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Research Article

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INTRACELLULAR SITE OF OPERATION AND SOME REGULATING FACTORS

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ABSTRACT The intracellular site of operation of the myeloperoxidase-H_2O_2-halide antibacterial system of granulocytes has been determined by utilizing measurements of the fixation of iodide to trichloracetic acid (TCA) precipitates of subcellular fractions, including intact phagocytic vesicles. $Na^{125}I$ was added to suspensions of guinea pig granulocytes in Krebs-Ringer phosphate buffer, and they were then permitted to phagocytize different particles. Phagocytic vesicles were formed by allowing cells to ingest a paraffin oil emulsion (POE) and collected by flotation on sucrose after homogenization. Measurement of ^{125}I bound to TCA precipitates of the different fractions and the homogenates disclosed that the lysosome-rich fraction obtained by centrifugation from control (nonphagocytizing) cells accounted for a mean 93.1% of the total cellular activity. With phagocytosis of POE, TCA-precipitable iodination increased two- to sevenfold, and the lysosomal contribution fell to a mean 36.9% of the total. The appearance of activity within phagocytic vesicles accounted for almost the entire increase seen with phagocytosis (a mean 75.7%), and iodide was bound within these structures with high specific activity. More iodide was taken up by cells than fixed, regardless of iodide concentration, and was distributed widely throughout the cell rather than selectively trapped within the vesicles.

The amount of iodide taken up and fixed varied considerably with the phagocytic particle employed. Yeast particles were found to stimulate iodination to a far greater degree than the ingestion of POE or latex. Such observations are consistent with the concept that the ingested particle is a major recipient of the iodination process. Measurements of metabolic activities related to the formation and utilization of peroxide by cells phagocytizing different particles were made and correlated with iodination. The findings suggest that mechanisms must exist within granulocytes to collect or perhaps even synthesize H_2O_2 within phagocytic vesicles to serve as substrate for myeloperoxidase. The simultaneous stimulation of other metabolic pathways for peroxide disposal and its release into the medium by phagocytizing cells is consistent with the high diffusability of this important bactericidal substance.

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INTRODUCTION

In mammalian granulocytes, an antimicrobial system has been defined in which H_2O_2, produced in increased amounts after phagocytosis, serves as a substrate for the lysosomal enzyme myeloperoxidase in an oxidative reaction lethal to many bacteria, fungi, certain viruses, and mycoplasmas (1). This system requires the participation of an oxidizable cofactor, such as a halide, and if the halide is iodide, its fixation to cellular protein, probably tyrosine residues, can be demonstrated (2). Concomitant with increased peroxide production, protein iodination by granulocytes increases markedly with phagocytosis (3). Both iodination and microbial killing are impaired to varying degrees in the presence of the

TABLE I

Protein Iodination in Homogenates of Resting Cells and Those Phagocytizing POE

Exp.	Resting $PB^{125}I$*	Phagocytizing $PB^{125}I$	Increase with phagocytosis
	cpm/mg protein/45 min	*cpm/mg/ protein/45 min*	%
I	1,027	7,742	770
II	9,063	52,652	580
III	3,440	7,500	220
Mean			510

* ^{125}I activity in a protein bound form as determined by counting TCA precipitates of cellular homogenates. The activity of the added ^{125}I in the reaction mixtures was 2.7×10^6 cpm in experiment I and 6×10^6 cpm in experiments II and III. No carrier NaI was added in experiments I and II, the carrier NaI concentration in experiment III was 20 μM.

myeloperoxidase inhibitors, azide and cyanide, or agents such as Tapazole (Eli Lilly and Co., Indianapolis, Ind.), which alter the intracellular organification of iodide (4). On the other hand, ascorbate can block iodination completely without affecting the antibacterial activity of granulocytes, indicating that fixation of iodide to organisms is not essential for the lethal effect (5). Furthermore, chloride ions may provide the major source of halide for the myeloperoxidase-H_2O_2 system, since they are present intracellularly in greater concentration than iodide (6). Nevertheless, the measurement of protein iodination by granulocytes is a convenient marker for the activity of the myeloperoxidase-H_2O_2 antimicrobial system within intact cells. In the present investigation, the iodination reaction has been utilized to localize the operational site of this system in subcellular fractions of both resting and phagocytizing granulocytes. In addition, some factors regulating this activity have been examined.

METHODS

Granulocytes. Granulocytes were harvested from peritoneal exudates induced in guinea pigs by 12% sodium caseinate injection, as previously described (7). Differential counting revealed that the final cell preparations in Krebs-Ringer phosphate buffer (KRP),[1] pH 7.4, contained more than 95% granulocytes, with the remainder of the cells consisting of macrophages and lymphocytes.

