Phagolysosomal pH of Human Neutrophils

By Petr Cech and Robert I. Lehrer

Human neutrophils sequester yeast cells or zymosan particles in two classes of phagocytic vacuoles: sealed and unsealed. The pH of sealed vacuoles was measured by a newly devised fluorometric procedure that used fluoresceinated zymosan particles, derived from *Saccharomyces cerevisiae*, as a pH probe. Five minutes after initiation of phagocytosis, sealed vacuoles were alkaline, exhibiting a pH of 7.80 ± 0.19 (mean ± SEM). Their acidity subsequently increased, so that the pH fell to 7.38 ± 0.25 after 15 min, 6.35 ± 0.35 after 30 min, and 5.68 ± 0.26 after 60 min. The implications of these findings for neutrophil microbicidal mechanisms are discussed.

EARLY STUDIES on vacuolar pH, typically performed by exposing phagocytes in vivo to microorganisms and indicator dyes, suggested that appreciable acidification (pH 3–5.5) occurred. More contemporary studies of this phenomenon in human neutrophils (PMNs) have led to seemingly disparate results. Mandell fed indicator-impregnated *Candida albicans* yeast cells to PMN monolayers and reported that 40%–60% of the ingested yeasts developed color changes consistent with a pH of 6–6.5 after 60 min. Jacques and Bainton performed similar studies with bakers yeast (*Saccharomyces cerevisiae*), but noted more vigorous acidification, reporting that 80%–88% of phagosomes in adherent neutrophils attained a pH of 4.5–5.0 after 60 min.

Recently, a quantitative spectrofluorometric method for measuring intravacuolar pH was developed by Okkuma and Poole and by Geisow et al. A particular virtue of this method was the use of dual excitatory wave lengths to ascertain a fluorescence ratio whose magnitude is proportional to pH. As will be shown in the present work, the fluorescence ratio is superior to measurement of absolute fluorescence at a single excitatory wavelength, because it was unaffected by the partial destruction of probe fluorescence by phagocytes noted in our system. Segal et al. adapted this procedure to examine the intraphagosomal pH of monolayered human PMNs and reported that phagocytic vacuoles were initially alkaline, exhibiting a pH of 7.75 after 2 min. Mild acidification subsequently occurred, so that the vacuolar pH was neutral after 15 min and 6.0–6.5 after 2 hr.

We recently reported the existence of two populations of phagocytic vacuoles, "sealed and unsealed," in human neutrophils that had ingested yeast particles and showed that the *Candida albicans* yeasts are killed poorly in vacuoles communicating with external medium ("unsealed" vacuoles). Because of the implications of this phagosomal heterogeneity for intraphagosomal pH and for systems involved in microbicidal activity, the aforementioned observations stimulated us to reexamine the intraphagosomal pH of human neutrophils.

MATERIALS AND METHODS

Neutrophils

Neutrophils were prepared from heparinized normal venous blood by a modification of Boyum's technique, made erythocyte-free by brief hypotonic lysis, and suspended in Ca²⁺-free Krebs-Ringer phosphate solution, pH 7.4, containing 1 g/liter each of glucose and bovine serum albumin (KRP) or Hanks' balanced salt solution (HBSS, Grand Island Biological Co., Santa Clara, CA).

Fluorescence Assay

Fluorescein isothiocyanate, isomer I (FITC, Sigma Chemical Co., St. Louis, MO) was coupled to zymosan particles (Sigma) by minor modifications of the method of Marshall et al. and Cochrane. Briefly, 30 mg of FITC was dissolved in 4.5 ml of 0.5 M sodium carbonate buffer, pH 9.0, and 19.5 ml of distilled water. Ninety milligrams of zymosan, suspended in 6 ml of water, was added and incubated for 30 min at room temperature with mixing. The resulting fluoresceinated zymosan (FZ) preparation, stored sterilely in plastic tubes at 4°C, remained useful for 1–2 mo. Prior to use, 1–2 ml of this preparation was washed by repeated centrifugation in 0.9% NaCl, until >99.5% of the total residual fluorescence was released by incubation at 37°C. This fluorescence was then redissolved in KRP to produce a solution having a fluorescent yield of 1% of their fluorescence in 2 hr.

