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STIMULATION OF THE HUMAN NEUTROPHIL SUPEROXIDE ANION-GENERATING SYSTEM WITH 1-*O*-HEXADECYL/OCTADECYL-2-ACETYL-SN-GLYCERYL-3-PHOSPHORYCHOLINE

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Abstract—1-*O*-Hexadecyl/octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AGEPC) stimulated a time- and concentration-dependent generation of superoxide anion (O_2^-) by human neutrophils. Maximum production of O_2^- occurred 60 sec subsequent to cell contact with AGEPC. Superoxide generation was reduced significantly if cells were not preincubated with cytochalasin B prior to exposure to AGEPC (0.01 to 10 μM). A time-dependent desensitization for O_2^- production was demonstrated in neutrophilis which were stimulated with AGEPC prior to contact with cytochalasin B. The rate and amount of O_2^- generated by AGEPC-activated neutrophils were enhanced significantly in the presence of extracellular calcium. However, incubation of neutrophils with ethyleneglycol-bis(β-amino-ethyl ether) *N*,*N*'-tetra-acetate (EGTA) in calcium-free medium had no effect on the O_2^--generating system. AGEPC-induced O_2^- production was suppressed by the glycolytic inhibitor 2-deoxy-D-glucose (2-DG) and the sulfhydryl reagents *N*-ethylmaleimide (NEM) and iodoacetic acid (1A). Sodium cyanide was inactive. Pretreatment of neutrophils with AGEPC reduced cell responsiveness to subsequent exposure to this stimulus. Desensitization of the O_2^--generating system activated with AGEPC appears to be stimulus specific for leukotriene B_4 (LTB_4) and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) in that these stimuli were capable of inducing O_2^- production by cells pretreated with AGEPC. However, neutrophils pretreated with AGEPC were desensitized to zymosan-activated serum (ZAS).

1-*O*-Hexadecyl/octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine (AGEPC, Platelet-Activating Factor), a mixture of acetyl glyceryl ether phosphoglycerides [1–3], is a lipid-derived mediator of systemic anaphylactic reactions which has been demonstrated to induce acute neutropenia and thrombocytopenia [4, 5]. AGEPC is released from a variety of cell types [6], including neutrophils [6, 7], and has been reported to stimulate human neutrophil aggregation [8], migration [8], adherence [9], and degranulation [10–12]. These observations, taken together with the recent findings of Humphrey *et al.* [13] whereby AGEPC induced increased vascular permeability in guinea pigs, suggest that AGEPC may function as a mediator of certain inflammatory reactions.

The purpose of this study was to further evaluate the effects of AGEPC on human neutrophils. In that the oxygen-derived free radicals generated by neutrophils have been implicated as putative mediators of the tissue injury associated with the inflammatory process [14], we investigated the capacity of AGEPC to stimulate the superoxide anion (O_2^-)-generating system of human neutrophils.

MATERIALS AND METHODS

Preparation of human neutrophils. Blood from normal human donors was drawn by venipuncture into 0.1 vol. of 3.8% citrate in conical plastic tubes. Neutrophils were purified employing standard techniques of dextran sedimentation, centrifugation on Ficoll/Hypaque, and hypotonic lysis. Final cell suspensions contained a minimum of 98% neutrophils, and viability of the neutrophils was always greater than 98% as determined by trypan blue exclusion.

Incubation conditions. Neutrophils (5×10^6) in 2 ml of phosphate-buffered saline (PBS), pH 7.4 containing 138 mM NaCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 0.1% glucose were incubated according to the various procedures described under Results. The calcium- and magnesium-free PBS utilized in several studies contained 2 and 3 parts/billion of contaminating calcium and magnesium, respectively, as determined by atomic absorption. These concentrations of calcium and magnesium have no effect on the models employed in these studies. After incubation the samples were centrifuged at 750 *g* (4°) for 3 min, and the clear supernatant fractions were assayed for superoxide anion.

Superoxide generation assay. Superoxide anion production by resting and stimulated human neutrophils was determined via the spectrophotometric assay of superoxide dismutase (SOD) inhibitable reduction of ferricytochrome *c* previously described [15, 16].

Enzyme assay. Lactate dehydrogenase (LDH) (EC 1.1.1.27) activity was determined as previously described [17].