Phagocytic particles. Latex particles 1.1 μm in diameter were obtained (Dow Chemical, U. S. A., Membrane Systems Div., Midland, Mich.), dialyzed overnight against distilled water, and suspended in KRP or Hanks' balanced salt solution, pH 7.4, at a concentration of 3×10^{10} particles/ml. Emulsions of paraffin oil particles (POE) stained with Oil red O dye were prepared in 2% bovine serum albumin-KRP by previously described methods (7). In experiments not involving subcellular fractionation, Fluorochemical Liquid (3M Co., Nuclear Products Div., St. Paul, Minn.) was substituted for POE to yield particles of higher density (1.765); because of its high density, washing of cells and protein after precipitation was facilitated. Baker's yeast (Fleischman's Yeast, Standard Brands Inc., New York) was killed by boiling, washed twice by centrifugation, and suspended in KRP at a final concentration of 1×10^8/ml. In some experiments the yeast was opsonized by incubation in 50% fresh guinea pig serum for 30 min at 37°C and washed once by centrifugation in KRP at 4°C before use.

Measurements of iodination in subcellular fractions. Final suspensions were prepared in 25-ml Erlenmeyer flasks and contained the following components: granulocytes in a concentration of 8–10 mg of cellular protein[2]/ml (Lowry, Rosebrough, Farr, and Randall technique [8]), 1.2 ml of POE (20% of the final volume), NaI (1.0–6.8×10^6 cpm/ml ^{125}I, with or without 20 μM carrier NaI), and KRP, containing 5.5 mM glucose to provide a final volume of 6.0 ml. The flasks were incubated at 37°C in a shaking water bath for 45 min, and the reaction was stopped by the addition of ice-cold 0.15 M NaCl containing 1 mM NaI and 1 mM sodium thiosulfate. Control (nonphagocytizing) cells were handled in an identical fashion, except that the POE was added at the end of the initial 45-min incubation period.

The cells were collected by low speed centrifugation, washed, homogenized in sucrose, and phagocytic vesicle, cytosol, and lysosome-rich pellet fractions were obtained by centrifugation on a discontinuous sucrose density gradient, as previously described (7). After collection of the fractions, a portion of each was saved for protein measurement and the extraction of Oil red O to quantitate intracellular distribution of the emulsion (7). The remainder of the fractions was prepared for measurements of protein-bound iodide by precipitation and washing (three times) with cold 10% trichloroacetic acid (TCA). These maneuvers were carried out in 12×75 mm plastic tubes (Falcon Plastics, Div. B-D Laboratories Inc., Los Angeles, (Calif.), which were dropped into larger tubes for determination of ^{125}I counts in the precipitates, by using a sodium iodide well crystal scintillation counter. In some experiments the total iodide in each fraction was measured by determining ^{125}I counts in washed portions which were not precipitated with TCA.

Measurement of iodination by cells phagocytizing different particles. Cell suspensions, with and without phagocytic particles, were established in 12×75 mm plastic capped test tubes (Falcon Plastics) and contained the following components in a 0.6 ml vol. of KRP: 10×10^6 granulocytes, approximately 500,000 cpm ^{125}I, and 10 nmol NaI (concentration of carrier iodide = 16 μM) and 0.1 ml of phagocytic particles. The latter were either 3×10^9 latex particles (ratio latex/cells = 300:1), 1×10^8 yeast (ratio yeast/cells = 10:1), or 0.1 ml of POE (16.7% of final volume). Previous studies have established that this concentration of POE is saturating for phagocytosis (9). With the multiplicities of latex and yeast particles per cells employed, 97.5–98.6% of the cells exhibited phagocytosis and contained an average of >50 latex or 4.5 yeast particles/cell after 30 min of incubation. Increasing

[1] *Abbreviations used in this paper:* KRP, Krebs-Ringer phosphate buffer; $PB^{125}I$, protein-bound ^{125}I; POE, emulsions of paraffin; TCA, trichloroacetic acid.

[2] 1 mg cellular protein = approximately 2×10^7 polymorphonuclear leukocytes.

