PHAGOCYTOSIS BY HUMAN LEUKOCYTES, PHAGOSOMAL pH AND DEGRADATION OF SEVEN SPECIES OF BACTERIA MEASURED BY FLOW CYTOMETRY

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SUMMARY. Phagocytosis by human leukocytes, phagosomal pH and degradation of seven species of bacteria were studied by a flow cytometric method. The percentage of phagocytosing leukocytes was similar for all bacterial strains examined, but *Salmonella typhi* and *Neisseria meningitidis* were more slowly phagocytosed than other bacteria. The phagosomal pH surrounding the different bacterial species 15 min after the start of phagocytosis were: *Streptococcus pneumoniae* 4·4; *N. meningitidis* 4·9; *Str. pyogenes* 5·1; *Staphylococcus aureus* 5·2; *Escherichia coli* 5·3; *S. typhi* 5·4; and *Klebsiella pneumoniae* 5·7. For longer incubation periods, the phagosomal pH remained nearly constant. *Staph. aureus*, *E. coli* and *S. typhi* were the most readily degraded of the species tested. The proteins of all bacteria were degraded more rapidly than their DNA as determined by measurements of the loss of fluorescein-isothiocyanate-fluorescence and ethidium bromide-fluorescence, respectively. The rate of degradation varied from one bacterial species to another. The degradation of proteins and DNA was maximal for bacteria residing in a phagosomal environment estimated to be between pH 5·2 and 5·4.

INTRODUCTION

With the use of various species of bacteria it has been demonstrated that differences in microbial structure may affect the interaction between phagocyte and microorganism (Elsbach, 1980). Capsular material may inhibit phagocytosis and surface proteins may influence ingestion and intracellular killing (Mims, 1982). Some authors have claimed that certain bacterial components, such as DNA, remain relatively intact after phagocytosis (Elsbach, 1980) whereas others have assumed that ingested microorganisms are almost completely degraded by phagocytosing cells (Steinman and Cohn, 1974). During phagocytosis the phagosomal pH becomes acidic, but there is still some controversy about the degree of acidification (Rous, 1925; Mandell, 1970; Jacques and Bainton, 1978; Segal *et al.*, 1981).

We have recently developed a flow cytometric (FCM) method for the study of phagocytosis by leukocytes (Bassøe, Solsvik and Laerum, 1980; Bassøe *et al.*, 1983b; Bjerknes and Bassøe, 1983). The assay is based on the phagocytosis of fluorescein-

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isothiocyanate (FITC)-labelled bacteria, and the discrimination and quantification of free bacteria, phagocytes and non-phagocytic cells by the simultaneous measurement of single-cell FITC-fluorescence and narrow-angle light scatter. Bacterial FITC-fluorescence, measured by FCM, is a sensitive indicator of pH and the fluorescence of phagocyte-associated bacteria is related to phagosomal pH (Bassøe *et al.*, 1983a). After phagocytosis, degradation of bacterial proteins and DNA can be estimated by FCM (Bassøe, 1984).

The present investigation is a systematic comparison of phagocytosis by human leukocytes, phagosomal pH and bacterial cell degradation with seven species of bacteria.

MATERIALS AND METHODS

Leukocytes. Heparinised (10 U/ml) human blood samples (10 ml) from each of 21 healthy volunteers were mixed with 3 ml of Macrodex 6% w/v (mol. wt 70 000; Pharmacia, Uppsala, Sweden) and allowed to sediment for 1 h at 20°C. After centrifugation of the leukocyte-rich plasma (400g for 5 min), the erythrocytes were lysed with NH₄Cl 0.8% w/v. The leukocytes were then washed in NaCl 0.85% w/v, supplemented with bovine serum albumin (BSA) 2% w/v (Sigma Chemicals, St Louis, MO, USA) and resuspended in Hanks's balanced salt solution (HBSS) (Flow Laboratories, Irvine) containing BSA 2% w/v. Total leukocyte counts were made with a Coulter Counter (Coulter Electronics, Harpenden) and differential counts by FCM measurements of cells stained with acridine orange (Hartman-Leddom Company, Philadelphia, PA, USA) by the method of Melamed *et al.* (1972) (*vide infra*). The fraction of non-lymphocytic cells, i.e., polymorphonuclear neutrophil leukocytes (PMNLs) and monocytes, was measured and the suspensions adjusted to 10⁷ non-lymphocytic cells/ml.