Washed FZ particles were opsonized in 1 ml of AB serum for 30 min at 37°C, washed twice in 0.9% NaCl, and suspended in KRP or HBSS. In a few experiments, as described in the text, KRP was modified to contain 0.16 mM instead of 16 mM phosphate buffer. FZ concentration was determined by direct hemocytometer counts or by spectrophotometric measurements, with equivalent results.

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Phagocytosis was initiated by mixing 5 x 10⁶ PMNs and 1 x 10⁶ opsonized FZ in 2 ml of KRP in small capped plastic tubes (Falcon no. 2054, Oxnard, CA), that were incubated and rotated (30 rpm) for various times at 37°C. At intervals, triplicate 200-μl samples (A, B, and C) were removed for fluorescence measurements. A 10-μl sample was transferred to a glass slide, mixed with 10 μl of 2 mM N-ethyl maleimide in phosphate-buffered saline, coverslipped, and examined with fluorescence epi-illumination to determine the proportion of FZ that was not cell-associated.

Sample A was mixed with an equal volume of aqueous 1% sodium deoxycholate, pH 7.4, to lyse the leukocytes and entrap the liberated FZ in a viscous clear gel. Sample B was mixed with an equal volume of KRP containing 10% swine skin gelatin, type I (Sigma). Sample C was centrifuged for 60 sec in an Eppendorf Model 1200 Microfuge to deposit both cells and FZ, and the supernatant was removed and mixed with an equal volume of KRP-gelatin, as for sample B. The deoxycholate or gelatin gel-stabilized samples (A or B) were transferred to 6 x 50 mm borosilicate glass tubes (Kimble Corp., Owens, IL) that served as microcuvettes, and fluorescence was measured in an Aminco Bowman spectrophotofluorimeter (American Instrument Co., Silver Spring, MD) thermostatted at 37°C and adapted to hold the microcuvettes. Stabilization of the samples in gelatious matrices prevented settling and agglutination of the particles, affording stable and reproducible measurements of fluorescence. It was our practice to obtain samples A, B, and C in duplicate.

One-millimeter entrance and exit slits, each corresponding to a bandpass of 5.5 nm, were used. The instrument’s sensitivity was adjusted so that 10⁻⁶ M fluorescein in 0.1 M NaOH read 80% of maximum on the 10 scale with an excitation (F′) of 485 nm and an emission of 530 nm. The instrument was blanked at 450 nm with a solution of PMNs in gelatin-KRP buffer, at the concentrations employed in our assays. As the same blank cuvette then registered a reading of 0.1–0.2 relative intensity units at F′ 485, experimental readings at F′ 485 were corrected by subtracting this value.

PMNs in KRP-gelatin solution remained 95%-100% viable, judged by trypan blue exclusion. From the geometry of our spectrophotofluorimeter and the conditions in our microcuvette, we calculated that the fluorescent signal recorded in our experiments arose from approximately 5 x 10⁶ FZ particles. The procedure used to calculate the intracellular pH, and the rationale of its corrections for noningested FZ, destroyed and released FITC, and FZ in “unsealed vacuoles” will be presented in the Results section. The percentage of “sealed” and “unsealed” vacuoles was routinely examined with heat-killed C. albicans and methylene blue, as recently described. In addition, we determined the percentage of cell-associated FZ in unsealed vacuoles. Because methylene blue did not quench the fluorescence of FZ unless concentrations that were toxic to neutrophils were employed, we employed a final concentration of 0.8% trypan blue for these studies. Although it did not stain PMNs, 0.8% trypan blue completely quenched the fluorescence of extracellular FZ, or in companion experiments, that of FITC-labeled heat-killed C. albicans yeasts. Intracellular FZ or FITC-C. albicans in sealed phagosomes maintained their fluorescence in the presence of 0.8% trypan blue. The principle of differential fluorescence quenching to distinguish intracellular from extracellular microorganisms has been described by Hed et al.⁸⁻¹³

Our final method for assaying unsealed vacuoles by fluorescence quenching entailed incubating PMNs with opsonized FZ or FITC-C. albicans, then mixing 50 μl of the suspension with 50 μl of 1.6% trypan blue in phosphate-buffered saline. Twenty microliters of this mixture was placed on a microscope slide, coverslipped, and examined by phase-epifluorescent microscopy. One hundred neutrophils were examined, and the total number of fluorescent particles contained within them was recorded (B). Another sample of the phagocyte mixture was counted without addition of stain, and the number of fluorescent particles/100 PMN was also noted (A). The percentage of cell-associated particles in sealed vacuoles was calculated as: (B/A) x 100.

