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A DISPLACEABLE SURFACE-BOUND SUPEROXIDE STIMULATING FACTOR ON CIRCULATING HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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SUMMARY: Human polymorphonuclear leukocytes isolated from blood by dextran sedimentation and hypotonic lysis produce superoxide in the absence of added surface membrane stimulating agents, which can be inhibited by chemokinetic concentrations of N-formyl-methionyl-phenylalanine and arachidonic acid, though higher concentrations stimulate superoxide production. Inhibition also occurs with a variety of biological proteins in low concentration, the hormones hydrocortisone and thyroxine, and protease inhibitors. The production of superoxide by these cells appears to depend upon a heat-labile non-dializable factor which can be displaced from the cell surface in its active form by repeated washings, or by incubation with N-formyl-methionyl-phenylalanine.

INTRODUCTION

The production of superoxide radicals by intact polymorphonuclear leukocytes *in vitro* occurs in response to a variety of particulate and soluble stimuli including detergents, chemotactic agents, and fatty-acids (1-3), which agents apparently initiate superoxide production at the surface membrane by a mechanism involving chymotrypsin-like proteases (4). Superoxide production by unstimulated polymorphonuclear leukocytes is negligible if the cells are obtained by a method involving density gradient centrifugation (2) but is quantitatively significant if the cells are obtained by dextran sedimentation and hypotonic lysis alone (5). This disparity in superoxide production suggests that the density gradient procedure displaces or inactivates a factor on the cell surface which was present *in vivo*, and which is preserved by the sedimentation and hypotonic lysis method. This report confirms the presence of such a factor on human polymorphonuclear leukocytes.

MATERIALS AND METHODS

Heparinized whole human blood was mixed with two volumes of 3% dextran in isotonic sodium chloride and allowed to separate for one hour. The plasma layer was drawn off and centrifuged for ten minutes at 400 x g. The cells were washed once to remove platelets by suspending with isotonic sodium chloride containing 2 mM Tris-HCl, pH 7.4, and were then centrifuged for a further ten minutes at

Abbreviations:

N-Formyl-methionyl-phenylalanine = F-Met-Phe
N-Benzoyl-L-tyrosine ethyl ester = BTEE
p-Tosyl-L-arginine methyl ester = TAME
L-1-Tosyl-amido-2-phenylethyl-chloromethyl ketone = TPCK
Phenylmethylsulfonyl-fluoride = PMS

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400 x g. Residual erythrocytes were lysed by resuspending the cells in 0.2% sodium chloride for 30 seconds at 25°C followed by an equal volume of 1.5% sodium chloride. After a ten minute centrifugation at 400 x g the pellet was normally suspended in an isotonic sodium chloride buffer containing 1 mM potassium chloride, calcium chloride, magnesium sulfate, and sodium azide as a cytochrome oxidase inhibitor, with 2 mM Tris-HCl, and 2.8 mM dextrose at pH 7.4.

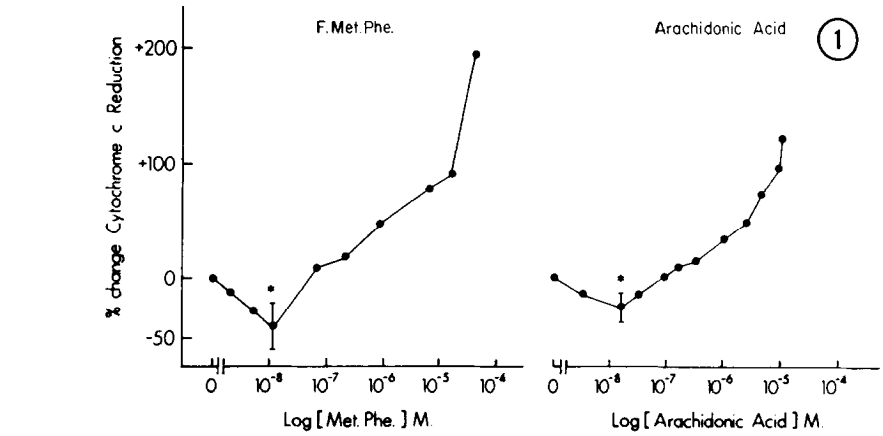
Superoxide production was assayed by the measurement of superoxide dismutase inhibitable cytochrome c reduction (6). All incubations were performed in plastic disposable test tubes at 37°C with gentle shaking in a water bath. At zero time cells were added to the buffer to give a final concentration of 2.5×10^6 cells/ml with 40 nmol/ml cytochrome c in a final volume of 2 ml. A 500 µl aliquot was removed immediately into 500 µl of ice cold saline to prevent cytochrome c reduction. After the selected thirty minute incubation period a further 500 µl aliquot was similarly removed. Blanks for spectrophotometric analysis were prepared identically to test samples with the exclusion of cytochrome c. All samples were centrifuged at 400 x g for ten minutes and the supernatant removed for measurement of absorbance at 550 nm at 25°C. Calculations of the amount of cytochrome c reduction using a molar extinction coefficient of 21×10^3 (7) for the difference between the absorption of reduced minus oxidized cytochrome c. Water soluble additives were dissolved in buffer and appropriate dilutions made prior to cell addition. Where relatively water insoluble additives were used they were dried under nitrogen in the test tube prior to addition of buffer and then vortexed and incubated for fifteen minutes prior to the addition of cells.