Serum. Pooled fresh, human serum from 10 healthy volunteers was stored in 1-ml portions at -80°C and, immediately before use, 1 ml of freshly thawed serum was diluted with 3 ml of HBSS containing BSA 2% w/v.

Bacteria. The strains of bacteria used were: *Staphylococcus aureus* S-209; *Streptococcus pyogenes* S-84 (β -haemolytic, group A); *Str. pneumoniae* type 1; *Klebsiella pneumoniae* CF10; *Neisseria meningitidis* NCTC10025; *Escherichia coli* S-618 and *Salmonella typhi* HJ. The bacteria were cultured overnight in Penassay Broth (Difco), washed twice with NaCl 0.85% w/v, killed by heat (60°C for 1 h) and labelled with FITC as described by Bassøe *et al.* (1983b). All preparative solutions were filtered through filters of 0.2- μ m pore size to avoid contamination. The bacteria were counted by FCM with leukocytes as internal reference (Bassøe *et al.*, 1983b) (*vide infra*) and adjusted to 10⁹ bacteria/ml in HBSS. Portions were stored at -80°C and thawed at room temperature immediately before each experiment.

Incubation. Leukocyte suspension (0.5 ml), bacterial suspension (0.1 ml) and diluted serum (0.4 ml) were mixed in disposable plastic tubes (12 $\times$ 75 mm) to give a ratio of 20 bacteria/non-lymphocytic cell and a final concentration of serum of 10% w/v. The tubes were incubated at 37°C for 15, 30 and 60 min with end-over-end rotation to promote contact between bacteria and leukocytes. Phagocytosis was terminated by the addition of 3 ml of ice-cold NaCl 0.9% w/v containing EDTA 0.02% w/v. The suspensions were then analysed by FCM.

Flow cytometry. A Cytofluorograf Ortho 50H, interfaced to a model 2150 Computer (Ortho Diagnostic Instruments, Westwood, USA), with an excitation wavelength of 488 nm was used. For differential counting of acridine orange-stained leukocytes, green fluorescence was measured at 515–575 nm and red fluorescence at 600–650 nm (Melamed *et al.*, 1972). For the phagocytosis assay, FITC-fluorescence was measured at 515–575 nm and narrow-angle light scatter at 488 nm (Bassøe *et al.*, 1983b; Bjerknes and Bassøe, 1983). When studying the intracellular degradation of bacteria, bacterial FITC-fluorescence was measured at 515–575 nm and ethidium bromide-fluorescence at 600–650 nm (Bassøe, 1984).

Phagocytosis. After phagocytosis, free bacteria, phagocytes and non-phagocytic cells were discriminated and quantified by the combined measurement of single-cell FITC-fluorescence and narrow-angle light scatter (Bassøe *et al.*, 1980; Bassøe *et al.*, 1983b; Bjerknes and Bassøe,

1983). From these measurements the following parameters were calculated (Bassøe *et al.*, 1983b):

1. *The percentage of phagocytosing leukocytes* was the number of fluorescent leukocytes divided by the total leukocyte count and multiplied by 100;
2. *The bacteria-per-phagocyte numbers* were the differences between the initial and final numbers of free bacteria per phagocytosing leukocyte.

Phagosomal pH. The fluorescence of FITC-labelled bacteria is a sensitive indicator of pH, because the bacterial fluorescence intensity is reversibly reduced when the environmental pH becomes acidic (Bassøe *et al.*, 1983a). Thus, ingested FITC-labelled bacteria show less fluorescence than extracellular bacteria (Bassøe *et al.*, 1983a). Because only minor amounts of bacteria and fluorescent bacterial degradation products are released from the phagocytes within the incubation periods studied, the reduction of FITC-fluorescence of the phagocyte population corresponds to the phagosomal acidification (Bassøe *et al.*, 1983a).

