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INHIBITION OF HUMAN NEUTROPHIL AGGREGATION BY ALBUMIN

RELATIONSHIP WITH CYTOSKELETON REORGANIZATION

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Abstract—Albumin, at concentration normally present in plasma (~600 μM), significantly inhibited leukotriene B_4 formation induced by a receptor mediated (fMet-Leu-Phe) and a receptor independent (calcium ionophore A23187) stimuli in human neutrophils. The inhibition of leukotriene B_4 synthesis was accompanied by a concomitant reduction of neutrophil aggregation. In addition, this plasma protein prevented the increase in F-actin content of neutrophils stimulated with fMet-Leu-Phe and A23187, thus suppressing actin polymerization. These data indicate that albumin profoundly affects biochemical and functional aspects of neutrophils suggesting, for this plasma protein, a regulatory role in the overall pattern of the inflammatory reaction.

Activation of circulating neutrophils by the complement component C_5a, formylated oligopeptides and calcium ionophores, triggers a cascade of events which are closely associated in time. Increase in intracellular calcium levels, activation of phospholipid turnover, release of arachidonate metabolites, mainly via the 5-lipoxygenase pathway, and superoxide anion generation are among the early responses of stimulated neutrophils [1]. Coupled to these biochemical events is the expression of discrete cellular dynamic functions such as adhesion, directed and random migration, chemotaxis, aggregation and phagocytosis. During these processes, continuous changes in cytoskeleton assembly occur, involving the transition of actin filaments from the monomeric state (G-actin) to a more organized structure (F-actin) [2, 3]. Albumin has been shown to inhibit superoxide anion formation by neutrophils stimulated by *cis*-unsaturated fatty acids and to reverse morphological changes induced by these fatty acids [4]. In addition, in the presence of this plasma protein, phagocytosis of rabbit neutrophils has been found to be effectively inhibited [5]. In this paper, we have addressed the question whether albumin, at concentrations normally present in plasma, affects neutrophil aggregation and leukotriene B_4 (LTB_4)† formation induced by a receptor mediated (*N*-formyl-methionyl-leucyl-phenylalanine, fMet-Leu-Phe) and a receptor-independent (calcium ionophore A23187) stimuli. In addition, the effects of this plasma protein on the state of actin polymerization, following the addition of the two stimuli, were investigated.

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† Abbreviations: LTB_4, leukotriene B_4; fMet-Leu-Phe, *N*-formyl-methionyl-leucyl-phenylalanine; BSA, bovine serum albumin.

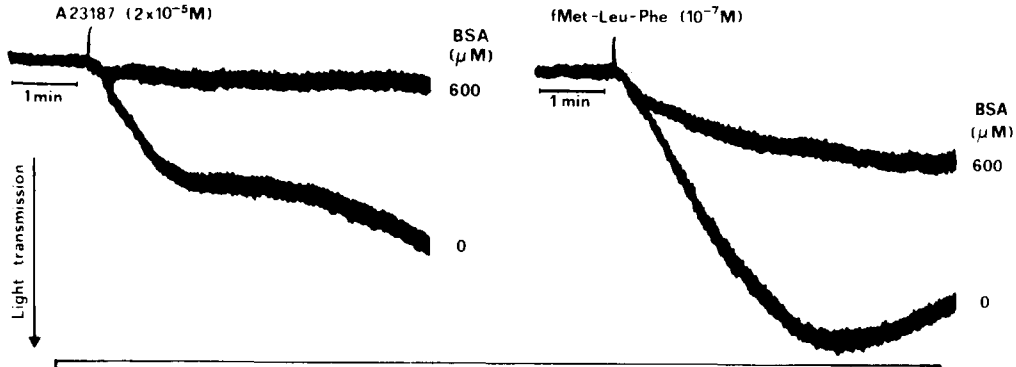
MATERIALS AND METHODS

Materials. Phosphate buffered saline, PBS (Flow Labs, Irvine, U.K.), Ficoll-Paque, Dextran T-500 (Pharmacia, Uppsala, Sweden), bovine serum albumin (BSA) and *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) (Sigma Chemical Co., St Louis, MO), calcium ionophore A23187 (Calbiochem, Behring Corp., La Jolla, CA), NBD-phallacidin(nitrobenzo-oxadiazole-phallacidin) (Molecular Probes, Junction City, OR).

Neutrophil isolation. Cells were isolated from human peripheral blood by sequential dextran sedimentation and Ficoll-Paque gradient centrifugation [6]. Contaminating erythrocytes were eliminated by hypotonic lysis. The purity of neutrophil suspensions was 95–97%. Isolated cells were resuspended at 10^7/ml in PBS containing 0.05% glucose with or without BSA.

