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Authors

Joiner, KA
Ganz, T
Albert, J
et al.

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The Opsonizing Ligand on *Salmonella typhimurium* Influences Incorporation of Specific, but Not Azurophil, Granule Constituents into Neutrophil Phagosomes

Keith A. Joiner,* Tomas Ganz,§ John Albert,‖ and Daniel Rotrosen‡

*Laboratory of Parasitic Diseases and ‡Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892; §Pulmonary Division, University of California at Los Angeles School of Medicine, Los Angeles, California 90024; and ‖Division of Molecular Virology and Immunology, Georgetown University, Rockville, Maryland 20852

Abstract. Phagosomes were purified from human neutrophils ingesting *Salmonella typhimurium* opsonized with adsorbed normal human serum or with rabbit IgG. Constituents within the phagosome were endogenously labeled by supplying the cells with ^{125}INa during phagocytosis. Lactoferrin and vitamin B_{12} binding protein (TC1 and TC3), markers for specific granules, were present in the phagosomes from neutrophils ingesting *S. typhimurium* opsonized with IgG but were 3.5- to 5-fold less prominent in phagosomes from cells phagocytosing *Salmonella* bearing C3 fragments only. In contrast, iodinated azurophilic granule components, most prominently defensins, were the major constituents in phagosomes prepared under both opsonization conditions. Furthermore, labeled complement (CR1 and CR3) and immunoglobulin (FcγRIII) receptors were incorporated in the phagosome regardless of the ligand mediating phagocytosis. These results suggest that the ligand–receptor interactions mediating phagocytosis influence incorporation of neutrophil-specific granule contents into phagosomes.

The biochemical composition of neutrophil phagosomes is incompletely characterized. Although it is generally accepted that the membrane of the phagocytic vesicle is derived from the plasma membrane of the neutrophil, the extent to which the phagosome is modified during or after the process of internalization is not well understood. In particular, the influence of variations in the ligand–receptor systems involved in the phagocytic process upon the ultimate composition of the phagosome is largely unknown.

Earlier studies characterized the enzyme content of phagocytic vacuoles, examined transport functions of the neutrophil plasma membrane after phagocytosis, and demonstrated that plasma membrane constituents are incorporated into the phagosomes (9, 20, 50, 51, 54, 57, 58). Experiments with surface labeled polymorphonuclear leukocytes (PMN)[1] and macrophages have demonstrated losses in plasma membrane constituents during phagocytosis (20, 50, 57, 58) and, in several cases, the appearance of these molecules in the phagocytic compartment has been documented. In at least one instance, differential loss of functional surface receptor activity for the Fc portion of IgG (Fc receptor) with no loss of functional surface C3b receptor activity was suggested during phagocytosis of an antibody-coated particle by macrophages (39). There have been fewer attempts to purify and characterize either the membrane or soluble contents of the phagocytic vacuole. In macrophages, the composition of endocytic vesicle and phagosome membranes resembles the plasma membrane (34, 37). Purified macrophage phagosomes contain complement and Fc receptors (38) as well as large amounts of actin and intermediate filaments (56). Recently, Skubitz and Kinkead (49) characterized neutrophil phagosomes containing serum-opsonized paraffin oil. Using the neutrophil-specific monoclonal antibody AHN-1, which recognizes the lacto-*N*-pentofucose moiety (36), proteins consistent in molecular weight with the C3b receptor, CR1, and members of the Cd18/Cd11 family, CR3, LFA-1, and p150,95 (43), were identified in the phagocytic vesicle.

We have purified and characterized PMN phagosomes containing *Salmonella typhimurium*. Emphasis was placed on identifying and comparing the majority of the components within the phagosome under the different opsonization conditions. Using the technique of endogenous iodination (26, 28) to label components of interest, we found that phagocyte receptors and azurophil granule components, particu-

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K. Joiner's present address, where reprint requests should be sent, is Division of Infectious Disease, P. O. Box 3333, Yale University School of Medicine, New Haven, CT 06510.

[1] *Abbreviations used in this paper*: DFP, diisopropyl fluorophosphate; Fc, Fc portion of IgG; MPO, myeloperoxidase; NHS, normal human serum; NPGB, nitro phenyl guanidino benzoate; PMN, polymorphonuclear leukocytes.

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larly defensins, were incorporated into the phagosome and indiscriminantly iodinated, regardless of the ligand coating *S. typhimurium*. For defensins, this represents the first evidence that these microbicidal peptides enter the phagolysosome during phagocytosis of bacteria. In contrast to the results with azurophil granule markers, iodinated lactoferrin and vitamin B_{12} binding protein (TC1/TC3), markers for neutrophil-specific granules, were incorporated more extensively into phagosomes containing IgG-sensitized *S. typhimurium* than when adsorbed normal human serum (NHS) was used as the coating ligand. These are the first results to suggest that inclusion of secondary granule constituents into neutrophil phagosomes is influenced by the ligand on the particle being ingested.