For determination of phagosomal pH, the FITC-fluorescence signals of extracellular free bacteria and phagocytes were gated to make separate histograms, and the mean fluorescence of free bacteria and phagocytes determined (Bassøe *et al.*, 1983a). The phagosomal pH was then calculated as described below (Bassøe *et al.*, 1983a):

1. *Phagocyte fluorescence (bacterial units) (Nf)* was the mean FITC-fluorescence of the phagocyte population divided by that of the free bacteria (fe) and multiplied by 100;
2. *The mean relative fluorescence of single phagocyte-associated (f) and free bacteria* was calculated from the relationship $f/fe = Nf/N$ where N is the mean number of bacteria per phagocytosing leukocyte;
3. *Phagosomal pH* was estimated from the f/fe values, using a standard curve of the relationship between FITC-fluorescence and environmental pH as described previously (Bassøe *et al.*, 1983a).

Degradation of bacteria. When bacteria are stained with FITC under standardised conditions, the fluorescence is closely proportional to their total protein content (Bassøe, 1984). Bacterial DNA can be stained with ethidium bromide (EB) and the DNA content quantified from bacterial EB-fluorescence (Bassøe, 1984).

In the phagosome, ingested bacteria are exposed to enzymes which digest the micro-organisms. When ingested bacteria are liberated from the phagocytes, reduced staining by EB is observed, and this has been used as an estimate of intracellular degradation of bacterial DNA (Bassøe, 1984). After liberation of ingested FITC-labelled bacteria by disruption of the phagocytes, a permanent loss of bacterial FITC-fluorescence is revealed. This reduction of FITC-fluorescence has been used as an estimate of intracellular degradation of bacterial proteins when measured at a pH of 7.4 (Bassøe, 1984).

To liberate the phagocyte-associated bacteria, the plasma membrane and the cytoplasm of the leukocytes were dissolved with the detergent Nonidet (NP40). After incubation for 15, 30 and 60 min, 100 μ l of each phagocytic suspension was added to 5 ml of a solution containing the detergent, RNAase (Sigma) and EB (Sigma) 1 mg/ml (Bassøe, 1984). Bacteria mixed with leukocytes immediately before the addition of detergent-EB solution served as controls. The intracellular degradation of phagocytosed bacteria was estimated, as described by Bassøe (1984), as follows:

1. *Degradation of bacterial proteins (%)* was the mean FITC-fluorescence of the bacteria in the phagocytic suspensions (F) divided by that of the controls (F_0) and multiplied by 100;
2. *Degradation of bacterial DNA (%)* was the mean EB-fluorescence of the bacteria in the phagocytic suspensions (F) divided by that of the control bacteria (F_0) and multiplied by 100;
3. *Crude phagosomal protease or DNAase enzymatic activity (E)* was calculated from the loss of bacterial FITC- or EB-fluorescence by the formula:

$$E = -\frac{1}{t} \ln \frac{F}{F_0}$$

where t is the incubation time.

Statistical methods. Significance of correlation was determined by a student's t -test or by Wilcoxon's rank-sum test for paired samples. All measurements were performed at least three times on three different days with leukocytes from different volunteers.

RESULTS

Phagocytosis

The initial rate of phagocytosis of *S. typhi* and *N. meningitidis* was lower than that for the other bacteria (table I). However, the maximal number of *S. typhi* per phagocyte, measured after 60 min, was only slightly lower than that for *Str. pyogenes*, *Str. pneumoniae* and *K. pneumoniae* ($0.05 > p > 0.02$), *Staph. aureus* ($0.02 > p > 0.01$) and *E. coli* ($0.01 > p > 0.001$). In addition, the maximal number of *N. meningitidis* per phagocyte, measured after 60 min, was similar to that of *Str. pyogenes*, *Str. pneumoniae* and *K. pneumoniae*. The fraction of phagocytosing leukocytes was about 70% for all species of bacteria examined and subpopulations of phagocytes with different FITC-fluorescence were not observed.