**Effect of External pH and Buffer Concentration on Assay**

In some experiments, PMNs and opsonized FZ were incubated together in a 5-ml siliconized water-jacketed reaction vessel (model 20013, Pierce Chemical, Rockford, IL) with access ports for a small pH electrode (Radiometer, Copenhagen, Denmark) and for intermittent addition of microliter quantities of 0.1 N NaOH to maintain external pH at 7.4 ± 0.02 U. The vessel contained a Teflon-coated magnetic stirring bar and was maintained at 37°C by a circulating water bath, allowing us to study uptake of opsonized FZ in minimally buffered KRP (0.16 mM phosphate) at constant external pH.

At intervals after addition of FZ, 400-μl samples were removed and mixed with an equal volume of standard or minimally (0.16 mM) buffered KRP containing 10% gelatin at pH 4–10. Half of this sample was immediately transferred to 6 x 50 mm glass microcuvettes for fluorescence measurements, and the remainder was used to measure extracellular pH with a combined glass electrode and to check cell viability and phagocytosis.

**Standard Curves**

In addition to generating standard curves with free, opsonized, or glutaraldehyde-fixed FZ (Fig. 1), we also devised a standard curve using phagocytic, glutaraldehyde-fixed PMNs that had ingested opsonized FZ (Fig. 2). The latter were made by incubating PMNs and opsonized FZ for 30 min at 37°C, as described above, centrifuging the mixture at 180 g for 5 min, then resuspending the PMN-FZ in 1.25% (v/v) glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 30 min at 4°C. The fixed PMN-FZ were thereafter washed twice with KRP and suspended in gelatin-stabilized buffers of various pH.

**Oxygen Consumption**

Neutrophils and opsonized FZ (final concentrations, 1 and 2 x 10⁷/ml, respectively) were introduced into a 1.2-ml water-jacketed chamber that contained a Clarke-YSI electrode and a small magnetic stirring bar. PMNs with FZ were run simultaneously as a control. Measurements of oxygen consumption were made with a Gilson Medical Electronics Dual Channel Oxygraph, Model K-1C, at 37°C for 60 min.

**RESULTS**

**Standard Curves**

FITC-labeled zymosan particles (FZ) fluoresced, after excitation at either 485 or 450 nm, in direct proportion to their concentration and the fluorescence ratio (FR), i.e., light emitted by FZ after excitation at 485 nm/light emitted following excitation at 450 nm, remained constant over a range of FZ concentrations from 1.5 to 10 x 10⁷ FZ/ml (data not shown). We selected a concentration of 2.5 x 10⁷ FZ/ml for our studies. As shown in Fig. 1, FR was linearly related to pH, between pH 4.5 and approximately 8.0. Neither glutaraldehyde fixation nor opsonization by serum significantly affected the relationship between FR and pH. The inset in Fig. 1 demonstrates that free FITC
and samples A and C of our assays follow the same FR/pH relationship manifested by the main figure.

FZ sequestered within neutrophil vacuoles might display different fluorescent properties than FZ in free suspension. To examine this possibility, we fixed PMNs containing ingested FZ with glutaraldehyde, reasoning that the subcellular compartments of such fixed nonviable PMNs would manifest the ambient extracellular pH. Data obtained with these glutaraldehyde-fixed preparations are shown in Fig. 2. Note that
a linear relationship between FR and pH persisted, but that the curve was slightly shifted from that shown in Fig. 1. In the pH range between 6.0 and 8.0 (FR = 3.6–5.5), pH values derived from any given value of FR differ by less than 0.3 pH units, when Figs. 1 and 2 are compared. At more acid pH values, the discrepancy increases, approximating 0.7 pH units at pH 4. As will be seen subsequently, most of our experimentally determined values for intravacuolar pH fell between pH 6 and 8, where the two standard curves were essentially equivalent. We elected to use the relationships between FR and pH obtained with glutaraldehyde-fixed phagocytic PMNs to analyze our data, and prepared similar standard curves for each new batch of FZ.