Preparation of zymosan-activated serum (ZAS). ZAS was prepared by adding zymosan to freshly prepared human serum (2 mg/ml) containing 250 μM

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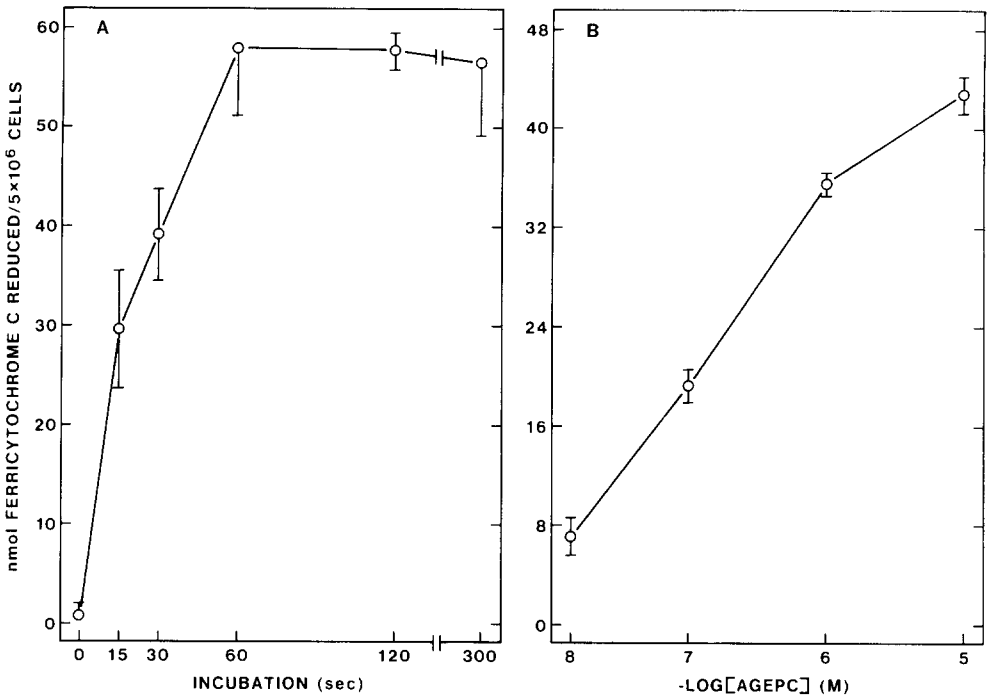

Fig. 1. Superoxide anion generation by human neutrophils exposed to AGEPC versus (A) time of incubation and (B) concentration of AGEPC. Neutrophils (5×10^6) were preincubated with cytochalasin B (5 μg/ml) for 10 min at 37° followed by exposure to AGEPC (1 μM) for the time periods indicated. Neutrophils were also incubated with the stipulated concentrations of AGEPC for 5 min. The spontaneous generation of O_2^- in buffer alone was: 5.2 ± 0.2 nmoles ferricytochrome *c* reduced/5×10^6 cells. Data represent the mean ± S.E.M. of three experiments.

epsilon-amino-*n*-caproic acid. Following 30 min of incubation at 37°, the serum was rendered free of zymosan particles by centrifugation at 3000 *g* (4°) for 10 min. ZAS was added to the reaction mixtures in a final concentration of 25% (v/v).

Source and preparation of reagents. AGEPC (Calbiochem-Behring, La Jolla, CA) was dissolved in 0.15 M NaCl containing 2.5 mg/ml bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Leukotriene B_4 (prepared by Dr. F. H. Lincoln of The Upjohn Co.) and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (Sigma) were prepared in methanol and dimethyl sulfoxide (DMSO) respectively. Cytochalasin B (Aldrich Chemical Co., Milwaukee, WI) was dissolved in ethanol. Iodoacetic acid and 2-deoxy-D-glucose (Sigma) were prepared in PBS. *N*-Ethylmaleimide (Sigma) and sodium cyanide (J. T. Baker Chemical Co., Phillipsburg, NJ) were prepared in ethanol and PBS respectively. Zymosan and epsilon-amino-*n*-caproic acid were obtained from Sigma. All compounds were soluble under the defined incubation conditions, and they produced no alteration in pH of the medium. The small amounts of DMSO, ethanol, and methanol (final concentration of 0.05%) employed as vehicle did not affect cell viability or superoxide anion generation.