TABLE II

*Distribution of Protein and Protein Iodination in Subcellular Fractions of Granulocytes before and after Phagocytosis of POE**

Fraction	Status of cells	Exp.	Protein	Percent of total protein recovered	$PB^{125}I$	Percent of total $PB^{125}I$ recovered	Specific activity $PB^{125}I$	Percent increase with phagocytosis
			mg		*cpm*		*cpm/mg protein*	
Pellet	Resting	I	8.7	40.7	23,744	95.3	2,728	—
		II	4.1	50.7	55,632	90.9	13,569	—
				Mean 45.7		93.1		
	Phagocytizing	I	5.6	33.1	27,749	28.3	5,474	200
		II	2.9	42.0	101,264	31.0	34,919	260
		III	3.3	26.0	72,900	51.5	24,250	—
				Mean 33.7		36.9		230
Supernate	Resting	I	11.6	54.5	984	4.0	86	—
		II	3.9	48.1	4,992	8.2	1,280	—
				Mean 51.3		6.1		
	Phagocytizing	I	9.8	57.9	4,512	4.8	463	540
		II	3.4	49.3	14,836	4.6	4,364	340
		III	9.1	70.0	20,500	15.0	2,285	—
				Mean 59.1		8.1		440
Phagocytic vesicles	Resting	I	1.0	4.8	187	0.8	257	—
		II	0.1	1.2	614	1.0	6,230	—
				Mean 3.0		0.9		
	Phagocytizing	I	1.5	9.0	65,165	67.1	44,171	17,000
		II	0.6	7.7	219,901	64.5	366,505	6,000
		III	0.5	4.0	49,000	31.5	98,500	—
				Mean 6.9		54.4		11,500

* For resting cells, recoveries of protein from the fractions of the homogenate values in experiments I and II were 94.5 and 105% and those of $PB^{125}I$ 107.5 and 84.5%, respectively. For phagocytizing cells, protein recoveries of the homogenate values in experiments I, II, and III were 99.5, 93.3, and 84.0% and those of $PB^{125}I$ 73.9, 86.3, and 121.7%, respectively.

‡ ^{125}I activity in a protein-bound form, as determined by counting TCA precipitates. The number of counts added in each experiment are given in Table I.

the multiplicities did not increase the number of particles per cell at this time. Appropriate particle-free and cell-free controls were set up in parallel, and in some preparations, sodium azide or potassium cyanide (both in 1 mM final concentration) was added to inhibit myeloperoxidase activity. The cell suspensions were rotated end over end at 37°C, and the reaction was stopped after a 60-min incubation period by the addition of 2 ml of ice-cold 0.15 M NaCl containing 1 mM NaI and 1 mM sodium thiosulfate. 2 ml of cold 20% TCA was added, and the resulting precipitates were freed of inorganic iodide by centrifugation and washing three times with ice-cold 10% TCA, as described previously (10). All final counts of the activity of ^{125}I were corrected for cell-free background activity.

Measurement of [1-^{14}C]glucose oxidation and O_2 consumption. The oxidation of [1-^{14}C]glucose to $^{14}CO_2$ by cell suspensions phagocytizing different particles was measured by previously published techniques (10). The number of cells and particles in each suspension was identical to that described above for comparative iodination, although the volume of suspending medium was increased to 1.5 ml, and in flasks containing POE, the volume added was 0.2 ml (13.3% of final volume).

Oxygen consumption was measured by using a Clark oxygen electrode connected to an oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) and recorder (Fisher Scientific Co., Silver Spring, Md.). The cell suspension in KRP (3.0 ml) contained 20×10^6 granulocytes (6.7×10^6/ml), and after a 20-minute equilibration period at 37°C, the phagocytic particles (0.1 ml volume) were added through the side port. Ratios of particles to cells were as follows: yeast 10:1, latex 750:1, POE 6% of final volume.[8] Oxygen consumption was then measured over the next 30–40 min, and the results expressed as maximal rates of O_2 consumed/min per 10^7 cells.

RESULTS

Fixation of iodide to a protein-bound form by cells ingesting POE. When POE were added to the granulocyte suspensions, medium iodide was converted to a TCA-precipitable form at rates which were significantly greater (2.2- to 7.7-fold) than those seen with non-phagocytizing control cells (Table I). When granulocyte myeloperoxidase activity was inhibited with sodium

[8] At this concentration the content of POE may have been subsaturating with respect to the initial rate of phagocytosis. This concentration was used additions via the port into the incubation chamber were limited to 0.1 ml.