Cells prepared as described were called once washed cells. In some experiments cells were washed in an identical fashion up to six times. If the initial washings were to be used as buffer the first wash was performed with buffer and this was centrifuged to remove any residual cells prior to use. When washings were dialyzed this was performed at 4°C against buffer for forty-eight hours. Statistical comparisons were performed using the paired t test.

N-formyl-methionyl-phenylalanine was obtained from Andrulis Research Corporation (Bethesda, Md.). All other chemicals were obtained from Sigma (St. Louis, Mo.). The soidum salt of L-thyroxine was used in the hormone studies in which cells were preincubated with the hormones for thirty minutes prior to exposure to test additives.

RESULTS AND DISSCUSSION

Dose curves for the superoxide-dependent reduction of cytochrome c were performed with the two chemokinetic agents, F-Met-Phe and arachidonic acid (8,9). The results are shown in Figure 1 where it may be seen that with once washed cells, obtained by dextran sedimentation and hypotonic lysis, concentrations of these agents which this laboratory has previously reported as being chemokinetic result in inhibition of superoxide production compared with that produced by unstimulated cells. Maximal inhibition was statistically significant and occured at a concentration of about 10^{-8}M for both agents (F-Met-Phe $df=4$; $t=3.83$; $p<.01$: Arachidonic acid $df=4$; $t=4.1$; $p<.01$). Higher concentrations of these agents result in increased superoxide production. An increase with F-Met-Phe has been previously reported (2), though not the increase with arachidonic acid, which is known to cause neutrophil degranulation (10). This biphasic curve parallels our reported observations of the biphasic effect of these agents on sodium potassium



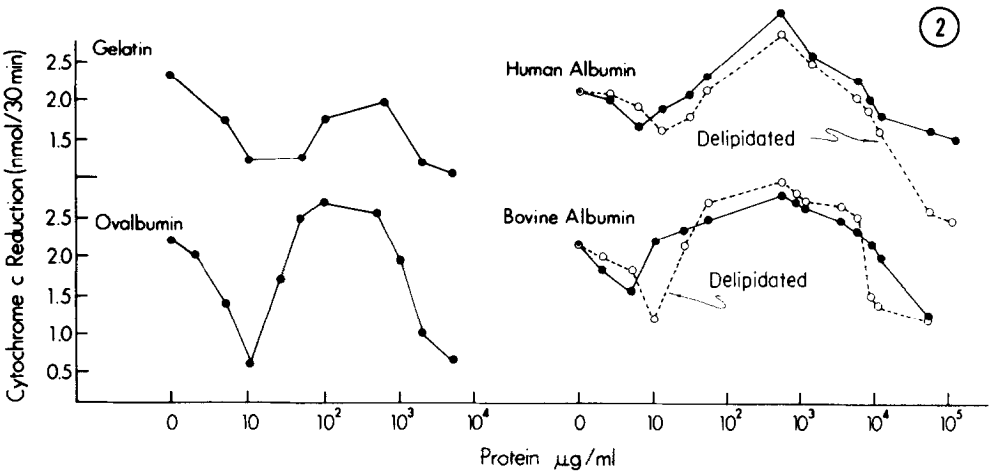


Figure 1: Effect of F-Met-Phe and arachidonic acid on superoxide production by polymorphonuclear leukocytes. All results are the mean of three experiments expressed as percent difference from control. * $p<.01$ by paired t test of original values. M±SD of percent difference at greatest inhibition is shown.

Figure 2: The influence of various concentrations of delipidated and non-delipidated bovine and human serum albumin, gelatin and ovalbumin upon the superoxide-dependent reduction of cytochrome c by polymorphonuclear leukocytes.

ATPase (11), and is also comparable to the differing effect of chemotactic and suprachemotactic concentrations of various agents upon ingestion of sensitized erythrocyte by polymorphonuclear leukocytes (12). The data is presented as percer tage change from unstimulated cells because of starting point variability with different cell batches, though the slopes obtained with all experiments were essentially similar. The starting point was usually in the order of 2 nmoles/30 minutes/2.5 x 10^6 cells which corresponds well with a previous report using the same isolation technique (5). A dose curve with digitonin which is known to

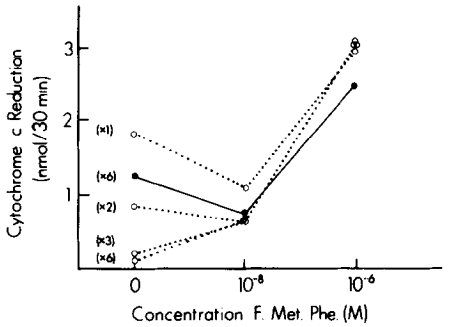


Figure 3: The influence of repeated washing upon cytochrome c reduction with and without the addition of inhibitory (10^{-8}M) or stimulatory (10^{-6}M) concentrations of F-Met-Phe. Cells washed six times were incubated with (●---●) or without (o···o) washings from once washed cells.

stimulate superoxide production (1) showed no inhibition at low concentration (data not shown).