RESULTS

AGEPC-stimulated superoxide anion generation by human neutrophils versus time of incubation and concentration of AGEPC. AGEPC induced a time- and concentration-dependent generation of O_2^- by cytochalasin B-treatment human neutrophils (Fig. 1). Superoxide production was rapid ($T_{\frac{1}{2}} \simeq 30$ sec) with maximum generation demonstrated with 10 μM AGEPC. Neutrophils pretreated with cytochalasin B generated 34.2 ± 5.5 nmoles of $O_2^-/5 \times 10^6$ cells when exposed to AGEPC. However, neutrophils incubated with AGEPC in the absence of cytochalasin B produced 10.8 ± 1.0 nmoles of O_2^-.

There was no significant release of cytoplasmic LDH (<7% of total activity discharged with 10 μM AGEPC) during the entire incubation period, which indicates that AGEPC activation of the O_2^--generating system is a selective, non-cytotoxic process.

Effect of the time of addition of cytochalasin B on AGEPC-induced superoxide anion generation by human neutrophils. The data in Fig. 2 demonstrate that maximum O_2^- production by AGPEC-stimulated neutrophils was achieved when cells were exposed to cytochalasin B 5 min prior to or simultaneously with AGEPC. Submaximal O_2^- generation was observed when neutrophils were exposed to cytochalasin B for 10–30 min prior to contact with stimulus, and a significant reduction in O_2^- production occurred when cytochalasin B was added at various time periods after AGEPC (Fig. 2).

Influence of divalent cations and ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetra-acetate (EGTA) on superoxide anion production by AGEPC-treated human neutrophils. Cytochalasin B-treated neutro-

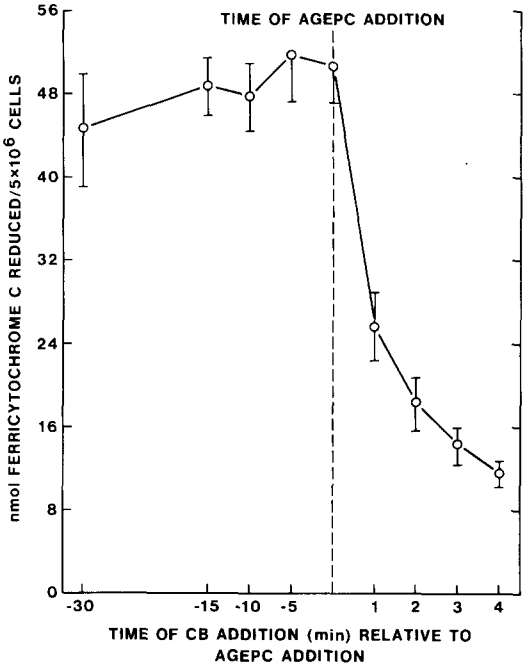

Fig. 2. Effect of the time of addition of cytochalasin B on AGEPC-induced superoxide anion production by human neutrophils. Neutrophils (5×10^6) were incubated with cytochalasin B (5 μg/ml) before (−30, −15, −10, or −5 min), simultaneously with, or after the addition of AGEPC (1 μM). Total incubation time was 35 min. The spontaneous generation of O_2^- in buffer alone was: 3.2 ± 0.9 nmoles ferricytochrome *c* reduced/5×10^6 cells. Data represent the mean ± S.E.M. of three experiments.

phils in calcium- and magnesium-free PBS reduced 17.7 ± 2.1 nmoles of ferricytochrome *c* when exposed to AGEPC as compared to 35.0 ± 5.7 nmoles of ferricytochrome *c* reduced in the presence of both divalent actions (Table 1). Neutrophil contact with AGEPC in PBS containing only calcium resulted in a level of O_2^- generation which equaled that observed when calcium and magnesium were both present. The enhancement of O_2^- production by extracellular calcium was concentration-dependent in that 12.2 ± 1.0, 17.4 ± 3.9, 24.2 ± 2.1, 31.2 ± 6.8, and 43.9 ± 2.0 nmoles of ferricytochrome *c* were reduced by AGEPC-treated neutrophils in the presence of 0, 0.15, 0.3, 0.6 and 1.2 mM extracellular calcium, respectively. In the presence of magnesium but not calcium, the quantity of O_2^- generated was essentially no different than that seen when both divalent cations were excluded from the incubation medium.

