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ON THE STOICHIOMETRY OF OXYGEN METABOLISM IN POLYMORPHONUCLEAR LEUKOCYTES

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Summary

The stoichiometry of oxygen uptake to superoxide output has been examined in neutrophilic polymorphonuclear leukocytes which have been stimulated by a particulate (serum-treated-zymosan) and a soluble (N-formylmethionyl-leucyl-phenylalanine) agent.

It has been shown that superoxide production by neutrophils does not necessarily accompany oxygen uptake during phagocytosis. Substantial superoxide-stimulating-activity (possibly complement fragment C3b) is derived from serum which has been stored at -20° but not from serum stored at 4°. N-formylmethionyl-leucyl-phenylalanine stimulates a much higher conversion of oxygen to superoxide than does serum-treated-zymosan.

It has been well established that four major metabolic events occur during phagocytosis by neutrophilic polymorphonuclear leukocytes - an increase in oxygen uptake (1-5), superoxide and hydrogen peroxide output (3,5-11) and hexose monophosphate shunt activity (2,3). The four events are collectively termed the "respiratory burst" and are believed to be involved in microbicidal activity since phagocytosis itself can occur in the absence of oxygen. All four events are closely related since superoxide and hydrogen peroxide are successive reduction states of oxygen and the reducing potential for the first of these reactions is believed to come from NADPH provided by the hexose monophosphate shunt (12-14).

$$NADPH + 2O_2 \xrightarrow{\text{NADPH oxidase}} NADP^+ + 2O_2^- + H^+$$

$$2O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

The respiratory burst may be activated by both particulate (opsonised bacteria and zymosan) and soluble agents (phorbol myristate acetate, a variety of ionophores, the complement peptide C5a, fluoride ion and the chemotactic peptide N-formylmethionyl-leucyl-phenylalanine)(9,15-17).

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The stoichiometry of oxygen uptake to superoxide output during the respiratory burst is uncertain but it is clear that a significant proportion of the oxygen taken up is returned to the system as superoxide (5-7,10,18).

The aim of the present work was to compare the stoichiometry of oxygen uptake to superoxide output in neutrophils during the respiratory burst initiated on the one hand by a particulate, phagocytosable stimulus (serum-treated-zymosan) and on the other hand by a soluble non-phagocytosable stimulus (N-formylmethionyl-leucyl-phenylalanine).

Materials and Methods

Bovine serum albumin, cytochrome C (type VI, horse heart), N-formylmethionyl-leucyl-phenylalanine and zymosan A (all Sigma), N-ethylmaleimide (Merck), phosphate buffered saline (Oxoid), Ficoll-Paque and Dextran T-500 (both Pharmacia) were commercially available.

Neutrophils: Neutrophils from healthy female volunteers aged 19 to 59 years (average age 36) were separated as follows, siliconised glassware being used throughout. Whole blood (5 ml, anticoagulated with EDTA) was defibrinated with a fritted glass rod for 10 min, mixed with phosphate buffered saline (5 ml, without Ca^{2+} or Mg^{2+}), layered onto Ficoll-Paque (6.6 ml) and centrifuged at room temperature for 40 minutes at 400 x g. Plasma, lymphocytes and excess Ficoll-Paque were removed and the erythrocyte-neutrophil pellet diluted to three times its volume with a solution of Dextran (3%) in phosphate buffered saline (without Ca^{2+} or Mg^{2+}). The erythrocytes were allowed to sediment (about 30 minutes), the neutrophil layer was then withdrawn, centrifuged at room temperature for 10 minutes at 200 x g and the supernatant removed. Contaminating erythrocytes were removed from the neutrophil pellet by exposure to water (0.75 ml) for 15 seconds followed by restoration of isotonicity with phosphate buffered saline (7 ml, without Ca^{2+} or Mg^{2+}). When one hypotonic lysis failed to remove the erythrocytes, the procedure was repeated a second time with only a 10 second exposure to water. The neutrophils were centrifuged at room temperature for 10 minutes at 200 x g, the supernatant withdrawn and the cells resuspended in phosphate buffered saline (1 ml, containing Ca^{2+} (0.9 mM) and Mg^{2+} (1 mM)). The preparations contained >95% neutrophils (Wrights stain) which were >90% viable by Trypan Blue exclusion.

