1968) J. Exp. Med. 127, 693–709.
[4] Abita, J. P. and Morgat, J. L. (1980) FEBS Lett. 111, 14–18.
[5] Henson, P. M., Zanolari, B., Schwartzman, N. A. and Hong, S. R. (1978) J. Immunol. 121, 851–855.
[6] Showell, H. J., Williams, D., Becker, E. L., Naccache, P. H. and Sha'afi, R. I. (1979) J. Reticuloendothel. Soc. 25, 139–150.
[7] Naccache, P. H., Showell, H. J., Becker, E. L. and Sha'afi, R. I. (1979) J. Cell. Physiol. 100, 239–250.
[8] O'Flaherty, J. T., Kreutzer, D. L. and Ward, P. A. (1979) Exp. Cell Res. 120, 31–37.
[9] Kitagawa, S., Takaku, F. and Sakamoto, S. (1980) J. Immunol. 125, 359–364.
[10] Issekutz, A. C., Lee, K. and Biggar, W. D. (1979) Infect. Immun. 24, 295–301.
[11] Van Epps, D. E. and Garcia, M. L. (1980) J. Clin. Invest. 66, 167–175.
[12] Kitagawa, S., Takaku, F. and Sakamoto, S. (1980) J. Clin. Invest. 65, 74–81.
[13] Schiffmann, E., Aswanikumar, S., Venkatasubramanian, K., Corcoran, B. A., Pert, C. B., Brown, J., Gross, E., Day, A. R., Freer, R. J., Showell, A. H. and Becker, E. L. (1980) FEBS Lett. 117, 1–7.
[14] Petroski, R. J., Naccache, P. H., Becker, E. L. and Sha'afi, R. I. (1979) Am. J. Physiol. 237, C43–C49.
[15] Hirata, F., Corcoran, B. A. Venkatasubramanian, K., Schiffmann, E. and Axelrod, J. (1979) Proc. Natl. Acad. Sci. USA 76, 2640–2643.
[16] Snijderman, R., Pike, M. C. and Kredich, N. M. (1980) Mol. Immunol. 17, 209–218.
[17] Fehr, J. and Dahinden, C. (1979) J. Clin. Invest. 64, 8–16.