Factors Influencing Fluorescence Measurements

During preliminary investigations, we identified four factors whose contribution to the fluorescent signal of our assay mixtures warranted attention. These factors were: (1) incomplete uptake of FZ by PMNs, (2) destruction of FZ fluorescence by PMNs, (3) release of fluorescent molecules into the supernatant, and (4) the existence of a class of incompletely sealed phagocytic vacuoles whose contents could exchange relatively freely with the external medium. Measurements of the distribution of cell-associated FZ into sealed and unsealed vacuoles were made by observing fluorescence quenching by trypan blue. We noted virtually identical proportions of unsealed vacuoles when ingestion of heat-killed C. albicans and FZ were compared (Table 1).

Table 1. Comparison of Fluorescence Quenching and Trypan Blue Staining Procedures in Determination of Percent Unsealed Vacuoles in Phagocytic Neutrophils

<table>
<thead>
<tr>
<th>Subject</th>
<th>Fluorescence Quenching</th>
<th>Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FZ</td>
<td>FITC-C. albicans</td>
</tr>
<tr>
<td>1</td>
<td>33.7 ± 3.6</td>
<td>42.3</td>
</tr>
<tr>
<td>2</td>
<td>36.8 ± 4.0</td>
<td>40.8</td>
</tr>
<tr>
<td>3</td>
<td>46.8 ± 4.3</td>
<td>44.3</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>38.7 ± 4.0</td>
<td>40.5 ± 2.2</td>
</tr>
</tbody>
</table>

Table 2 shows data pertaining to these four factors. Phagocytosis of opsonized FZ, although substantial, was incomplete. Approximately 3% of the particles remained unassociated with PMNs at each time point. The small signal contributed by the free extracellular zymosan would have an FR characteristic of the extracellular pH, 7.4. Destruction of fluorescence occurred and was quantitated from the “A” (deoxycholate-treated) samples, described in Materials and Methods. This was rapid in onset, with a mean decrease in fluorescence of 25.3% after 5 min and 32.5% after 60 min. Although this would have imposed a major obstacle to recording intravacuolar pH had we used absolute values of fluorescence at a single excitatory wavelength, it did not confound the dual-wavelength FR approach that was used. As already shown, the residual neutrophil-associated FZ fluorescence (Fig. 2) maintained an FR/pH relationship approximating that of the original FZ preparation (Fig. 1). Release of fluorescence from FZ to a nonsedimentable extracellular form also occurred. This accounted for approximately 3% of the initial FZ fluorescence at 5 min and approximately 14% after 60 min and must have been consequent to degradation of FZ by phagocytic PMNs. Negligible spontaneous elution of soluble fluoresceinated molecules from FZ occurred in cell-free mixtures. Excitation and emission spectra of the solubilized fluoresceinated material closely resembled that of FITC or FZ, so that the FR of this material also “reported” the pH of the extracellular medium.

Calculation of Intravacuolar pH

The overall fluorescent signal from our assay cuvettes arises from four sources: (1) FZ in sealed vacuoles, (2) FZ in unsealed vacuoles, (3) uningested FZ, and (4) free (non-FZ-associated) fluorescent material. By measuring the overall fluorescent signal and the magnitude of any three of its four components, one can calculate the signal emanating from the fourth.

This approach is illustrated by Tables 3 and 4, which contain data and calculations referable to a single time
nce (B) 4, part C, and the algebraic process is given in Table 4, calculate the

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fluorescence from free FITC (C).

unsealed vacuoles are shown in Table

point of one experiment. The five measured values A, B, C, m, and n are shown in Table 3, part A. The frequency of uningested FZ (n) was determined by
direct phase-fluorescent microscopy. The proportion of unsealed vacuoles was measured with heat-killed C. albicans and methylene blue. Fluorescent measure-
ments at F'485 are denoted by A, B, or C; those at F'450 are symbolized by the corresponding primes.