Incubation of neutrophils with EGTA prior to treatment with cytochalasin B had no effect on AGEPC-induced O_2^- production (Table 1). In addition, O_2^- generation was not attenuated if EGTA was present during the entire preincubation and incubation periods.

Effect of metabolic inhibitors on AGEPC-elicited superoxide anion generation by human neutrophils. The glycoytic inhibitor 2-deoxy-D-glucose (2-DG) suppressed O_2 production by cytochalasin B-treated neutrophils exposed to AGEPC (Table 2). Further, 2-DG was more effective in the absence of glucose. The sulfhydryl reagents *N*-ethylmaleimide (NEM) and iodoacetic acid (1A) also inhibited AGEPC-induced O_2^- generation. Sodium cyanide, an uncoupler of oxidative phosphorylation, had no effect on O_2^- production by AGEPC-stimulated neutrophils (Table 2).

Desensitization and cross desensitization of the human neutrophil superoxide anion-generating response. The data in Table 3 indicate that neutrophils pretreated with AGEPC were essentially unresponsive (desensitized) to a subsequent exposure to AGEPC with respect to O_2^- generation. Desensitization was also demonstrated with LTB_4, FMLP, and ZAS. Neutrophils pretreated with FMLP were significantly less responsive to the effect of LTB_4, whereas prior exposure to LTB_4 did not affect

Table 1. Effects of divalent cations on AGEPC-elicited superoxide anion generation by human neutrophils

Experimental condition*	O_2^- generation (nmoles ferricytochrome *c* reduced/5 min/5×10^6 cells)
No divalent cations	17.7 ± 2.1†
0.6 mM Ca^{2+} + 1.0 mM Mg^{2+}	35.0 ± 5.7
0.6 mM Ca^{2+}	37.4 ± 6.9
1.0 mM Mg^{2+}	18.6 ± 2.7
Control‡	25.0 ± 1.8
EGTA (2 mM)	23.6 ± 2.8

* Neutrophils (5×10^6) were preincubated with cytochalasin B (5 μg/ml) for 10 min in PBS with or without divalent cations. The cells were further incubated for 5 min with AGEPC (1 μM). The spontaneous generation of O_2^- in buffer alone was 4.2 ± 1.5 nmoles ferricytochrome *c* reduced per 5 min per 5×10^6 cells.

† Data represent the mean ± S.E.M. of two to three experiments.

‡ Neutrophils (5×10^6) were preincubated with or without EGTA for 10 min in calcium-free PBS. The cells were subsequently washed and incubated with cytochalasin B (5 μg/ml) for 10 min followed by a 5-min incubation with AGEPC (1 μM).

Table 2. Effects of metabolic inhibitors on AGEPC-elicited superoxide anion generation by human neutrophils

Agent*	Glucose (5 mM)	O_2^- generation† (nmoles ferricytochrome *c* reduced/1 min/5 × 10^6 cells)
Control	+	33.9 ± 0.4‡
	−	31.4 ± 7.8
2-deoxy-D-glucose (5 mM)	+	21.2 ± 3.2 (37)§
	−	10.6 ± 2.7 (66.1)
Iodoacetic acid (0.1 mM)	+	12.8 ± 3.5 (54.4)
N-ethylmaleimide (0.005 mM)	+	3.6 ± 2.8 (89.6)
Sodium cyanide (0.25 mM)	+	30.1 ± 1.9 (10.6)

* Neutrophils (5 × 10^6) were preincubated with cytochalasin B (5 μg/ml) for 10 min in the presence and absence of the respective metabolic inhibitors. Experiments involving 2-DG were performed in the presence and absence of glucose. The cells were then incubated with AGEPC (1 μM) for 1 min.

† The spontaneous generation of O_2^- in buffer alone was: 3.9 ± 0.6 nmoles ferricytochrome *c* reduced per 1 min per 5 × 10^6 cells.

‡ Data represent the mean ± S.E.M. of two to three experiments.

§ Numbers in parentheses indicate the percent inhibition of O_2^- production.

the capacity of neutrophils to express a typical O_2^--generating response to FMLP. In addition, cells exposed to ZAS were desensitized by pretreatment with AGEPC, FMLP, and LTB_4. However, cells exposed to the three aforementioned stimuli were not desensitized by pretreatment with ZAS (Table 3). Thus, cross desensitization was not observed with these stimuli.