TABLE I

Phagocytosis by human leukocytes of seven species of bacteria measured by flow cytometry

Species	Mean number* of bacteria/phagocytosing leukocyte $\pm$ SEM after incubation for		
	15 min	30 min	60 min
<i>Staph. aureus</i>	18.6 $\pm$ 1.1	19.7 $\pm$ 0.1	19.7 $\pm$ 0.3
<i>E. coli</i>	18.2 $\pm$ 1.2	19.1 $\pm$ 0.4	19.7 $\pm$ 0.2
<i>Str. pyogenes</i>	18.9 $\pm$ 0.5	19.3 $\pm$ 0.3	19.3 $\pm$ 0.4
<i>Str. pneumoniae</i>	18.4 $\pm$ 0.6	18.5 $\pm$ 0.6	19.1 $\pm$ 0.3
<i>K. pneumoniae</i>	18.4 $\pm$ 1.0	18.8 $\pm$ 0.3	19.2 $\pm$ 0.5
<i>N. meningitidis</i>	14.3 $\pm$ 0.3	16.2 $\pm$ 0.8	18.3 $\pm$ 1.0
<i>S. typhi</i>	13.8 $\pm$ 1.3	16.1 $\pm$ 0.3	17.7 $\pm$ 0.3

*Mean of three separate assays.

Phagosomal pH

After a period of 15 min, the phagosomal pH surrounding *Str. pneumoniae* was estimated to be 4.4, which was significantly lower than that for all the other bacteria (table II). The phagosomal environment of *K. pneumoniae* had the highest pH, namely

TABLE II

Phagosomal pH surrounding seven species of bacteria measured by flow cytometry

Species	Mean pH* $\pm$ SEM after incubation for		
	15 min	30 min	60 min
<i>Str. pneumoniae</i>	4.4 $\pm$ 0.1	4.5 $\pm$ 0.2	4.4 $\pm$ 0.1
<i>N. meningitidis</i>	4.9 $\pm$ 0.2	5.0 $\pm$ 0.1	4.9 $\pm$ 0.3
<i>Str. pyogenes</i>	5.1 $\pm$ 0.3	5.1 $\pm$ 0.3	5.1 $\pm$ 0.4
<i>Staph. aureus</i>	5.2 $\pm$ 0.2	5.3 $\pm$ 0.1	5.2 $\pm$ 0.1
<i>E. coli</i>	5.3 $\pm$ 0.1	5.4 $\pm$ 0.4	5.3 $\pm$ 0.1
<i>S. typhi</i>	5.4 $\pm$ 0.3	5.5 $\pm$ 0.2	5.4 $\pm$ 0.1
<i>K. pneumoniae</i>	5.7 $\pm$ 0.2	5.6 $\pm$ 0.1	5.5 $\pm$ 0.2

*Mean of three separate assays.

c. 5.7, and the other species of bacteria resided in phagosomal environments of intermediate pH. During longer incubation periods, the phagosomal pH remained nearly unaltered for the seven species of bacteria examined (table II).

Degradation of bacterial protein

For each species of bacteria examined, the mean FITC-fluorescence of bacteria from disrupted phagocytes at a pH of 7.4 decreased with increasing incubation time (figs 1 and 2) indicating intracellular degradation of bacterial proteins. After a period of 60 min, the FITC-fluorescence of *E. coli* and *Staph. aureus* was reduced by 45% and 44%, respectively, indicating that the proteins of these two bacteria were more readily broken down than the proteins of any other bacteria examined ($0.02 > p > 0.01$). For incubation periods longer than 15 min, *K. pneumoniae* showed more protein degradation than *N. meningitidis* and *Str. pneumoniae* ($0.01 > p > 0.001$). The proteins of the other species of bacteria were digested to the same extent (fig. 2).

