Note

Hydroxylation of Phenylalanine by Stimulated Polymorphonuclear Leukocytes—Attempts to Detect Hydroxyl Radicals—

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Upon exposure to appropriate stimuli, polymorphonuclear leukocytes (PMNL) undergo what is termed "the respiratory burst," a coordinated group of metabolic events whose purpose is the production of microbicidal oxidizing agents. This respiratory burst results in the production of large quantities of superoxide (O_2^-) and H_2O_2 , which may then interact to form hydroxyl radicals ($OH^\cdot$) and singlet oxygen (1O_2).^{1~3)} The $OH^\cdot$ is one of the strongest oxidizing agents known, and may take a significant part in the bactericidal activity of PMNL.¹⁾ $OH^\cdot$ has been detected by EPR spectroscopy⁴⁾ and gas chromatography,^{5,6)} in conjunction with spin-trapping and ethylene production from methional, respectively, but these methods are expensive or complicated. Liquid chromatography, which has now found its way into almost all laboratories, is not yet adapted for the detection of $OH^\cdot$ generated in media containing RMNL.

We have demonstrated with chemical systems and high-performance liquid chromatography (HPLC) that $OH^\cdot$ can mediate the formation of *o*-, *m*-, and *p*-tyrosines from phenylalanine, but H_2O_2 , O_2^- , and 1O_2 can not.^{7~9)}

In this report, we attempted to detect $OH^\cdot$ which may be generated during the respiratory burst of PMNL, using phenylalanine as a $OH^\cdot$ trapping agent, and HPLC.

Guinea-pig PMNL were obtained from casein-induced peritoneal exudates as described previously.¹⁰⁾ The *o*- and *m*-tyrosines derived from phenylalanine were measured by HPLC under the conditions described previously.¹¹⁾

The incubation of phenylalanine with PMNL in the presence of phorbol myristate acetate (PMA) as a stimulus caused the formation of 69 ± 10 pmol/ 10^7 cells/60 min of *o*-tyrosine and 45 ± 7 pmol/ 10^7 cells/60 min of *m*-tyrosine (Fig. 1). Although *p*-tyrosine was also formed, it could not be measured because of a large amount of *p*-tyrosine already in the cells. Tyrosines were also formed when F-Met-Leu-Phe (FMLP) and F^- were used as stimuli, and in

both cases the ratios of *o*- and *m*-tyrosines were similar to that obtained from PMA-stimulated cells. Figure 2 shows the courses of the formation of tyrosines (*o*- + *m*-) by PMA-, F^- , and FMLP-stimulated PMNL. With F^- , a pronounced delay was observed; there is also a pronounced delay between the addition of F^- and the onset of O_2^- formation.¹²⁾ Formation tyrosine isomers was greatly reduced if the stimulus was omitted from the reaction mixture, demonstrating that for tyrosine formation PMNL had to be activated, *i.e.*, oxidizing agents generated during the respiratory burst were required. More tyrosines were formed in proportion to cell numbers added between 10^6 and 10^7 cells/ml using PMA as a stimulus (not shown). Since the formation of tyrosines from phenylalanine is specifically mediated by $OH^\cdot$,^{7~9)} it is very possible that activated PMNL produced $OH^\cdot$ which can react with phenylalanine.

To obtain further evidence as to the nature of the

FIG. 1. Chromatogram of the Reaction Mixture.

A) The reaction mixtures containing phenylalanine (1 mM), PMNL (10^7 cells), glucose (5 mM), $CaCl_2$ (0.5 mM) and PMA ($0.16 \mu M$) in 1.0 ml of Krebs Ringer phosphate buffer were incubated at $37^\circ C$ with shaking. After 60 min of incubation, $200 \mu l$ of trichloroacetic acid (6 M) was added. After centrifugation, $200 \mu l$ of the supernatants were directly injected into HPLC.

B) Same as A), except for no external addition of phenylalanine, $CaCl_2$, glucose, or PMA.