Materials and Methods

Buffers and Reagents

The following buffers and reagents were used in these experiments: Hanks' balanced salts solution (HBSS) containing 0.15 mM $CaCl_2$ and 1 mM $MgCl_2$; iodination buffer, consisting of HBSS containing 0.3 mM $CaCl_2$, 2 mM $MgCl_2$, and 2×10^{-6} M NaI; lysis buffer, consisting of 2% NP-40, 0.5% Na deoxycholate, 100 mM NaCl, 10 mM Tris, 0.5% NaN_3, 50 μM nitro phenyl guanidino benzoate (NPGB) (Sigma Chemical Co., St. Louis, MO), 20 μM leupeptin (Boehringer Mannheim Biochemicals, Indianapolis, IN), and 20 μM pepstatin (Calbiochem-Behring Corp., La Jolla, CA); relaxation buffer, consisting of 100 mM KCl, 10 mM Pipes, 3 mM NaCl, and 3.5 mM $MgCl_2$, pH 7.38; and relaxation buffer containing 2.5 mM EGTA.

Bacteria, Growth Media, and Metabolic Labeling

S. typhimurium, strain RG108 (kindly provided by Dr. Robert Goldman, Abbott Laboratories, North Chicago, IL) was used for all experiments. Characteristics of this strain and conditions for growth, metabolic labeling with [^{35}S]methionine, or surface iodination have been described previously (15, 22).

Antibodies and Antisera

Rabbit anti-human lactoferrin (Cappel Laboratories, Malvern, PA) and rabbit anti-mouse IgG1 and anti-kappa light chain (Bio-Rad Laboratories, Richmond, CA) were purchased. Rabbit anti-TC1 and -TC3 was kindly provided by Dr. Robert Allen, University of Colorado, Denver, CO. This antiserum also recognizes lactoferrin, as determined by ELISA and immunoblot, although the ELISA titer was 64- to 128-fold lower than the titer of antilactoferrin (Joiner, K. A., unpublished observations). mAb IB4 (IgG2a), directed against the common β chain (Cd18) of CR3, LFA-1, and p150,95, was kindly donated by Sam Wright (Rockefeller University, New York). mAb 1B4 (IgG2a), recognizing CR1, was provided by John O'Shea (National Institutes of Health, Bethesda, MD). mAb 44D (IgG1,kappa), also directed against CR1, was a gift from Victor Nussenzweig (New York University, New York). mAb 3G8 (IgG1,kappa), recognizing FcγRIII, was generously provided by Howard Fleit (State University of New York, Stony Brook, NY).

Phagocytosis

Neutrophils were prepared using Ficoll-Hypaque, dextran sedimentation, and hypotonic lysis and were treated with the protease inhibitors diisopropyl fluorophosphate (DFP), NPGB, pepstatin, and leupeptin, as reported before (2, 8, 22). Bacteria were opsonized exactly as described (22) by incubation in either (*a*) 10% NHS or C8D (serum deficient in the eighth component of complement), both adsorbed before use (22–24) with *S. typhimurium* to remove natural antibodies, or (*b*) in 50 μg/ml of IgG directed against the RG108 outer membrane. The conditions were chosen in initial experiments designed to achieve equivalent phagocytosis when the two ligands were compared. Unlabeled bacteria or metabolically labeled ^{35}S-bacteria were mixed with PMN for phagocytosis at a final bacteria-to-cell ratio of 50:1 as described previously. In experiments in which endogenous iodination was done using unlabeled bacteria, ^{125}INa was added (1 mci/10^8 PMN), and phagocytosis was performed in iodination buffer. Reactions were stopped, noningested bacteria were removed, and cell viability was determined as before (22).

Preparation of Neutrophil Phagosomes Containing *S. typhimurium*

Neutrophils containing *S. typhimurium* were suspended in relaxation buffer at 4°C and ruptured in a nitrogen cavitation bomb after a 20-min exposure to N_2 at 350 psi (7). The homogenate was centrifuged at 80 *g* for 5 min at 4°C to remove unbroken cells. The supernatant was applied to a continuous sucrose gradient (34 ml) ranging from 28 to 75% sucrose, layered over 3 ml of 75% sucrose in 25 × 89-mm Ultraclear tubes (Beckman Instruments, Inc., Fullerton, CA). Gradient tubes were centrifuged at 25,000 rpm for 1 h at 4°C in an SW28 rotor in an L8 M70 ultracentrifuge (Beckman Instruments, Inc.). The gradients were fractionated at a flow rate of 2 ml/min, and the A_{280} of the gradient fractions was measured continuously (Instrumentation Specialities Co., Lincoln, NE). In some experiments, the density of selected fractions was calculated from the refractive indicies of the sample.

Selected fractions were analyzed by SDS-PAGE using the Laemmli system (29). Autoradiography (^{125}I) was performed either on dried gels or after electrophoretic transfer (53) of the constituents in the gel to polyvinylidene difluoride membranes (Immobilon; Millipore Continental Water Systems, Bedford, MA).