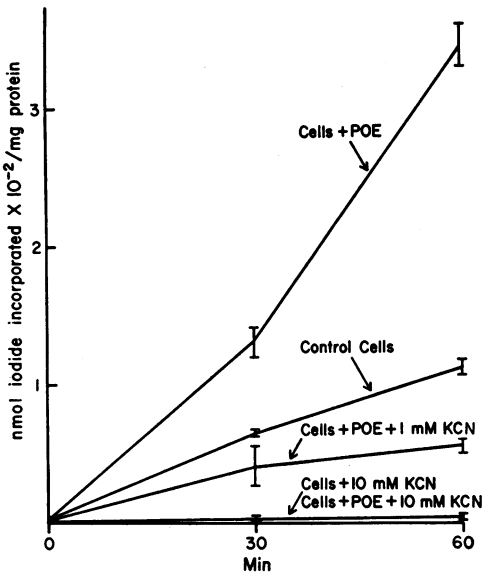

FIGURE 1 Rates of protein iodination by nonphagocytizing (control) cells and by cells ingesting 2% albumin-Fluorochemical liquid of 1.765 density in the presence and absence of the myeloperoxidase inhibitor KCN. Each line is the mean of duplicate determinations, as noted by the brackets.

azide or potassium cyanide, protein iodination was blocked (Fig. 1).

Distribution of protein iodination in subcellular fractions. The distribution of protein and protein-bound ^{125}I ($PB^{125}I$) activity in subcellular fractions of the homogenates presented in Table I are given in Table II. Protein recoveries from resting cell fractions averaged 99.8% of the homogenates and those of $PB^{125}I$ activity paralleled this (mean = 96.0%). With phagocytizing cells, recoveries of both remained high (mean for protein = 92.3%, and for $PB^{125}I$ = 94.0%), but were somewhat more variable. This variability was due in part to difficulties in handling the adherent high specific-activity phagocytic vesicle fraction. As noted, in resting cells the lysosome-rich pellet accounted for a mean 45.7% of cellular protein and almost all iodination activity (93.1%). With phagocytosis, the specific activity of protein iodination in the homogenates increased a mean 5.1-fold, the contribution of the lysosomal pellet to this activity fell to 36.9% and that of the phagocytic vesicle fraction rose from 0.9% to a mean 54.4%. The activity in the supernates remained low (6.1–8.1% of the total) in both resting and phagocytizing cells, despite their accounting for over half the cellular protein content (means 51.3 and 59.1% for resting and phagocytizing cells, respectively). Although only a mean 6.9% of cellular protein was found within the vesicles collected from phagocytizing cells, iodide was fixed to protein in greatest extent therein and with the highest specific activities (4- to 10.5-fold greater than the lysosomal pellets and 43- to 95-fold greater than the supernates). In fact, when the data are expressed in terms of the contribution that each fraction made to the *increment* in activity observed with phagocytosis, the phago-

TABLE III

Contribution of Subcellular Fractions to the Increment in Iodination Occurring with Phagocytosis

Exp	Increment in $PB^{125}I$* activity above resting cells with phagocytosis	Contribution of fractions to the phagocytic increment‡		
		Pellet	Supernate	Phacogytic vesicles
	cpm/mg protein		%	
I	6,715	18.1	4.9	77.2
II	43,589	18.6	3.9	77.7
III	4,060	—	—	72.1
Mean		18.4	4.4	75.7

* ^{125}I activity in a protein-bound form as determined by counting TCA precipitates. Numbers represent the difference between the specific activities of the homogenates for phagocytizing and resting cells, as depicted in Table I.

‡ This value was calculated by determining the difference between the resting and phagocytic activities of each fraction per mg of cellular protein from the percentages given in Table I and then calculating the percent contribution of each fraction to the increment in iodination seen with phagocytosis. In the third experiment, the value for the phagocytic vesicle fraction of resting cells was assumed to be negligible (1% or less of the total activity, see Table I). The value obtained for this fraction from phagocytizing cells was used to represent the increment in iodination seen with phagocytosis.

TABLE IV

*Iodide Fixation to Protein by Cells Phagocytizing Different Particles**

Phagocytic particles	Resting cells fixation	Phagocytizing cells fixation
	nmol (%)‡	*nmol* (%)
Yeast	0.088 (69%)	1.06 (80.5%)
Latex	0.088 (69%)	0.17 (91.0%)
POE	0.028 (39%)	0.053 (61.0%)

* Reaction flasks contained 35×10^6 polymorphonuclear leukocytes (PMN), 16 μM NaI, and 500,000 cpm ^{125}I and particles (yeast/cells = 5:1, latex/cells = 200:1, POE 0.5 ml) in 3 ml of KRP. Both total ^{125}I and TCA-precipitable iodide were measured after a 60-min incubation period.

‡ Values are the mean of duplicate determinations for TCA-precipitable iodide expressed as nmol/10×10^6 cells, with numbers in parentheses indicating the percent of total intracellular iodide this bound fraction represents. The yeast and latex assays were run simultaneously.