Serum-Treated-Zymosan: Zymosan A (50 mg) was boiled with phosphate buffered saline (5 ml, without Ca^{2+} or Mg^{2+}) for one hour, centrifuged and resuspended in phosphate buffered saline (1 ml, without Ca^{2+} or Mg^{2+}). Pooled human AB serum (3.5 ml, fresh frozen at -20°) was added and incubated at 37° for 30 minutes. The serum-treated-zymosan was centrifuged off and resuspended in phosphate buffered saline (without Ca^{2+} or Mg^{2+}) at a concentration of 50 mg/ml. It was made freshly as required.

N-formylmethionyl-leucyl-phenylalanine: A stock solution (1 mM) was made up in dimethyl sulphoxide and kept frozen. Dilute solutions (1 μM and 10 μM) were made as required by diluting with water.

Medium: The medium used in all metabolic experiments was a modified Hanks' balanced salt solution supplemented with glucose 0.1% and bovine serum albumin 0.1%.

Oxygen uptake studies: To neutrophil suspension (0.3 - 0.7 x 10^6 cells in 0.05 or 0.1 ml) in a Clark-type oxygen electrode (Rank Bros.) was added medium (0.95 or 0.9 ml) and the cells incubated at 37° for 15 minutes to establish the endogenous rate of oxygen consumption. Serum-treated-zymosan (0.02 ml) or N-formylmethionyl-leucyl-phenylalanine (0.01 ml of 10 μM) was then added and the increase in oxygen consumption followed for 5 minutes.

Superoxide output following stimulation by serum-treated-zymosan: To neutrophil suspension (0.3 - 0.7 x 10^6 cells in 0.05 or 0.1 ml) was added medium (0.58 or 0.53 ml) and cytochrome C (0.25 ml of 60 μM) and the solution equilibrated by shaking for 5 minutes at 37°. The reaction was started by addition of serum-treated zymosan (0.02 ml) and allowed to proceed at 37° for 5 minutes before being stopped by addition of N-ethylmaleimide (0.1 ml of 10 mM) and cooled in an ice bath for 10 minutes. Each reaction was accompanied by a blank reaction treated in exactly the same way except for the addition of serum-treated-zymosan. The reaction mixtures were then centrifuged and superoxide in the supernatant determined by increase in absorption at 550 nm (adjusted for the blank value) using the extinction coefficient of 21.1 mM^{-1} cm^{-1} for reduced cytochrome C (19).

Superoxide output following N-formylmethionyl-leucyl-phenylalanine stimulation: to neutrophil suspension (0.35 - 0.7 x 10^6 cells in 0.05 or 0.1 ml) in a cuvette was added medium (0.6 or 0.55 ml) and cytochrome C (0.25 ml of 60 μM) and the solution equilibrated at 37° for 5 minutes. The reaction was started by addition of N-formylmethionyl-leucyl-phenylalanine (0.1 ml of 1 μM) and the production of superoxide monitored continuously by cytochrome C reduction at 550 nm in a Cary 17 spectrophotometer at 37°.

Results and Discussion

Since it was known that increasing neutrophil concentration did not lead to a linear increase in superoxide production (7), it was decided to examine the stoichiometry of superoxide production at two different neutrophil concentrations.

In early studies zymosan was opsonised with serum which had been refrigerated (but not frozen) immediately following isolation. In these studies serum-treated-zymosan elicited a substantial increase in oxygen uptake (which was not followed for more than 30 min) but a very slight superoxide output which lasted only a few minutes and represented only 17% of the oxygen uptake during the first 5 minutes (Table 1).

By contrast zymosan which was opsonised with serum which had been frozen immediately following isolation provoked a very similar oxygen uptake

Table 1. Oxygen Uptake and Superoxide Output in Neutrophils Stimulated by Serum-Treated-Zymosan*

Neutrophils ($\times 10^6$)	Oxygen uptake nmoles/5 min/ 10^6 cells	Superoxide output nmoles/10^6 cells	Superoxide output as a percentage of oxygen uptake
$0.3 \pm 0.05^{\dagger}$ (3)	$16.9 \pm 2.3^{\dagger}$	$2.7 \pm 1.3^{\dagger}$	16
0.6 ± 0.1 (2)	23.2 ± 7.7	4.0 ± 1.5	17

* The zymosan was activated with human pooled AB serum which had been stored at 4° following isolation.

