A Possible Role of Arachidonic Acid in Human Neutrophil Aggregation and Degranulation

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Chemotactic factors stimulate neutrophils to aggregate and, in the presence of cytochalasin B, to degranulate. Recently, the authors found that arachidonic acid also stimulates human neutrophils to aggregate but does not stimulate cytochalasin-B–treated or untreated cells to degranulate. In this report the authors examined the effect of three blockers of arachidonic acid metabolism on these cellular responses. It was found that the arachidonic acid analog 5,8,11,14-eicosatetraynoic acid and indomethacin, but not aspirin, inhibited not only the arachidonic-acid–induced aggregation response but also the degranulation responses evoked by C5a or a synthetic oligopeptide chemotactic factor. These results suggest that arachidonic acid may be a precursor of bioactive metabolites that stimulate the aggregation and foster the degranulation responses of neutrophils. Thus, these metabolites may be mediators of neutrophil function. Agents that block their formation may thereby inhibit aggregation and degranulation. (Am J Pathol 96:799–810, 1979)

IN STIMULATING polymorphonuclear neutrophils (PMNs), many chemotactic agents first interact with stereospecific receptors: C5a, for instance, binds to one receptor, whereas oligopeptide chemotactins such as formyl-methionyl-leucyl-phenylalanine (FMLP) bind to a quite different receptor.[1-6] As a consequence of this binding, PMNs migrate, degranulate, and aggregate. It seems reasonable to presume that these PMN responses are directed by intracellular mediators that transduce chemotactic factor–receptor binding into cell function.

In response to thrombotic stimuli such as thrombin, adenosine diphosphate (ADP), and epinephrine, platelets aggregate. One particular fatty acid, 5,8,11,14-eicosatetraenoic acid (arachidonic acid) also stimulates this platelet response.[7-9] The analog 5,8,11,14-eicosatetraynoic acid (ETYA), indomethacin, and aspirin (ASA) block cellular arachidonic acid metabolism.[7,8,10-13] Each of these blockers inhibits the platelet response not only to arachidonic acid but also to thrombotic stimuli.[7-13] These results suggest, and further studies have confirmed,[14,15] that metabolic derivatives of arachidonic acid can stimulate platelet function and are involved in the platelet response to thrombotic stimuli. Exogenous arachidonic acid itself

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is the precursor of these metabolites when platelets are exposed to arachidonic acid; endogenous arachidonic acid is the precursor when these cells are exposed to thrombotic stimuli. In either event these bioactive derivatives appear to be intracellularly formed mediators of the platelet response.

Recently we found that arachidonic acid can also stimulate human PMNs to aggregate and that this action is blocked by ETYA.[16] We further studied this phenomenon and found that ETYA and indomethacin but not ASA inhibit not only this response but also the aggregation and degranulation response of PMNs to C5a and FMLP. These results suggest that metabolic derivatives of arachidonic acid may be intracellular mediators of the PMN response to chemotactins.

Materials and Methods

Reagents and Buffers

Butylated hydroxytoluene (BHT), ADP, indomethacin, and ASA were purchased from Sigma Chemical Company, St. Louis, Missouri. Epinephrine was purchased from Elkins-Sinn, Inc., Cherry Hill, New Jersey. Purified human thrombin was a generous gift of Dr. Leon Hoyer, Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut. Arachidonic acid, arachidic acid, 11,14,17-eicosatrienoic acid, and 8,11,14-eicosatrienoic acid were purchaesd from Nu-Chek Prep, Elysian, Minnesota; ETYA was a gift of Dr. W. E. Scott, Hoffman-La Roche, Inc., Nutley, New Jersey. Each fatty acid was the sodium salt and greater than 99% pure as assayed by thin-layer chromatography. These acids were dissolved (100 mM) in dimethyl sulfoxide (DMSO) containing 1.4×10^{-4} M BHT and stored at −70 C under nitrogen. Idomethacin and cytochalasin B (Aldrich Chemical Co., Milwaukee, Wisconsin) were also dissolved in DMSO and stored at −70 C. ASA was prepared fresh in DMSO or buffer before each experiment. The FMLP was obtained and used as previously described [4,17] and stored (1 mM) in DMSO at 4 C. The C5a was isolated from zymosan-activated normal human serums that had been pretreated with 1 M ϵ-aminocaproic acid. Purification procedures employed sequential column chromatography with CM-cellulose, Sephadex G-100, CM-cellulose C32, and CM-Sephadex C25 as described by Fernandez and Hugli.[18] The final reagent was dissolved in 5 mM phosphate-buffered (pH 7.2) 0.145 M saline and had chemotactic, degranulating, and aggregating activities when diluted 500-fold. A volume of 2 μl of this preparation per 1 ml of PMN suspension corresponded to 0.8 μg protein per ml and a calculated C5a concentration of 8×10^{-8} M. In the concentrations used here, neither the BHT (4.2×10^{-6} M or less), DMSO (0.1%, or less), nor the phosphate buffer (4.0% or less) influenced PMN function or viability. The cell-suspending buffer used for these studies was a previously described Hanks' balanced salt solution.[17]