Neutrophil aggregation. Neutrophil aggregation was performed using a modification [7] of the method originally described by Craddock *et al.* [8], in an Elvi aggregometer (Elvi Logos) connected with a linear recorder (Omniscribe). Stock solutions, in dimethylsulfoxide and ethanol for fMet-Leu-Phe and calcium ionophore A23187, respectively, were divided into aliquots, kept at −80° and dilutions freshly made in PBS. Neutrophil (7.5×10^6 cells/ml) aggregation was carried out in the presence of $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM) and followed for 5 min after the addition of the stimulus. The aggregation was quantified by measuring the area under the aggregation curve with a graphic tablet connected to a personal computer (Apple IIe, Apple Computer, CA, U.S.A.) and the results expressed in cm^2/min.

Determination of leukotriene B_4 (LTB_4) formation. Neutrophil samples (10^7 cells/ml) were preincubated at 37° (1,000 rpm stirring) for 1 min in the presence of 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Calcium ionophore A23187 (10^{-5} M) or fMet-Leu-Phe



BSA (600 μM)		
−	4.6±0.7	9.8±1.0
+	0.4±0.3**	3.2±0.4*

Fig. 1. Effect of BSA (600 μM) on neutrophil aggregation (cm^2/min) induced by calcium ionophore A23187 (2×10^{-5} M) and by fMet-Leu-Phe (10^{-7} M). In the upper panel representative tracings are reported. In the lower panel are the means ± SE of five separate experiments. (* $P < 0.005$; ** $P < 0.001$).

(10^{-7} M) were added as stimuli and incubations were carried out for 7 min. The incubation was then stopped by adding methanol and LTB_4 formation was quantified by a specific radioimmunoassay procedure, according to Salmon *et al.* [9]. A commercially available kit (Amersham) was used. For LTB_4 antibody characterization see manufacturer's indications.

Quantification of F-actin content. The procedure used was according to Howard and Oresajo [10], based on the NBD-phallacidin extraction assay. Briefly, the relative fluorescence intensity of methanol extracts from cells (10^6), incubated with either appropriate buffer or the stimulus for 2 min, was assessed by fluorimetry (excitation 465 nm, emission 535 nm) using a Perkin Elmer mod. LS-5 spectrofluorimeter. Relative F-actin content was expressed as described by Howard and Wang [11]. Incubations were performed at 37° using cells resuspended in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Cells were stimulated by 2×10^{-5} M A23187 or by 10^{-7} M fMet-Leu-Phe. The results were corrected for the autofluorescence from the unstained cells similarly treated except for the NBD-phallacidin staining.

RESULTS AND DISCUSSION

The aggregation of neutrophils induced by either A23187 or fMet-Leu-Phe was markedly inhibited by the presence of increasing concentrations of BSA in the incubation media (Figs 1 and 2). This inhibition, was more pronounced for cells stimulated with calcium ionophore A23187 (−91%) than that detected using fMet-Leu-Phe as stimulus (−67%). In accordance with data from the literature [12], in our experimental conditions, the synthesis of LTB_4, following incubation of neutrophils with A23187, was greater than that measured using fMet-Leu-Phe, indicating that the amount of LTB_4 formed by neutrophils is strictly dependent on the stimulus used. BSA at 600 μM concentration significantly reduced LTB_4 synthesis by neutrophils stimulated with A23187. Also, using fMet-Leu-Phe (10^{-7} M) as stimulus, BSA (600 μM) significantly inhibited LTB_4 formation, the degree of inhibition being, however, lower (−29%) than that achieved using A23187 (−54%) (Fig. 3). Incubation of cells with appropriate stimuli is known to induce rapid polymerization and redistribution of F-actin within the cells [3]. Under our experimental conditions, changes in F-actin content following incubation of neutrophils with either fMet-Leu-Phe or A23187 were comparable to those reported in the literature [11] (Table 1). Also, F-actin content determined 10 min after the addition of the stimulus, was decreased (−18 and −25% for A23187 and fMet-Leu-Phe, respectively) indicating that, at incubation times longer than 2 min, depolymerization occurred [11]. Relative F-actin content of neutrophils resuspended in buffer containing 600 μM BSA and stimulated either with calcium ionophore (2×10^{-5} M) or fMet-Leu-Phe (10^{-7} M) was significantly lower than that of cells incubated with buffer alone (Table 1). To avoid the possible interference of BSA with the extraction procedure, cell pellets were sonicated 10 min during the first extraction. Also, using this experimental procedure which allowed a complete extraction of the fluorescent probe by methanol, BSA (600 μM) completely prevented the increase in F-actin content of cells stimulated with the two considered stimuli. The possibility that BSA could bind the calcium ionophore A23187 thus lowering the concentration available for cell stimulation was ruled out on the basis of data reported in literature [13]. In addition, binding of fMet-Leu-Phe to intact neutrophils was performed in the presence of BSA. K_d values (4.0×10^{-8} M and 3.2×10^{-8} M fMet-Leu-Phe, in the absence and in the presence of 600 M BSA respectively, the difference being not statistically significant) allowed to exclude the hypothesis that BSA could interfere in the stimulus-receptor coupling. Taken together, these data indicate that BSA profoundly affects several functional, metabolic