TABLE V

Effect of Sodium Azide on Iodide Uptake and Fixation by Granulocytes before and after Yeast Phagocytosis

Status of cells		Iodide uptake mean±SE	Iodide fixation to protein mean±SE
Resting	Control	0.12±0.03* (3)‡	0.11±0.06 (5)
	+1 mM azide	0.09±0.01	0.07±0.02
	% inhibition by azide	27.3±8.0	35.7±15.0
Phagocytizing	Control	2.71±0.59 (4)	2.37±0.42 (6)
	+1 mM azide	0.13±0.02	0.06±0.02
	% inhibition by azide	95.1±1.3	99.6±1.5

* nmol/10^7 PMN/60 min. Mixtures contained 1×10^7 PMN, 500,000 cpm ^{125}I and 16 MM NaI, with or without opsonized yeast (5×10^7) and sodium azide (1 mM).

‡ Number of experiments are shown in parentheses.

cytic vesicles accounted for a mean 75.7% (Table III).

Relationship of iodide fixation to iodide uptake and distribution. To determine if the high specific activtiy of protein iodination within the phagocytic vesicle fraction could be explained by selective trapping of iodide within the vesicles, the distribution of not only protein-bound, but total iodide was measured in different fractions from cells which ingested POE. As shown in Fig. 2, total iodide distribution paralleled that of cellular

TABLE VI

*Estimated Relationship of Intracellular Total and Free Iodide to Extracellular Iodide under Different Conditions**

Status of cells	Ratio of total intracellular to extracellular iodide	Ratio of free intracellular to extracellular iodide
	per mg of cell wt	*μM*
Resting (5)‡	1.34 [0.91–1.61]	0.561 [0.185–0.785]
+1 mM azide (3)	1.10	0.345
Phagocytizing		
POE (2)	1.19 [0.78–1.60]	0.63 [0.61–0.65]
Latex (1)	2.40	0.31
Yeast (5)	28.4 [18.2–38.6]	6.00 [5.07–6.94]
Yeast + 1 mM axide (3)	2.23	1.20

* Starting medium iodide concentrations in all experiments ranged between 16 to 20 μM. Results, mean and [range], are given using the estimated intra- and extracellular iodide concentrations (see text for details) at the conclusion of a 60-min incubation period.

‡ Number of experiments are shown in parentheses.

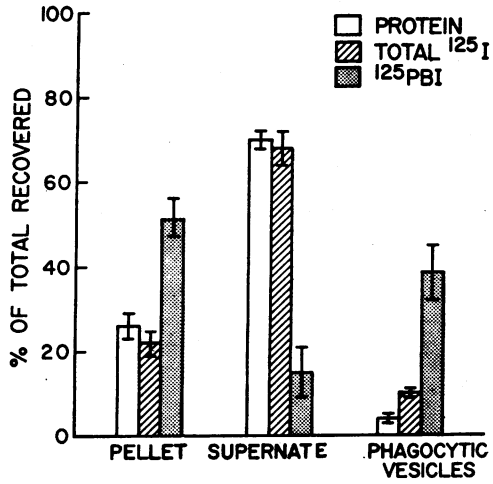

FIGURE 2 Comparative distribution of protein, total ^{125}I and PB^{125}I in subcellular fractions obtained from cells which had previously ingested 2% albumin-paraffin oil emulsion. Each bar represents the mean of duplicate determinations, and the brackets the range.

protein and was highest in the cell supernates followed by the pellets. The phagocytic vesicles accounted for only 5.0% of the total iodide within the cells. More iodide was taken up by the cells than was fixed to protein during phagocytosis (12.1% fixed of the total taken up in the experiment depicted, and 61.0% in a later experiment shown in Table III). 53.6% of iodide in the vesicles was bound to protein, vs. 35.1% in the pellet and only 3.2% in the supernate.

To determine if the type of particle significantly influenced the extent of iodide uptake and intracellular protein iodination, these values were measured in cells which had ingested POE as opposed to yeast or latex particles. Regardless of the particle ingested, the cells always took up more iodide than was fixed to protein (Tables IV and V). After phagocytosis of yeast there was an average of 10- to 20-fold more iodination than after latex or POE ingestion. During phagocytosis, iodide uptake, as well as fixation, was markedly inhibited in the presence of 1 mM sodium azide (Table V).