Degradation of bacterial DNA

The mean EB-staining of each species of bacteria was reduced with increasing incubation time (figs 1 and 2), indicating intracellular degradation of bacterial DNA. After a period of 60 min, the DNA of *E. coli* and *S. typhi* was degraded by 32% and 33%, respectively. The DNA of *E. coli* was more rapidly degraded than that of *K. pneumoniae* ($0.01 > p > 0.001$), whereas the DNA of *S. typhi* decayed more rapidly than that of *N. meningitidis* and *Str. pneumoniae* ($0.01 > p > 0.001$). For the other bacteria the observed differences were not significant (fig. 2).

Relative protein and DNA degradation

The decay of bacterial FITC-fluorescence was compared with the decay of EB-fluorescence. The results indicated that the proteins of *Staph. aureus*, *Str. pyogenes*, *K. pneumoniae* and *E. coli* were degraded more rapidly than their own DNA ($0.01 > p > 0.001$) (fig. 2). For the other bacteria, the rate of DNA degradation did not differ significantly from the rate of protein degradation. However, in all parallel experiments, bacterial proteins were more extensively digested than DNA (fig. 2).

Phagosomal enzyme activities

The rate constants of crude phagosomal protease and DNAase activities were estimated from the loss of bacterial FITC- and EB-fluorescence after incubation for 15, 30 and 60 min. The protease activities diminished by about 12% in the period from 15 to 30 min, and then by about 35% during the next 30 min ($p < 0.01$ using Wilcoxon's rank-sum test). The DNAase activities were compared at the time intervals 0–30 min and 30–60 min of incubation. The DNAase activities in the latter were about 25% less than in the former ($p < 0.01$ using Wilcoxon's rank-sum test).

FIG. 1.—Three-dimensional computer drawings showing FITC- and EB-fluorescence of *E. coli* at 0, 30 and 60 min after the start of phagocytosis. The combined FITC- and EB-fluorescence measurements, performed by FCM, were based on analysis of about 30 000 bacteria. The vertical axis represents the number of bacteria.

FIG. 2.—Human phagocyte degradation of bacterial proteins (●—●) and DNA (○—○) from seven species of bacteria measured by FCM. Each point represents the mean $\pm$ SEM of three experiments with leukocytes from three different individuals. The mean fluorescence values of bacteria mixed with non-phagocytosing leukocytes served as controls, and were set to 100%.

Phagosomal pH and degradation of bacteria

To compare the estimates of phagosomal pH with the digestion of bacterial proteins and DNA, the bacteria were arranged according to increasing values of phagosomal pH (fig. 3). As determined by the loss of FITC- and EB-fluorescence, *Staph. aureus*, *E. coli* and *S. typhi* were most readily degraded by human phagocytes. These bacteria resided in a phagosomal environment the pH of which was 5.2–5.4. The degradation of bacterial proteins and DNA was maximal within this range of phagosomal pH.

DISCUSSION

In the present investigation, FCM was used to study several parameters of phagocytosis by human leukocytes, phagosomal pH and the digestion of seven species of bacteria.

In parallel experiments, *N. meningitidis* and *S. typhi* were more slowly phagocytosed than the other bacteria. However, the percentage of phagocytosing leukocytes was similar for all the bacteria used and subpopulations of phagocytes, differing in FITC-fluorescence, were not observed. These findings indicate a lower rate of

FIG. 3.—The relationship between degradation of bacterial proteins (A) and DNA (B) and phagosomal pH measured by FCM. Each value represents the mean of three experiments with leukocytes from three different individuals. ○ = *Str. pneumoniae*; △ = *N. meningitidis*; □ = *Str. pyogenes*; ● = *Staph. aureus*; ▲ = *E. coli*; ■ = *S. typhi*; and x = *K. pneumoniae*.

phagocytosis of *N. meningitidis* and *S. typhi* by each phagocyte, and not the absence of phagocytosis or poor phagocytosis by a subpopulation of the leukocytes.