Neutrophils

Blood from normal human donors who had not ingested ASA, indomethacin, nor other anti-inflammatory agents for 10 days was layered over Ficoll-Hypaque discontinuous gradients to obtain leukocyte populations containing greater than 97% PMNs and fewer than 2 platelets per 100 leukocytes.[6,17]

Degranulation Assay

Human PMN suspensions (2600 cells/μl) were preincubated for 5 minutes with 5 μg/ml cytochalasin B and, where indicated, an inhibitor of arachidonic acid metabolism before incubation with a degranulating substance for another 5 minutes. The supernatant fluid of the PMN suspensions was then assayed for lysozyme, β-glucuronidase, and lactic acid dehydrogenase, as previously described.[4,19] In some experiments, the supernatant fluids of PMN suspensions studied in the aggregation assay (see below) were also examined for these enzyme activities.

Aggregation Assay

As was done in the degranulation assay, all reagents were made 37 C and pH 7.4 before use, and all experiments were performed at 37 C. A volume of a PMN suspension (4,600 cells/μl) was placed in a plastic vial and stirred continuously with a magnetic bar. After 4 minutes the suspension was made 1.4 mM Ca^{2+} and 0.7 mM Mg^{2+} by the addition of the chloride salts. One minute thereafter, a small volume of an aggregating substance was added to the suspension. Where indicated, an inhibitor was added at a specified time during this sequence. Just before and at ¼, ½, 1, 2, 4, 8, and 15 minutes after adding an aggregating substance to the suspension, 20-μl samples were obtained and immediately examined for total particle and large particle concentration with a model ZBI Coulter Counter, as previously described.[17,19] Large particles were particles greater than 1.8 times the (volume) size of the unaggregated PMN. The results are reported as the large particle percentage (LPP) or the maximal change in the LPP (MLPP). The LPP is 100 times the large particle concentration divided by the total particle concentration; the MLPP is the highest LLP found at ¼, ½, 1, 2, 4, 8, or 15 minutes after adding an indicated substance to the PMN suspension minus the LPP found just before this addition.

Cell Toxicity

In order to determine whether the reagents used here were toxic to the PMNs, the supernatant fluids of cell suspensions studied in the degranulation assays or in the aggregation assays were examined for lactate dehydrogenase activity. None of the reagents in the concentrations and combinations used here induced significant increases in the lactic acid dehydrogenase activity of these fluids, even when they were incubated with the cells for 20 minutes.

Results

Aggregation

As reported previously, chemotactic substances [3,17,19] and arachidonic acid [16] aggregated the PMNs. Text-figure 1 shows this effect: 5×10^{-7} M FMLP, 2 μl/ml C5a, and 10^{-5} M arachidonic acid rapidly induced transient rises in the LPP of PMN suspensions. We have previously shown that these changes in the LPP reflect the formation of two- and three-celled PMN aggregates but not the cellular swelling which is induced by chemotactic substances.[17] Dose-response curves for FMLP showed that aggregation was not detected below 1.7×10^{-8} M, increased incrementally with increases in FMLP concentrations between 1.7×10^{-8} M and 5×10^{-7} M, and plateaued above 5×10^{-7} M.[3,17,19] The C5a reagent used was much