No direct calculations of cellular weight and water were made to enable a precise determination of intracellular iodide concentrations relative to the medium; however, approximate values were calculated from the available data. By multiplying the cell protein values by 10, approximate cell weights were obtained. Based on the assumption that water comprises 70% of cell weight, a figure for cell water was then derived. Medium iodide concentrations at the end of an incubation period were determined from the total amount of iodide taken up by the cells. This latter figure was also used to calculate intracellular iodide contents and concentrations

TABLE VII

*Interrelationships between Iodination and Other Oxidative Metabolic Activities of Resting and Phagocytizing Granulocytes**

Function	Resting cells	POE	Latex	Yeast‡
		% resting value		
Iodination	0.042±0.005§ (7)	229±30 (9)	408±170 (6)	4,120±1,000 (4)
Oxygen consumption	0.96±0.08‖ (4)	321±19 (3)	810±57 (3)	1,450±450 (4)
[1-^{14}C]glucose oxidation	19.7±5.7¶ (5)	207±38 (5)	646±116 (5)	1,020±240 (5)

* Values given are mean±SE of the number of experiments shown in parentheses under results. If only two experiments were done, the range is given.

‡ Yeast was opsonized by incubation in 50% normal guinea pig serum, as described in Methods.

§ nmol iodide fixed to protein/10^7 PMN/60 min.

‖ nmol oxygen consumed/6.7 × 10^6 PMN/min.

¶ nmol glucose converted to $^{14}CO_2$/10^7 PMN/60 min.

per milligram of cell weight and microliter of cell water. As shown in Table VI, when medium and intracellular iodide concentrations were compared under different conditions, little evidence for significant intracellular iodide accumulation was observed in the absence of phagocytosis. Mean total iodide contents averaged 1.34-fold above the medium, whereas the concentration of free intracellular iodide ions was below that in the medium. Yeast phagocytosis led to the most significant accumulations (a mean 28.4-fold) of intracellular iodide above that in the medium. The concentration of free iodide ion within cells during yeast ingestion was calculated to be 6.0-fold above the medium. Both total and free intracellular iodide concentrations were markedly suppressed to the levels found in the medium in the presence of sodium azide. During phagocytosis of particles other than yeast, no evidence for accumulation of nonorganified iodide ion above medium concentrations was observed. Increased total cellular iodide concentrations above the medium were thus related to their covalent fixation to TCA-precipitable residues in the cells.

Relationship of iodination to other oxidative activities of granulocytes. A series of experiments was then run to compare the ability of different particles to induce iodination as opposed to other oxidative activities reflecting the formation and metabolism of H_2O_2 by granulocytes. The results are shown in Table VII.

Ingestion of an average of 4.5 yeast/cell led to significantly greater stimulation of iodination than that shown by cells saturated with POE or containing a mean > 50 latex particles ($P < 0.01$, two-sample t test). Iodination by cells phagocytizing latex was higher on the average than that shown by cells ingesting POE; however, the means were not significantly different ($P > 0.1$), and in four of six experiments, the increments with phagocytosis were equal. Oxygen consumption and [1-^{14}C]glucose oxidation were also significantly higher for cells phagocytizing yeast or latex than those containing POE ($P < 0.01$ t test). Although the values obtained for these activities were higher on the average for yeast as opposed to latex phagocytosis, the difference between the means was not significant ($P > 0.1$).

Since it has been shown that these activities are dependent to a great extent on the initial rate of phagocytosis (9), a parameter that cannot be equilized for cells phagocytizing different particle types, a more meaningful set of comparisons obtains when the relative stimulation of these three activities by the phagocytosis of the different particles is examined. It was found that POE ingestion stimulated iodination and [1-^{14}C]glucose oxidation equivalently and not significantly differently from oxygen consumption. When latex was phagocytized, iodination was stimulated to a significantly lesser degree than oxygen consumption ($P < 0.05$), while [1-^{14}C]glucose oxidation was intermediate between the two, and was not statistically different from either. With yeast phagocytosis, a disproportionate increase in iodination occurred when compared with both oxygen consumption and [1-^{14}C]glucose oxidation ($P < 0.05$). Stimulation of [1-^{14}C]glucose oxidation was less than that of O_2 consumption, but the differences were not statistically significant.