There has been some controversy about the degree of phagosomal acidification during phagocytosis. Thus, early investigators reported that the phagosomal *pH* may become as low as 3.0 (Rous, 1925) whereas Mandell (1970), and later Segal *et al.* (1981), reported only mild acidification (*pH* 6.5) during phagocytosis. We found that the fall of phagosomal *pH* varied from one bacterial species to another and that the maximal phagosomal acidification ranged from about *pH* 4.4 to 5.7, confirming our own preliminary data (Bassøe *et al.*, 1983a) and those of other workers (Sprick, 1956; Jensen and Bainton, 1973; Jacques and Bainton, 1978; Geishow, D'Arcy-Hart and Young, 1981).

The fall in phagosomal *pH* following phagocytosis has been attributed to production of lactic acid (Klebanoff and Clark, 1978), to modification in Donnan equilibrium (Jensen and Bainton, 1973) or to the presence of H⁺-pumping ATPase (Jacques and Bainton, 1978). Yet other reports have indicated that degranulation into the phagosome may be of importance (Styrt and Klempner, 1982; Bassøe *et al.*, 1983a). Because the various species of bacteria were all heat-killed and then incubated with leukocytes from the same volunteers and with serum from a standard source, the observed differences of maximal phagosomal acidification with the different species can probably be attributed to their different bacterial properties. Thus, whatever mechanism is responsible for phagosomal acidification, it seems likely to be influenced by the surface properties of the particle to be ingested.

The acidic phagosomal environment is important for microbial killing. Intracellular killing by the peroxidase-hydrogen peroxide system depends on an acid *pH* (Klebanoff, 1975) and acidity promotes the rate of superoxide reduction to hydrogen peroxide (Stossel, 1974). However, phagosomal acidity *per se* may be bactericidal to some microorganisms, such as pneumococci (Klebanoff and Clark, 1978). In this connection it is interesting to note that the lowest phagosomal *pH* observed by us was that surrounding *Str. pneumoniae*.

It has been claimed that bacterial DNA undergoes little or no detectable degradation after phagocytosis (Cohn, 1963; Elsbach *et al.*, 1973; Elsbach, 1980). However, digestion of DNA-anti-DNA complexes by human PMNLs and monocytes is documented (Lamers, de Groot and Roos, 1982) and DNAase has been demonstrated in PMNLs (Baggiolini, 1972). In accordance with this, we found extensive reduction of EB-staining indicating degradation of bacterial DNA during phagocytosis.

As evaluated by loss of FITC- and EB-fluorescence, we observed significant variations in protein and DNA degradation among different bacterial species. The staining properties of bacteria have been claimed to correspond approximately to the degree of structural disorganisation that accompanies bacterial digestion. Thus, gram-positive bacteria are held to be more rapidly degraded than gram-negative bacteria (Elsbach, 1980). Our findings suggest no such systematic difference in either the rate or extent of protein and DNA degradation between gram-negative and gram-positive bacteria.

The bacteria that were most readily degraded during phagocytosis resided in a phagosomal *pH* of *c.* 5.2–5.4. The digestion of bacterial proteins and DNA was maximal within this range of *pH*, which is close to the *pH*-optimum for RNAase, DNAase and most phagosomal hydrolases (Barret, 1969).

With the use of the decay of bacterial FITC-fluorescence and EB-staining, respectively, as estimates of crude phagosomal protease and DNAase activities, both enzyme activities were found to have diminished with increasing incubation time. It has been reported that phagocytosing leukocytes inactivate their own phagosomal enzymes during phagocytosis (Voetman *et al.*, 1981), a suggestion in accord with our findings.

In conclusion, significant variations in the rate of phagocytosis by human leukocytes, phagosomal acidification and degradation of seven different species of bacteria were demonstrated. Thus, not only the ingestion but also phagosomal acidification and degradation seemed to depend on the species of bacterium interacting with the phagocyte. The observed differences should be taken into consideration when examining phagocytic functions in patients. The FCM technique used here is sensitive and allows the demonstration of small differences among different bacterial species.

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