The equations used to calculate the signals arising from sealed and unsealed vacuoles are shown in Table 4, part C, and the algebraic process is given in Table 4, part D. Equation 1 states that observed total fluorescence (B) equals fluorescence from sealed vacuoles (x) + fluorescence from unsealed vacuoles (y) + fluorescence from free FITC (C) + fluorescence from extracellular FZ [n(A − C)], and may be simplified to the form shown in equation 2 by letting a = B − [C + n(A − C)]. Equation 3 states that the fluorescence measured in the deoxycholate lysate (A) arises from all of the formerly ingested particles (d) plus contributions from formerly uningested FZ [n(A − C)] and free FITC (C). Equation 4 is equivalent to equation 3, merely defining d in terms of the measured variables.

Equations 3 and 4 establish how much fluorescence cell-associated FZ would manifest if the vacuolar pH was 7.4. Since unsealed vacuoles are presumed to be in equilibrium with the extracellular medium at pH 7.4, it follows (equation 5) that fluorescence from FZ in unsealed vacuoles (y) equals the proportion of FZ in unsealed vacuoles (m) times d. By substituting this value for y in equation 2, we derive equation 6, which defines the fluorescence arising from sealed vacuoles in terms of the measured parameters.

By following the same process for measurements made at 450 nm, x' and y' can be obtained, allowing calculation of the fluorescence ratio from sealed vacuoles (x'/x) or unsealed vacuoles (y'/y) by equation 7. Equation 8 demonstrates how fluorescence ratio

Table 3. Measured and Derived Fluorescent Values Used in Calculating Intravacuolar pH

<table>
<thead>
<tr>
<th>Symbol(s)</th>
<th>F'485</th>
<th>F'450</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, A'</td>
<td>3.715</td>
<td>0.775</td>
</tr>
<tr>
<td>B, B'</td>
<td>2.33</td>
<td>0.51</td>
</tr>
<tr>
<td>C, C'</td>
<td>0.725</td>
<td>0.145</td>
</tr>
<tr>
<td>m</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Equations and Calculations Used to Derive Intravacuolar pH

<table>
<thead>
<tr>
<th>C. Equations</th>
<th>D. Calculations</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) B = x + y + C + n(A − C)</td>
<td>(5) y = md</td>
</tr>
<tr>
<td>(2) x + y = a</td>
<td>(6) x = a − md</td>
</tr>
<tr>
<td>(3) A = d + C + n(A − C)</td>
<td>(7) FRu = x/x', FRw = y/y'</td>
</tr>
<tr>
<td>(4) d = A − C − n(A − C)</td>
<td>(8) pH = (FR + 1.02)/0.81 (Fig. 2)</td>
</tr>
</tbody>
</table>

The measured data displayed in Table 3 were used to solve the equations for fluorescence ratio (eq. 7) and consequently to derive the mean pH (eq. 8) of sealed vacuoles.

Neutrophils were incubated with opsonized FZ for 60 min, and the measurements listed in part A were obtained as described in the text and used to calculate the values shown in part B. Please consult both the text and Table 4 for details of the equations and calculations used. The symbol prime (') denotes measurements made at 450 nm.
is converted to pH by reference to standard curves generated by experiments such as that illustrated in
Fig. 2 (FR = 0.81 pH + 1.02).

These calculations are illustrated in Table 4, part D, showing vacuolar acidification after 60 min by normal
neutrophils in a single experiment. The FRaw of 4.748
corresponds to a pH of 7.16 in unsealed vacuoles. The
FR of 3.038 corresponds to an “average” pH of 5.01 in
the sealed vacuole.

**Extracellular Buffering Capacity and pH**

In addition to the four factors already enumerated that
influenced the fluorescent signal arising from
phagocytic vacuoles containing FZ, we evaluated the
effects of extracellular buffering capacity on these
measurements. In three experiments, we found that
the signals were independent of the buffer concentra-
tion (16 mM or 0.16 mM KRP) present during the
phase of ingestion or measurement (data not shown).
We obtained similar results when HBSS replaced
KRP in other experiments (not shown).

**Intravacuolar pH of Human Neutrophils**

As shown in Fig. 3, the pH of sealed human neutro-
phils phagocytic vacuoles containing FZ was biphasic.
During the initial postphagocytic period, the mean pH
was mildly alkaline, 7.8 ± 0.19 (mean ± SEM, n = 6),
after 5 min. We did not attempt earlier measurements,
preferring to wait until virtually all added FZ had
become ingested. By 15 min, the intravacuolar pH had
returned to near neutrality (7.38 ± 0.25). After 30
min, the mean pH was 6.35 ± 0.35, and by 60 min it
had fallen further to 5.68 ± 0.26. The mean pH of
unsealed vacuoles was 7.37 ± 0.03 throughout this
60-min period.