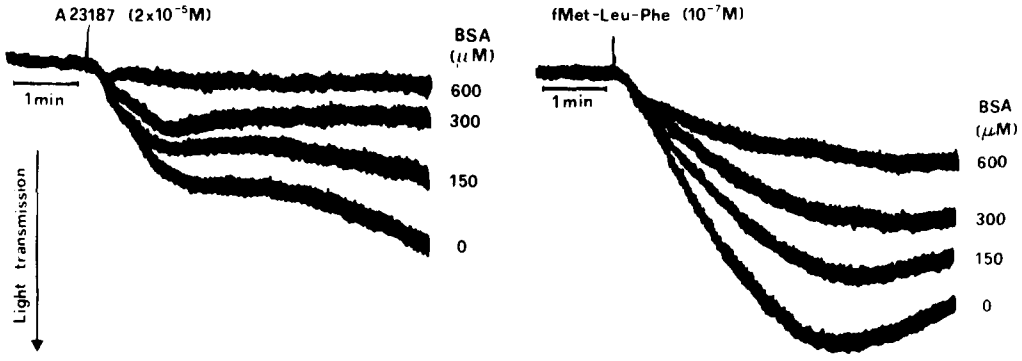


Fig. 2. Effect of increasing concentrations of BSA (0–600 μM) on neutrophil aggregation induced by calcium ionophore A23187 and by the chemotactic peptide fMet-Leu-Phe. An example.

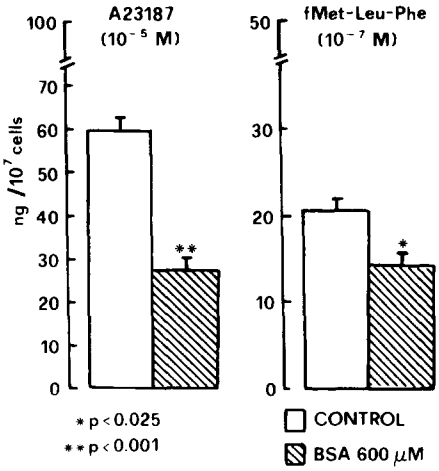


Fig. 3. Effect of 600 μM BSA on LTB_4 synthesis by neutrophils stimulated with calcium ionophore A23187 (10^{-5} M) and fMet-Leu-Phe (10^{-7} M).

Table 1. Effect of BSA on the amount of F-actin in stimulated human neutrophils

BSA (μM)	fMet-Leu-Phe (10^{-7} M)	A23187 (2×10^{-5} M)
0	25.3 ± 5.5	18.5 ± 1.9
600	3.7 ± 2.1	5.4 ± 3.0

F-actin content is expressed as percent increment relative to control. Values are the mean ± SE of ten experiments.

and biochemical aspects of the neutrophil response which are of relevance in the overall pattern of the inflammatory reaction. The reduced synthesis of LTB_4, observed in the presence of BSA, may result in a reduced cellular response in terms of aggregation. In fact, LTB_4 is known to modulate neutrophil function and in particular, this 5-lipoxygenase metabolite has been shown to act both as calcium ionophore [12] and as activator of neutrophil aggregation [14], lysosomal enzyme release [12] and chemotaxis [15]. The inhibition of LTB_4 synthesis by BSA may involve several aspects of arachidonate metabolism. In fact, this protein is known to bind, with high and low affinity, long chain polyunsaturated fatty acids, including arachidonate, which is the precursor of prostaglandins and leukotrienes [16]. On the other hand, BSA was shown to inhibit the synthesis of 5-HETE, LTB_4 and its isomers and ω-oxidation products from exogenous substrate in neutrophils stimulated with the calcium ionophore A23187 [13]. In addition, this plasma protein stabilizes several intermediate products of arachidonate oxidation, as the short living compound of the 5-lipoxygenase pathway, LTA_4 [17], thus in turn reducing the overall synthesis of LTB_4. In the presence of 600 μM BSA the reduction of neutrophil response to fMet-Leu-Phe and A23187, both in terms of aggregation and LTB_4 synthesis, was accompanied by more than 70% reduction in the relative F-actin content, suggesting again that a linkage between aggregation, formation of lipoxygenase products and actin polymerization exists. Presently, since it has been demonstrated that neutrophil glycoprotein complexes are involved in numerous adhesion-dependent functions, including aggregation [18, 19], further studies should address the interaction of albumin with neutrophil surface structure. In conclusion these data, together with other observations indicating that albumin may act in limiting the neutrophil-mediated tissue damage [20] suggest, for this plasma protein, a regulatory role in the development and amplification of the inflammatory response.

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