† Values are means ±SD; numbers in brackets indicate the number of experiments, each of which was performed in duplicate. In each case measurements of oxygen uptake and superoxide output were performed on the same preparation of neutrophils.

to the first experiments but a much greater superoxide output (50-60% of oxygen uptake during the first 5 minutes) (Table 2). Furthermore, the production of superoxide in these experiments continued for about 20 minutes. Thus the greater superoxide output was not a consequence of greater oxygen uptake.

The stoichiometry of superoxide output in the latter experiments confirmed recent values (7,10) and was considerably higher than earlier results on both human and animal neutrophils (5,6). In accord with previous observations (7) it was noted that superoxide output per cell tended to decrease with increasing neutrophil concentration but there was reasonable agreement between the observed stoichiometries at both cell concentrations.

Studies with cytochalasin B have demonstrated that superoxide production in human neutrophils can occur without phagocytosis taking place and merely requires contact of the stimulus with the phagocyte surface (18). Present results show the converse namely that phagocytosis can occur with virtually no superoxide output. It has been suggested that the superoxide stimulating activity produced by exposure of serum to bacteria is largely present in the

Table 2. Oxygen Uptake and Superoxide Output in Neutrophils Stimulated by Serum-Treated-Zymosan* and by N-Formylmethionyl-leucyl-phenylalanine

	Serum-Treated-Zymosan			N-Formylmethionyl-leucyl-phenylalanine		
Neutrophils ($\times 10^6$)	oxygen uptake nmoles/5 min/ 10^6 cells	superoxide output nmoles/5 min/10^6 cells	superoxide output as a percentage of oxygen uptake	oxygen uptake nmoles/10^6 cells	superoxide output nmoles/10^6 cells	superoxide output as a percentage of oxygen uptake
0.35 ± 0.09†	23.0 ± 6.7†	14.3 ± 3.7†	62	12.9 ± 3.5Φ	16.4 ± 5.0Φ	127
0.7 ± 0.18†	22.2 ± 5.6†	11.5 ± 2.9†	52	8.1 ± 3.0†	10.3 ± 2.5†	127

* The zymosan was activated with human pooled AB serum which had been frozen (-20°) immediately after isolation.
† means ±SD of six experiments, each of which was performed in duplicate.
Φ means ±SD of three experiments, each of which was performed in duplicate.
In each case measurements of oxygen uptake and superoxide output due to stimulation of serum-treated-zymosan and N-formylmethionyl-leucyl-phenylalanine were performed on the same preparation of neutrophils.

serum rather than associated with the microorganism (20). Present work indicates that substantial superoxide stimulating activity, although derived from serum, is associated with the zymosan particles (since there was no serum present in the assay system) and that it is destroyed by storing the serum at 4^{o} rather than -20^{o}. This activity may be complement fragment C3b which is believed to have a major role in particle recognition and phagocytosis by neutrophils (16,21).

By contrast with the serum-treated-zymosan reactions, stimulation of neutrophils by N-formylmethionyl-leucyl-phenylalanine produced a rapid, but short-lived (2-4 minutes) burst of superoxide and an equally short-lived burst of oxygen uptake. The brief nature of this reaction confirmed previous observations with N-formylmethionyl-leucyl-phenylalanine on neutrophils (22) and was in marked contrast to the longer stimulation observed with other soluble activators such as complement fragment C5a and phorbol myristate acetate (16). The stoichiometry of the reaction indicates a much higher conversion of oxygen to superoxide than was the case with stimulation by serum-treated-zymosan although not quite as high as that recently observed in the reaction of N-formylmethionyl-leucyl-phenylalanine with alveolar macrophages (23). It is to be noted that although both oxygen uptake and superoxide output per cell decreased with increasing neutrophil concentration, there was good agreement in the stoichiometry.

It may be concluded from these results that superoxide production is associated with the chemotaxis of (effect of N-formylmethionyl-leucyl-phenylalanine) and the contact of a suitably opsonised particle with neutrophils. The opsonin which stimulates phagocytosis does not appear to be the same as that which stimulates superoxide output. Thus it appears that superoxide output is not an automatic consequence of oxygen uptake during phagocytosis.

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