It was of interest to compare these kinetics to those
of oxygen consumption induced by exposing neutro-
phils (10⁷/ml) to twice that number of opsonized FZ in
an oxygen electrode. The values for O₂ consumption
(nmole/10⁷ neutrophils/min) were as follows: unstimu-
lated, 1.1 ± 0.1; 5 min, 8.7 ± 0.9; 15 min, 6.0 ± 1.0;
30 min, 3.9 ± 0.8; and 60 min, 2.4 ± 0.8 (mean ±
SEM, n = 3). Maximal oxygen consumption occurred
between 1.2 and 3.2 min after addition of FZ and had a
rate of 16.9 ± 1.2 nmole/10⁷/min. Thus, the period of
intravacuolar alkalosis corresponded to the period of
maximal “respiratory burst” activity, and the later
vacuolar acidification occurred when oxidative met-
abolism had returned to near resting rates. Examina-
tion of the rate of destruction of fluorescence by
neutrophils (Table 2) indicated that 77.8% of the
destruction had already occurred by 5 min, during the
period of maximal oxidative metabolism. We made no
attempt to determine the nature of the destructive
process.

**DISCUSSION**

The recent introduction of covalently bound, fluo-
rescent pH probes that can be measured quantitatively
promised to overcome some of the limitations inherent
in the older visual techniques of assessing vacuolar pH.
Ohkuma and Poole used FITC-labeled dextran, inter-
nalized by pinocytosis, to demonstrate that the pH of
secondary pinolysosomes in murine peritoneal macro-
phages was approximately 4.7.4 Geisow et al. con-
firmed their results, and by coupling fluorescein to
Saccharomyces cerevisiae with a carbodiimide ligand,
modified the procedure so that it could be applied to
phagolysosomes.5 They reported that the phagocytic
vacuoles of murine peritoneal macrophages were tran-
siently alkaline (pH 7.75) 2 min after induction of
phagocytosis. Thereafter, the vacuoles rapidly acid-
ified, reaching a pH of approximately 5.8 after 7 min
and finally equilibrating at a pH of 5.4 ± 0.2 after
15–20 min. Segal et al.6 used fluorescein-labeled
Staphylococcus aureus to assess the intravacuolar pH
of human neutrophils in monolayer culture and
reported the initial intraphagosomal pH to be alkaline
(pH 7.75 after 2 min), with gradual acidification to pH
7.0 after 15 min and pH 6.0–6.5 after 2 hr.

Our own experiences with fluorescent probe meth-
ology suggest that while fluorescent particles may
provide elegant pH probes, they must be used with an
understanding of the biologic complexities of phago-
cytes. We identified several important factors that
contributed to the fluorescent signal emerging from
suspensions of normal neutrophils and fluorescent
zymosan (FZ). These included: destruction of fluo-
rescent molecules by postphagocytic leukocytic events,
solubilization of fluoresceinated molecules from FZ
and their release into the external medium, incomplete

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*Fig. 3. Intraphagosomal pH of normal human neutrophils. Neutrophils from 6 healthy subjects were incubated with opson-
ized FZ for 60 min, and fluorescent analysis of intravacuolar pH
was performed. Solid circles represent the pH of sealed vacuoles,
which constituted approximately 60% of the total. Open circles
represent the average pH of sealed plus unsealed vacuoles.*
uptake of FZ by neutrophils (minor), and the existence of two classes of phagocytic vacuoles, sealed and unsealed. Because we determined a fluorescent ratio (FR) that correlated with pH, rather than absolute values of fluorescence intensity in our measurements, destruction of 25%–33% of the overall fluorescence by phagocytic neutrophils did not compromise the integrity of our measurements. We devised procedures to measure the other factors quantitatively, and our experimental results allowed derivation of the FR arising from sealed phagocytic vacuoles, which, in our system, comprised approximately 60% of the total vacuole population.