DISCUSSION

Previous studies have documented that during phagocytosis, granulocytes can concentrate iodide from the extracellular medium and convert it to a covalent form which is precipitated with TCA (3). As in the thyroid, this conversion is impaired by inhibitors of iodide transport (4). However the most marked inhibition is observed when the heme moiety of myeloperoxidase is inactivated by azide or cyanide (4) or when there is a genetic impairment in the formation of its substrate, hydrogen peroxide, as in chronic granulomatous disease of childhood (3). Both conditions are associated with reduced granulocyte bactericidal activity (1, 11). While the number and type of residues iodinated have not been quantitated in the present or previous investigations, such data are available for erythrocytes enzymatically iodinated by similar mechanisms. Using glucose and glucose oxidase as a peroxide-generating system, Hubbard and Cohn (12) were able to demonstrate that several peroxidases, including myeloperoxidase, can stimulate iodide incorporation into erythrocyte membrane components precipitated with TCA. Analysis of these components revealed that 10–15% were extractable with lipid solvents; however, virtually all organic iodide was recovered as monoiodotyrosine. This indicates that this

amino acid, when present in protein or lipoprotein cellular components, is the chief receptor for the iodination reaction. Although not precisely documented, it seems highly likely that organic iodide precipitated by TCA treatment of granulocytes must also largely reside in tyrosine residues found in protein or lipoprotein cellular components. In support of this, Stolc has documented that human granulocytes form not only monoiodo-, but also diiodotyrosine and some thyroxine during phagocytosis in the presence of iodide concentrations similar to those employed in the present investigation (13). Analysis of the increased conversion of iodide to an organic form during phagocytosis thus affords a measurement of intracellular H_2O_2, iodide, myeloperoxidase, and protein interaction.

Using this measurement, the studies reported here demonstrate that the major intracellular operational site of the granulocyte myeloperoxidase-H_2O_2-halide antimicrobial system is the phagocytic vesicle. It was shown that after ingestion of particles contained in a paraffin oil-albumin emulsion, protein iodination was stimulated significantly by phagocytosis, and was blocked by inhibitors of myeloperoxidase. When subcellular fractions were examined, the small amount of activity found in resting cells was localized almost exclusively to the lysosome-rich pellet. Previous studies (7) using the same techniques employed in the present investigation have demonstrated that this fraction contains over 80% of cellular myeloperoxidase activity. After phagocytosis, almost the entire increment in iodination could be recovered from the phagocytic vesicle fraction where iodide was fixed to protein in high specific activity. It has previously been demonstrated that this fraction contains about 17% of cellular myeloperoxidase activity, representing delivery from the lysosomal fraction (7). Despite finding about 20% of cellular myeloperoxidase activity in the cytosol of the cell (7), the marked lack of iodination found within this fraction indicates that in intact cells, H_2O_2 and halide are not utilized by myeloperoxidase in this location, and its detection there may, in fact, represent an artifact of preparation.

Klebanoff's investigations have indicated that the major reactants of the myeloperoxidase-H_2O_2-iodide system must be present in close proximity in order for iodination and antimicrobial activity to occur (2). As noted, myeloperoxidase reaches the vesicles by degranulation from the lysosomal fraction after POE ingestion. More iodide was taken up by cells than that fixed to protein, and its distribution followed that of cellular protein. Its covalent fixation to protein occurred almost exclusively within the phagocytic vesicles, however, presumably reaching these structures by diffusion through the cytoplasm. In agreement with earlier published findings, no apparent intracellular accumulation of iodide was observed by resting cells (4), whereas during phagocytosis, medium iodide was trapped and concentrated within granulocytes. Estimated intracellular concentrations of free iodide ion were less than those in the medium, and uptake as well as fixation was markedly inhibited in the presence of the myeloperoxidase inhibitor, sodium azide. These findings suggest that the increased uptake of iodide with phagocytosis is dependent to a large extent on the provision of a "sink" by the iodination reaction, which traps and binds iodide to protein within the phagocytic vesicles.

Protein iodination was observed to vary with the type of particle ingested and was significantly higher during phagocytosis of yeast than during either latex particle or POE ingestion. This is presumably due in part to the higher protein content of yeast as opposed to the other particles and is consistent with the concept that the ingesed particle provides a major source of receptors for iodination (14). In keeping with this idea, Klebanoff has published electron radiomicrographs which demonstrate localization of iodide grains in the region of ingested bacteria (15). That ingested proteins may not serve as the sole receptors for iodination is suggested by the observation of small but significant amounts of iodination in association with the myeloperoxidase-rich lysosomal pellet of non-phagocytizing cells and the ability of latex particles in protein-free medium to stimulate iodination when phagocytized. In the case of the former, it is conceivable that iodination may be occurring with phagocytic vesicles, preformed by ingestion of the caseinate used to induce the peritoneal exudates. With the latter, it seems quite possible that, in addition to proteins within the vesicle or vesicle membranes potentially serving as recipients for iodination, a certain amount of extracellular iodination may occur due to release of H_2O_2 and myeloperoxidase into the medium. In this regard, preliminary experiments utilizing a highly sensitive technique for the detection of free H_2O_2 (16) have indicated that appreciable amounts of this compound are released from granulocytes during phagocytosis of latex (17). In contrast, little or no increase in peroxide release was observed with POE ingestion. Release of myeloperoxidase during phagocytosis of latex has also been documented (18), and with provision of substrate H_2O_2 in the medium, iodination of extracellular proteins and cellular debris may well occur. This concept is strengthened by earlier observations of significantly more iodination by phagocytizing granulocytes when protein in the form of whole serum or serum albumin is added to the extracellular medium (4). Furthermore, in preliminary studies, significant iodination of granulocytes has been observed in the absence of phagocytosis when peroxide was generated in the extracellular medium with glucose oxidase and glucose (19).