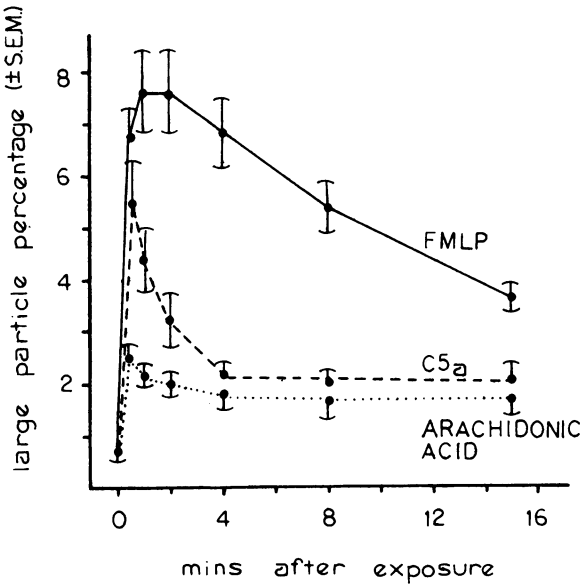
purer than C5a preparations previously used: 2 μl/ml of this reagent contained 0.8 μg protein/ml and an estimated C5a concentration of about 8×10^{-8} M. Aggregating effects of this preparation were detected in concentrations as low as 0.2 μl/ml and increased incrementally with increases in C5a up to 2 μl/ml, the highest concentration used. Arachidonic acid did not aggregate the cells below 10^{-6} M, induced increasing effects between 10^{-6} and 10^{-5} M, and appeared to inhibit the maximal response when used in concentrations above 10^{-5} M. The three aggregating substances, therefore, appeared to induce somewhat different PMN responses; arachidonic acid was able to induce only low levels of aggregation. Additionally, the effects of C5a appeared more reversible than those of FMLP, which, in turn, appeared more reversible than those of arachidonic acid (see Text-figure 1).

Degranulation

The solid lines of Text-figure 2 show dose-response curves for β-glucuronidase and lysozyme release of cytochalasin-B–treated PMNs exposed to 1.7×10^{-8} to 10^{-6} M FMLP and 1.7 to 10 μl/ml C5a. In concentrations of 10^{-4} to 10^{-8} M, arachidonic acid did not degranulate the cells regardless of whether the cells were pretreated with cytochalasin B (not shown).

Specificity of Arachidonic Acid Action

The following agents in the concentrations given were examined for

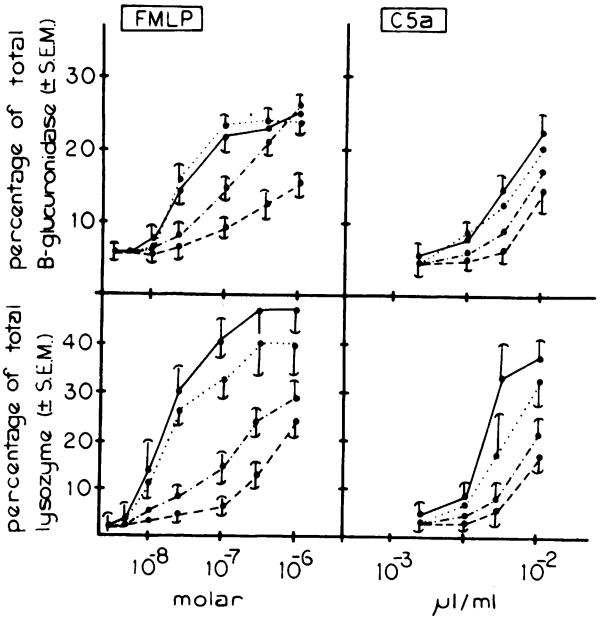


TEXT-FIGURE 1—Large particle percentage of neutrophil suspensions found after adding 5×10^{-7} M FMLP, 2 μl/ml C5a, or 10^{-6} M arachidonic acid. Each of the values found after the addition are significantly greater ($P < .01$, Student unpaired t test) than the 0 time value. Each point is the mean of at least 12 separate experiments.

their effect on PMNs: ETYA (10^{-6} to 3.3×10^{-5} M); 11,14,17-eicosatrienoic, 8,11,14-eicosatrienoic, and eicosanoic acids (10^{-6} to 10^{-5} M); ADP and epinephrine (10^{-6} to 3.3×10^{-4} M); thrombin (10 U/ml); indomethacin (10^{-5} to 10^{-4} M); and ASA (10^{-6} to 5×10^{-3} M). None of these reagents aggregated or degranulated the cells. Thus, neither structurally similar conjoiners of arachidonic acid nor agents that specifically aggregate platelets aggregated or degranulated PMNs. We conclude that the action of arachidonic acid has a high degree of structural specificity and that platelet contamination (about 40 platelets/μl cell suspension) does not underlie the PMN responses found here.