Since low concentrations of protein enhance chemotaxis in vitro, experiments were performed with and without various proteins as shown in Figure 2. Human and bovine albumins, ovalbumin and gelatin showed similar effects on superoxide production, with inhibition at low concentrations similar to that seen with the chemokinetic agents and also a marked inhibition at high concentration. Delipidation had no significant effect.

Other investigators have reported that a variety of substances including albumin, immunoglobulin, and complement are retained on the cells following isolation and can be removed by repeated washing (13). It is possible that the differences in superoxide production by cells isolated with differing techniques can be explained by a differential removal of a superoxide stimulating agent from the cell surface during preparation. The influence of cell washing is shown in Figure 3 where cells were incubated alone or with inhibitory or stimulatory concentrations of F-Met-Phe following one to six washes. Washing had no effect with stimulatory concentrations suggesting that the cell had not been damaged by multiple washes. In unstimulated cells washing progressively reduced superoxide production, which could be restored by the addition of washings from once washed cells. This suggests the presence of a soluble superoxide stimulating factor which is removable by washing and which we found to retain its activity following dialysis at 4°C for forty-eight hours but to loose its activity with heating at 80°C for ten minutes. Since this factor is displaced by washing it is also possible that the inhibitory effect of the chemotactic agents was due to displacement of this factor. Buffer from cells incubated with inhibitory concentrations of F-Met-Phe was dialyzed to remove the chemotactic peptide and was then examined for superoxide stimulating activity. This activity was present in medium from cells treated with F-Met-Phe as shown in Table 1.

TABLE 1

Effect on cytochrome c reduction by polymorphonuclear leukocytes of cell washing and the addition of washings, hormones and protease inhibitors.

Cell Washes	Additions	Cytochrome c reduction*
1	Nil	2.12 ± 0.31 (3)
1	BTEE 100 μM	1.05 ± 0.14 (2)
1	BTEE 200 μM	0.21 ± 0.19 (2)
1	TAME 2.5 mM	1.49 ± 0.23 (2)
1	TAME 5.0 mM	1.18 ± 0.20 (2)
1	Hydrocortisone 10^{-7}M	1.17 ± 0.35 (3)
1	Hydrocortisone 10^{-6}M	0.68 ± 0.19 (3)
1	L-Thyroxine 10^{-7}M	1.09 ± 0.42 (3)
1	L-Thyroxine 10^{-6}M	0.97 ± 0.44 (3)
6	Nil	0.10 ± 0.04 (3)
6	Washings**	1.70 ± 0.29 (3)
6	Washings + TPCK 5 μM	0.61 ± 0.33 (2)
6	Washings + PMS 1 mM	0.30 ± 0.19 (2)
3	Nil	0.52 ± 0.30 (3)
3	Dialized washings**	0.59 ± 0.28 (2)
3	Dialized washings (F-Met-Phe)***	2.83 ± 0.51 (2)

*nmoles/30 minutes/2.5 x 10^6 cells. Mean ± SD. Numbers in parenthesis refer to number of experiments.

**Washings from x1 washed cells.

***Washings from x1 washed cells incubated for 30 minutes with 10^{-8} M F-Met-Phe.

We have confirmed the observations of Kitagawa et al (4) that various protease inhibitors and synthetic substrates for serine proteases inhibit superoxide production by polymorphonuclear leukocytes in the presence of a variety of stimuli. The addition of washings from once washed cells failed to produce increased superoxide production in the presence of the protease inhibitors, BTEE, TAME (Table 1), TPCK and soybean trypsin inhibitors, inhibited superoxide from once washed unstimulated cells. The hormones hydrocortisone and thyroxine had a similar effect. This supports previous observations with hydrocortisone (14,15) while the effect of thyroxine has not been previously reported.

From these observations we have concluded that circulating human polymorphonuclear leukocytes possess a surface factor which stimulates superoxide production which can be removed by washing, incubation with F-Met-Phe and density gradient centrifugation, and which is heat-labile and dializable. This factor activates, or is itself, a chymotrypsin-like protease. It is likely that F-Met-Phe and other proteins competitively displaces this factor at low concentrations of anions result in other effects on the cell membrane in a fashion similar to that observed with digitonin. The polyphasic effect observed with protein on superoxide production is not understood. However, it is not unlike the biphasic effect

seen with the smaller anions, arachidonic acid and F-Met-Phe. The fact that high concentrations of protein inhibit superoxide release offers a potential defense mechanism *in vivo* against untoward superoxide release by polymorphonuclear leukocytes circulating in blood.

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