We found, in confirmation of the report by Segal et al.,⁶ that sealed vacuoles initially demonstrated a mild alkalosis, manifesting a pH of 7.80 ± 0.19, 5 min after initiation of phagocytosis. Acidification, i.e., attainment of a pH below 7.0, first appeared between 15 and 30 min after ingestion, concomitant with the waning of respiratory burst activity. By 60 min, the mean pH of sealed vacuoles was 5.68 ± 0.26. This value resembles the equilibrium pH values reported for murine peritoneal macrophages containing Saccharomyces cerevisiae (5.4 ± 0.2), but is lower than the values reported by Segal et al. for human neutrophils containing fluoresceinated Staphylococcus aureus (pH 6.0–6.5). Although technical factors, such as the use of monolayers or different particles, might explain these discrepancies, it should be noted that the values of Segal et al. were uncorrected for any effects of uningested bacteria, unsealed vacuoles, and released FITC. To the extent that any of these factors were present, they would tend to mask a lower pH signal from phagocytic vacuoles with a signal whose FR reflected the extracellular milieu.

Several aspects of neutrophil behavior may be influenced by intravacuolar pH. That sealed vacuoles are neutral to slightly alkaline for 15–30 min should favor the early action of granule and vacuolar membrane enzymes with concordant pH optima. Among the neutrophil enzymes optimally active at this pH range are NAD(P)H oxidase¹⁵–¹⁶ and various neutral proteases.¹⁵,¹⁶ One neutral protease, a chymotrypsin-like enzyme, has been reported to have antibacterial¹⁷,¹⁸ and antifungal activity¹⁹ in vitro. It is noteworthy that the protein’s bactericidal activity against S. aureus or Streptococcus faecalis is exhibited in vitro only at or above pH 7.0, conditions now shown to exist for at least 15 min after ingestion of zymosan particles, as in our study, or Staphylococcus aureus.⁶

The evident temporal dissociation between respiratory burst activity, maximal quite early in the postphagocytic response, and vacuolar acidification, which develops after attenuation or cessation of the burst, is of considerable interest in considering myeloperoxidase-mediated antimicrobial mechanisms. Myeloperoxidase, a well-characterized component of neutrophil primary (azurophil) granules, displays optimal antimicrobial activity in vitro at pH 4.5–5.0 in various cell-free model systems.¹ Inactive by itself, myeloperoxidase requires the simultaneous presence of H₂O₂ and halide ions to effect microbial killing. However, our data, and that of Segal et al., suggest that H₂O₂ production by intact neutrophils would be maximal when the phagocytic vacuoles have a pH between 7.0 and 7.8. Although human neutrophils acidified their vacuoles to an “average” pH of 5.7 after 1 hr, their H₂O₂-generating oxidative metabolism (measured indirectly as enhanced O₂ consumption) was <10% of its earlier maximal rate by this time. Thus, we conclude that myeloperoxidase operates in intact phagocytic vacuoles quite far from the conditions that support its optimal antimicrobial activity in model systems. Possibly, this partially accounts for the discrepancy between the potency of myeloperoxidase in such cell-free models and the relatively infrequent clinical sequelae²⁰–²⁵ attending its absence from intact neutrophils.

The coincidence of vacuolar alkalization and respiratory burst activity may well be a consequence of the alkalinizing effect of the dismutation of the superoxide anions produced by NAD(P)H oxidase during the respiratory burst.²⁶ This reaction may be written: 2O₂⁻ + 2H⁺ → H₂O₂ + O₂, or alternatively: 2O₂⁻ + 2H₂O → H₂O₂ + O₂ + 2OH⁻. Observations that neutrophils from subjects with chronic granulomatous disease, deficient in O₂⁻ production, acidify their vacuoles more promptly than normal⁶,²⁶ and lack the initial phase of alkalosis⁶ are consistent with this view. It is possible that such premature acidification deprives chronic granulomatous disease neutrophils of the antibacterial activities of their chymotrypsin-like cationic protein by rapidly achieving a pH below 7.0. Evidence that vacuolar alkalization by permeant buffers promotes staphylocidal activity by chronic granulomatous disease neutrophils has been advanced by Segal et al.⁶ but requires confirmation.

ACKNOWLEDGMENT

We thank Lenore E. Cohen for excellent technical assistance.

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Phagolysosomal pH of human neutrophils

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