Table 3. Desensitization and cross desensitization of the human neutrophil superoxide anion generation response by AGEPC and other stimuli

Desensitizing agent*†	Stimulus*†	O_2^- generation‡ (nmoles ferricytochrome *c* reduced/5 × 10^6 cells)
	AGEPC	16.0 ± 1.9§
	FMLP	73.6 ± 7.4
	LTB_4	15.2 ± 2.5
	ZAS	32.7 ± 5.2
AGEPC	AGEPC	3.0 ± 1.9
FMLP	FMLP	7.5 ± 3.2
LTB_4	LTB_4	3.5 ± 0.8
ZAS	ZAS	17.3 ± 5.0
FMLP	AGEPC	17.5 ± 1.2
AGEPC	FMLP	75.9 ± 11.6
LTB_4	AGEPC	32.5 ± 7.2
AGEPC	LTB_4	15.3 ± 2.7
ZAS	AGEPC	21.9 ± 5.0
AGEPC	ZAS	17.9 ± 3.3
LTB_4	FMLP	65.3 ± 13.9
FMLP	LTB_4	5.3 ± 2.2
ZAS	FMLP	119.4 ± 6.9
FMLP	ZAS	19.9 ± 5.8
ZAS	LTB_4	26.0 ± 2.8
LTB_4	ZAS	23.0 ± 4.0

* Neutrophils (5 × 10^6) were preincubated with DMSO (5 min), methanol (15 min), BSA-saline (5 min), normal serum (10 min), AGEPC (5 min), FMLP (5 min), LTB_4 (15 min) or ZAS (10 min). Cells were subsequently washed and incubated with cytochalsin B (5 μg/ml) for 10 min followed by exposure to the respective stimuli for the aforementioned time periods.

† The desensitizing and activating concentrations of the respective stimuli were: AGEPC (1 μM), FMLP (0.05 μM), LTB_4 (1 μM), ZAS (25%, v/v). However, in studies in which cells were exposed to a second addition of FMLP, the desensitizing concentration of FMLP was 100 μM.

‡ Spontaneous generation of O_2^- in buffer alone was: 3.1 ± 0.5 nmoles ferricytochrome *c* reduced/5 × 10^6 cells.

§ Data represent the mean ± S.E.M. of three to five experiments.

DISCUSSION

The data reported here indicate that AGEPC induces the generation of O_2^- by human neutrophils. Superoxide anion production was rapid, with maximum production occurring 60 sec following cell exposure to AGEPC. The rate of O_2^- generation correlates with the kinetics of AGEPC-induced granule exocytosis [8, 12]. The EC_{50} (concentration eliciting 50% of maximal O_2^- generation) for AGEPC is approximately 0.13 μM.

Superoxide anion production by AGEPC-stimulated neutrophils was reduced significantly, but not abolished, if cells were not pretreated with cytochalasin B prior to exposure to stimulus. Similar observations were reported for pepstatin A-elicited O_2^- generation [18] as well as H_2O_2 production by FMLP and phorbol myristate acetate (PMA)-treated neutrophils [19]. Further, optimal O_2^- production was observed when neutrophils were exposed to cytochalasin B 5 min prior to or simultaneously with AGEPC whereas the addition of cytochalasin B before (10–30 min) or after AGEPC resulted in a marginal and marked decrease, respectively, in O_2^- generation. Similar data were obtained with O_2^- production and degranulation elicited with various stimuli [12, 20, 21]. It would appear that contact between neutrophils and a stimulus such as AGEPC before addition of cytochalasin B elicits a state of unresponsiveness or desensitization, the nature of which is unknown at the present time. However, the marked enhancement of O_2^- generation which was observed when AGEPC-treated neutrophils were exposed to cytochalasin B may, in part, reflect an ability of cytochalasin B to elicit a surface recognition response in that neutrophils adhering to a surface will undergo granule exocytosis when exposed to C5a in the absence of cytochalasin B [22]. C5a has a requirement for cytochalasin B when exposed to neutrophils in suspension [21].