Effect of Inhibitors of Arachidonic Acid Metabolism

Figure 3 shows the effects of varying concentrations of ETYA, indomethacin, and ASA on the aggregation response of PMNs to 5×10^{-7} M FMLP, 2 μl/ml C5a, and 10^{-5} M arachidonic acid. ETYA and indomethacin were active in inhibiting each PMN response; ASA was not. Similar results were found when the period of preincubation was varied from 5 minutes to ½ or 0 minutes, or when the dose of aggregating substance was varied (not shown). ETYA and indomethacin were also able to block the degranulation response to FMLP and C5a; again, ASA was ineffective (Text-figure 2, interrupted lines). ETYA and indomethacin potencies in



TEXT-FIGURE 2—Effect of 10^{-5} M ETYA (-·-·-), 10^{-4} M indomethacin (------), and 5×10^{-3} M ASA (•••••) on the release of β-glucuronidase (*upper panels*) and lysozyme (*lower panels*) stimulated by varying concentrations of FMLP (*left side panels*) or C5a (*right side panels*). The *solid lines* are the results found when cells were preincubated without an inhibitor. Neutrophils were preincubated with cytochalasin B and the inhibitor for 5 minutes and then exposed to the chemotactic factor for another 5 minutes before the supernatant fluid was assayed. Each point is the mean of at least four separate experiments.

Table 1—Molar Concentration of ETYA and Indomethacin That Inhibits by 50% the Aggregation, β-Glucuronidase, and Lysozyme Release of Neutrophils Stimulated by FMLP, C5a, and Arachidonic Acid*

	Response and Inhibitor†					
	Aggregation		β-Glucuronidase release		Lysozyme release	
Stimulus	ETYA (M)	Indomethacin (M)	ETYA (M)	Indomethacin (M)	ETYA (M)	Indomethacin (M)
FMLP	2×10^{-6}	2×10^{-5}	6×10^{-6}	4×10^{-5}	4×10^{-6}	2×10^{-5}
C5a	1×10^{-6}	3×10^{-5}	3×10^{-5}	1×10^{-4}	2×10^{-5}	2×10^{-4}
Arachidonic acid	1×10^{-6}	3×10^{-5}	—	—	—	—

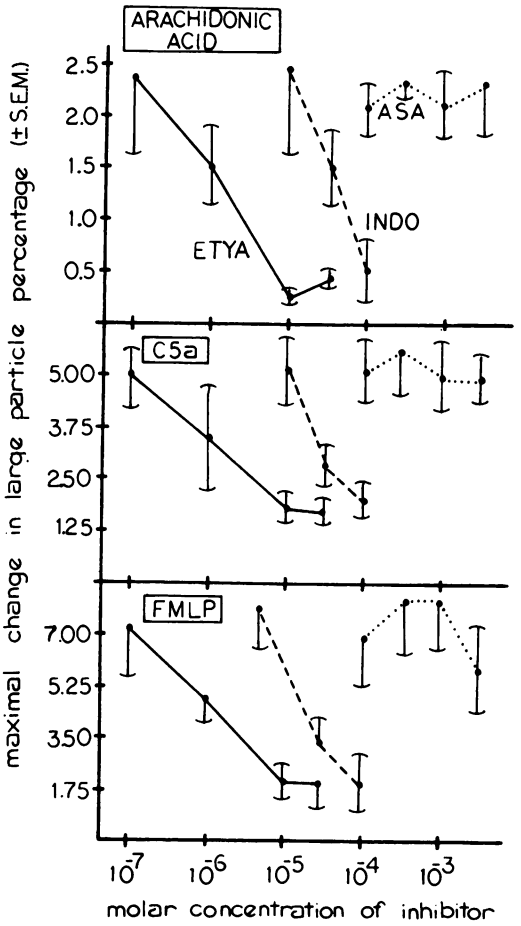
* Neutrophils were incubated with varying concentrations of ETYA or indomethacin for 5 minutes before exposure to 5×10^{-7} M FMLP, 2 μl/ml C5a, or 10^{-5} M arachidonic acid. The concentration of inhibitor that reduced the response by 50% was extrapolated from inhibitor dose-response curves such as those of Text-figure 3. Similar results were found with the use of lower stimulus concentrations.