In the experimental systems employed, then, the factors which regulate the extent of protein iodination by granulocytes include the uptake and diffusion of iodide, the delivery of myeloperoxidase to the phagocytic vesicle by degranulation, and the presence of appropriate receptors for iodide fixation. The key stimulus to this reaction, however, would appear to be the increased generation of H_2O_2 as substrate for myeloperoxidase by post-phagocytic metabolic events.

As a probe to study the interplay between these factors, different types of particles were employed as phagocytic substrate. Previous studies have established that rates of degranulation and oxidation of [1-^{14}C]glucose, and presumably oxygen consumption and iodination, are proportional to the initial rate of particle ingestion, reflecting common responses to membrane stimulation (9). Due to the major differences in the size and composition of the particles employed, it is impossible to compare on an absolute scale the rates of uptake. Therefore, this approach is most meaningful when one compares the effect of the same particle on different post-phagocytic metabolic processes. To control the effect of ingestion rate, sufficiently high particle concentrations were employed to ensure maximal stimulation of the various metabolic activities that were attainable with a given particle type. Phagocytosis of yeast particles led to a proportionally greater degree of iodination than did either latex or POE particles when compared to [1-^{14}C]-glucose oxidation and oxygen consumption. All three metabolic activities were proportional to each other during POE phagocytosis. In contrast, ingestion of latex led to proportionally less iodination than was seen with the other activities. Oxygen consumption (20) and [1-^{14}C]glucose oxidation (21) are related to H_2O_2 production, suggesting that this was not the factor limiting iodination of latex particles. There is no evidence that latex and yeast particles induce different degrees of degranulation (18), suggesting that this also was not the limiting factor. Furthermore, phagocytosis of all three particlels caused trapping of iodide from the medium in excess of that fixed to protein. Thus it seems likely that it is the lack of receptors for iodination on the surface of latex particles (14) which serves as the basis for the disassociation between iodination and the other metabolic activities which were stimulated by ingestion of this particle.

The parallel between the stimulation of iodination and, in particular, [1-^{14}C]glucose oxidation by ingestion of all the particle types presumably reflects the simultaneous utilization of peroxide by both myeloperoxidase and the enzymes (21) linking peroxide metabolism to the hexose monophosphate shunt. Whether peroxide is synthesized within phagocytic vesicles during particle ingestion and leaves to enter the surrounding cytoplasm (and extracellular medium) from this site, or enters the vesicles by diffusion from other (e.g., intracytoplasmic) sources, remains to be precisely defined. In this regard, however, the minimal increment in iodination of peroxidase-rich pellet protein with phagocytosis can be interpreted as indicating either little free diffusion of peroxide through the cytoplasm or successful competition for H_2O_2 against peroxidase by intracytoplasmic enzymes, including those linked to the hexose monophosphate shunt and catalase. In support of this concept, catalase has been shown to inhibit myeloperoxidase-mediated iodination and bactericidal activity in cell-free systems (2). An example of successful compartmentalization of myeloperoxidase-mediated iodination (and antimicrobial activity) within the phagocytic vesicle of the granulocytes may thus be afforded by the previous demonstration that these structures are devoid of catalase activity (7). The presence of enzymes within the cytosol for peroxide disposal presumably serves then to protect the cell from the potentially lethal effects of this powerful oxidant, particularly when coupled to peroxidase (22).

Finally, the observation in preliminary experiments (17) that free peroxide appears within the extracellular medium by as early as 15 s after contact between cells and particles suggests that it has arrived there from forming phagocytic vesicles or even from the plasma membrane at the time of particle contact. This is consistent with the hypothesis that the site of H_2O_2 production is plasma membrane which ultimately becomes the phagocytic vesicle. The intracellular localization of the iodination reaction to the phagocytic vesicle may then be further evidence of intravesicle production of this key substrate for normal granulocyte bactericidal activity.

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