The capacity of AGEPC to activate the neutrophil O_2^--generating system was enhanced significantly in the presence of extracellular calcium, a characteristic common to other stimuli [18, 23]. In addition, the quantity of O_2^- produced was unaffected by pretreating cells with EGTA prior to contact with AGEPC. Therefore, the generation of O_2^- by AGEPC and other stimuli in the absence of extracellular calcium may, as we have suggested previously [20, 23, 24], indicate that the availability of intracellular as opposed to extracellular calcium might be a rate-limiting component in the generation of O_2^- and other reactive oxygen species [19] by human neutrophils.

In addition to the data described here, reports from other laboratories have demonstrated the expression of an oxidative burst by neutrophils [8, 9] and other cell types [25–27] exposed to AGEPC. We report here that an investigation of the metabolic requirements for human neutrophil stimulation with AGEPC revealed suppression of O_2^- generation by the glycolytic inhibitor 2-DG. Further, AGEPC-elicited O_2^- production was inhibited by the sulfhydryl inhibitors NEM and 1A, indicating a requirement for free sulfhydryl groups. The cyanide-insensitive aspect of several neutrophil activities [12] is reflected in our finding that sodium cyanide had no effect on AGEPC-induced O_2^- generation.

Pretreatment of neutrophils with AGEPC rendered the cell unresponsive to subsequent exposure to this stimulus. The attenuated capacity of neutrophils to generate O_2^- in response to a second addition of stimulus is referred to as stimulus desensitization and has been demonstrated with other stimuli [20, 21]. These observations could be explained on the basis of "down regulation" of specific receptor populations [28]. According to this mechanism of ligand-elicited receptor modulation, exposure of cells to various ligands results in a decrease in the number of available receptors for a given ligand. Applying this concept to neutrophil-associated receptor–ligand interactions, Sullivan and Zigmond [29] reported that contact between neutrophils and the chemotactic oligopeptide *N*-formylnorleucylleucylphenylalanine eventuated in a decrease in the number of receptors for this ligand on the cell surface. Consistent with these observations we suggest that, following an initial exposure of neutrophils to AGEPC, the number of available membrane receptors/determinants is diminished, resulting in a decreased O_2^--generating response by neutrophils to a second addition of AGEPC. This decrease in receptor number (desensitization) may entail internalization with subsequent degradation of receptor–ligand complexes [30], interaction of neutrophils with reactive oxygen species generated during cell contact with various stimuli [31], or shedding of complexes from the cell surface. We have also demonstrated desensitization with the chemotactic tripeptide FMLP, zymosan-activated serum (ZAS, which contains the neutrophil activator C5a), and LTB_4, a 5-lipoxygenase product of arachidonic acid metabolism which stimulates various neutrophil functions [32]. Further, the finding that cross desensitization does not occur with these four stimuli indicates that they interact with different membrane receptors/determinants on/in the neutrophil. In this regard, specific binding sites/receptors for FMLP [33], LTB_4 [34], AGEPC [35], and C5a [36] have been identified on human neutrophils. Alternatively, this selectivity may indicate that there are subpopulations of neutrophils each possessing receptors with a specificity for a given ligand. Consistent with this hypothesis is the recent identification of neutrophil subpopulations utilizing monoclonal antibodies [37].

The stimulus specific nature of AGEPC-elicited desensitization of O_2^- generation is illustrated by the observation that cells pretreated with a desensitizing concentration of AGEPC are fully capable of eliciting a typical O_2^- generating response to FMLP and LTB_4. It is interesting to note that, while pretreatment of cells with LTB_4 has no effect on the cell response to FMLP, prior exposure of neutrophils to FMLP desensitized the cells to LTB_4. Although not an example of cross desensitization, a similar finding was reported by Showell *et al.* [38] working with rabbit neutrophils. We have also observed that pretreatment of cells with AGEPC, FMLP or LTB_4 desensitized the neutrophils to ZAS. However, prior exposure of cells to ZAS resulted in an enhanced O_2^--generating response of neutrophils to AGEPC,

FMLP and LTB_4. There is as yet no explanation for these observations.

We have described the capacity of AGEPC, a putative mediator of inflammation, to activate the human neutrophil O_2^--generating system. Stimulation of this neutrophil function with AGEPC is an energy-requiring and intercellular calcium-dependent process. Because evidence is accumulating which suggests that the reactive oxygen species generated by neutrophils are associated with neutrophil-mediated tissue injury, AGEPC represents a relevant and useful tool for investigating neutrophil function.

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