† For enzyme release cells were also preincubated with 5 μg/ml cytochalasin B for 5 minutes.

inhibiting the C5a-induced degranulation response were an order of magnitude lower than their potencies in inhibiting FMLP-induced aggregation and degranulation responses and the C5a-induced and arachidonic-acid–induced aggregation responses (Table 1). Similar results were found for lower concentrations of each stimulus (not shown).

Discussion

PMNs contain arachidonic acid bound to their phospholipid membranes [20-22]; this arachidonic acid, as well as exogenous arachidonic acid,[23-25] can be metabolized into bioactive derivatives. Phagocytosing PMNs, for instance, synthesize prostaglandins,[25-27] thromboxanes,[28-30] and



TEXT-FIGURE 3—Effect of varying concentrations of ETYA, indomethacin (INDO), and ASA on the maximum change in the large particle percentage of neutrophil suspensions exposed to 10^{-5} M arachidonic acid, 2 μl/ml C5a, or 5×10^{-7} M FMLP. Neutrophils were preincubated with the inhibitor for 5 minutes before exposure to the aggregating substance. Note that the abcissa scales are different for each stimulus. Each value is the mean for at least six separate experiments and the lowest values for the three ETYA and INDO curves are significantly ($P < .01$, Student unpaired t test) below the values found for cells not treated with an inhibitor. These latter values are 2.3 ± 0.5 (for arachidonic acid), 5.2 ± 1.0 (for C5a) and 7.7 ± 0.9 (for FMLP).

oxidized fatty acids [23] from endogenous or exogenous arachidonic acid, and this synthesis is inhibited by blockers of arachidonic acid metabolism.[23-29,31] Certain prostaglandins,[32,33] thromboxanes,[34,35] and oxidized fatty acid derivatives of arachidonic acid [36-42] have been reported to stimulate or enhance PMN function. It may be that one or more of these derivatives mediates the PMN response to chemotactins.

We found that arachidonic acid stimulated PMNs to aggregate in a manner somewhat similar to that of two different chemotactic factors (Text-figure 1). Each of these three agents rapidly induced more or less transient PMN aggregation responses. Each agent was inhibited by ETYA and indomethacin but not by ASA (Text-figure 3). The potency of each blocker did not appear to depend on the type of aggregating stimulus (Table 1). Studies on the degranulation response of cytochalasin-B–treated PMNs (or cytochalasin-B–untreated PMNs) showed different results. Arachidonic acid did not stimulate PMN degranulation. Nevertheless, ETYA and indomethacin but, again, not ASA were able to block the PMN degranulation responses stimulated by C5a and FMLP (Text-figure 2). The potencies of these inhibitors in blocking FMLP-induced degranulation paralleled their potencies in blocking FMLP-induced, C5a-induced, and arachidonic-acid–induced aggregation; however, they were an order of magnitude weaker in blocking C5a-induced degranulation (Table 1). Many of these data are compatible with the hypothesis that metabolic derivatives of arachidonic acid may stimulate certain PMN responses and be involved in the cellular response to chemotactic factors, ie, that these derivatives may represent one type of intracellular mediator of PMN function.

The data reported here neither prove nor fully support the above hypothesis. Arachidonic acid aggregated but did not degranulate the PMNs, and this aggregation response was more sustained (albeit lower in magnitude) than the chemotactic factor-induced response (Text-figure 1). Arachidonic acid did not duplicate the activity of chemotactins. If our underlying hypothesis is correct, these results suggest that metabolic derivatives of arachidonic acid may be necessary but not sufficient to induce degranulation and optimal aggregation; these postulated derivatives may be only one set of intracellular mediators that must act in concert with other mediators, such as cytosolic Ca^{2+} [19] or cyclic nucleotides,[32] in order to produce physiologic PMN function. Because ETYA and indomethacin were relatively impotent in inhibiting C5a-induced degranulation, this response may involve a different combination or sequence of generation of intracellular mediators, compared with the other cell responses. Finally, ASA, which is known to block prostaglandin production

in phagocytosing PMNs [27,31] did not significantly inhibit any of the PMN responses studied here (Text-figures 2 and 3 and Table 1). Although longer preincubation periods or higher drug concentrations might have uncovered an inhibitory action, the relative lack of effect of this drug is disturbing. It may be that nonprostaglandin derivatives of arachidonic acid metabolism are involved in the PMN responses found here. We believe that the data underscore the need to further define the role of arachidonic acid and its derivatives in PMN function.

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