C) A synthetic mixture of reference compounds. Peak: 1=*p*-tyrosine (6 pmol); 2=*m*-tyrosine (6 pmol); 3=*o*-tyrosine (6 pmol); 4=phenylalanine (12 nmol).

FIG. 2. Tyrosine Formation from Phenylalanine by PMNL as a Function of Time.

Phenylalanine was incubated with PMNL under the conditions described in the legend of Fig. 1(A), except for using PMA (0.16 μ M) (●), NaF (20 mM) (△) and FMLP (0.1 mM) (×) as stimuli or without stimulus (○). Samples were withdrawn at the indicated times and assayed as described in the legend of Fig. 1(A).

oxidizing species, the effects of $\text{OH}^\cdot$ scavengers on the tyrosine formation by PMA-stimulated PMNL were examined (Table I). $\text{OH}^\cdot$ scavengers such as benzoate, mannitol, thiocyanate, and thiourea effectively prevented tyrosine formation, suggesting the generation of $\text{OH}^\cdot$ in the medium containing PMA-stimulated PMNL.

Using this tyrosine formation from phenylalanine, the roles of O_2 and H_2O_2 in $\text{OH}^\cdot$ formation in the medium containing PMNL were examined by testing the effects of superoxide dismutase (SOD) and catalase on the reaction (Table I). SOD was an effective inhibitor of tyrosine formation, implicating O_2^- in $\text{OH}^\cdot$ generation. Catalase was much less effective. McCord and Day demonstrated O_2^- and H_2O_2 dependent production of $\text{OH}^\cdot$ catalyzed by Fe^{3+} -ethylenediaminetetraacetic acid (EDTA) complex,¹³⁾ via the reaction of the net Haber-Weiss phenomenon.¹⁴⁾ The addition of Fe^{3+} -EDTA to our system greatly increased the tyrosines. Catalase was a more effective inhibitor of tyrosine formation by the reaction system with Fe^{3+} -EDTA than SOD, in contrast to the results observed in the reaction system without Fe^{3+} -EDTA (Table I). These findings, at first sight, may suggest that H_2O_2 is not a main precursor of $\text{OH}^\cdot$ in the medium containing PMNL.

We have demonstrated using phenylalanine as a $\text{OH}^\cdot$ trapping agent and HPLC that the generation of $\text{OH}^\cdot$ occurred during the respiratory burst of PMNL. HPLC may be used to trace $\text{OH}^\cdot$ generated in the medium.

TABLE I. EFFECTS OF VARIOUS SUBSTANCES ON TYROSINE FORMATION FROM PHENYLALANINE BY STIMULATED PMNL

Reaction mixtures contained phenylalanine (1 mM), PMNL (10^7 cells), PMA (0.16 μ M), glucose (5 mM), CaCl_2 (0.5 mM), and the specified amount of various substances in 1.0 ml of Krebs Ringer phosphate buffer. Incubation was at 37°C for 30 min with shaking. Reaction mixtures were assayed as described in the legend of Fig. 1.

Substances added	Conc. (mM)	Tyrosine formation (o- + m-) (% of control 100)
None (control)		
Sodium benzoate	100	42 ± 9
	50	62 ± 12
Mannitol	100	37 ± 8
	50	73 ± 15
Sodium thiocyanate	50	37 ± 9
Thiourea	10	68 ± 9
	1	97 ± 6
Urea	10	99 ± 5
Superoxide dismutase	100 μ g/ml	10 ± 6
	50 μ g/ml	19 ± 10
	20 μ g/ml	33 ± 8
Catalase	100 μ g/ml	55 ± 15
	50 μ g/ml	69 ± 10
	20 μ g/ml	90 ± 9
Bovine serum albumin	100 μ g/ml	99 ± 8
Fe^{3+} -EDTA	0.1	4700 ± 535
+ superoxide dismutase	100 μ g/ml	183 ± 29
+ Catalase	100 μ g/ml	92 ± 21

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