In some experiments, phagosome fractions were analyzed by two-dimensional electrophoresis according to the method of Hochstrasser et al. (18). Samples from endogenously labeled (^{125}INa) phagosome pools were precipitated with 10% TCA at 4°C for 10 min. Precipitates were washed with cold absolute ethanol, solubilized in 10% SDS containing 2.3% DTT, and then diluted 1:5 in ampholyte buffer and electrophoresed as described (18).

To identify iodinated granule proteins within phagosomes, selected sucrose gradient fractions were treated with unlabeled KI (1 mM final concentration) and were acidified by the addition of acetic acid to 5% final concentration to dissolve granule proteins. The solution was cleared by centrifugation at 12,000 *g* for 10 min, and an aliquot of the supernatant was analyzed by electrophoresis in 12.5% acid-urea polyacrylamide gels. Aliquots of the supernatant were also concentrated by vacuum centrifugation (Speed-Vac; Savant Instruments, Hicksville, NY) and analyzed by 10–20% gradient SDS-PAGE.

In Vitro Iodination of Purified Defensins by H_2O_2-myeloperoxidase (MPO)-^{125}I

Individually purified defensins HNP-1, HNP-2, and HNP-3 were prepared as described previously (13). MPO for these experiments was a generous gift of Dr. Inge Olsson (University of Lund, Lund, Sweden). Iodination was performed by mixing 8 μg MPO, 7.5 μg each of HNP-1, HNP-2, and HNP-3, 0.5 mM H_2O_2, and 25 μCi of carrier-free K-^{125}I (Amersham Corp., Arlington Heights, IL) in a final volume of 200 μl of 0.34 M sucrose, 10 mM Na phosphate, pH 7.4. After a 30-min incubation at 37°C, the mixture was treated with acetic acid to 5% final concentration and analyzed on 12.5% acid-urea polyacrylamide gels.

Autoiodination in PMN Granule-enriched Fractions

Granule-enriched fraction was prepared by homogenization of 10^8 PMN in 0.34 M sucrose, 10 mM Na phosphate, pH 7.4, in a Potter-Elvehjem homogenizer (Thomas Co., Philadelphia, PA) until >90% cells were broken as judged by phase-contrast microscopy. Intact cells, nuclei, and cell debris were removed by centrifugation at 200 *g* for 10 min. The granules were collected by centrifugation in an Eppendorf microcentrifuge (Brinkmann Instruments Co., Westbury, NY) at 14,000 *g* for 10 min and resuspended in 1 ml of the same buffer. A 100-μl aliquot of this suspension was mixed with 10 μCi of K^{125}I, and H_2O_2 was added to 0.5 mM. The samples were incubated for 30 min at 37°C with another equal addition of H_2O_2 at 15 min. In some experiments, radioiodination was terminated before electrophoretic analysis by the addition of unlabeled potassium iodide and sodium thiosulphate to 1 mM final concentration each. After addition of acetic acid to 5% final concentration, an aliquot of the sample was analyzed by 12.5% acid-urea PAGE or lyophilized and analyzed by 10–20% SDS-PAGE.

Immunoprecipitation of Radiolabeled Constituents from Extrinsically Labeled Bacteria and from Plasma Membrane and Phagosome Peaks Purified on Sucrose Density Gradients

All immunoprecipitation experiments were performed in the same manner. Pools of defined volumes from gradient fractions were mixed one part sample to two parts lysis buffer and rotated at 4°C overnight. Pools of equal volume (proportional to cell equivalents) were used in all immunoprecipitation experiments comparing results with different ligands. The detergent-insoluble residue was removed by centrifugation at 12,500 *g* for 30 min, and the supernatant was "precleared" by rotation on Sepharose 4B in lysis buffer at a ratio of 200 μl of resin to 1 ml of supernatant. Then 10 μl of the appropriate antiserum or ascites or 100 μl of monoclonal tissue culture supernatant was added, and the mixtures were rotated for an additional 12-16 h at 4°C. When the primary antibody was rabbit antiserum or a mouse monoclonal antibody other than IgG1, 20 μl of protein A-sepharose was added. When the primary antibody was a monoclonal of the IgG1,kappa isotype, 20 μl of protein A-Sepharose bearing rabbit anti-mouse IgG1 and rabbit anti-mouse kappa light chain was added. Rotation at 4°C was continued for an additional 4 h. Samples were washed five times in lysis buffer, the final pellet was solubilized in 50 μl of SDS-PAGE sample buffer, and 40 μl of sample was loaded per lane for analysis by SDS-PAGE. Since the volume of the starting pool for immunoprecipitation and the volumes of antiserum, protein A-sepharose, and SDS-PAGE sample buffer were equivalent for all samples, the relative intensities of bands on SDS-PAGE autoradiograms can be directly compared for different opsonization conditions.

Electron Microscopy of Fractions from Sucrose Density Gradients

Samples from gradient fractions were pooled, diluted 1:2 in relaxation buffer, and centrifuged at 25,000 rpm at 4°C in an SW55 rotor (Beckman Instruments, Inc.) for 30 min. The supernatant was discarded, and the pellet was fixed in a mixture of paraformaldehyde and glutaraldehyde in cacodylate buffer followed by osmium tetroxide in cacodylate buffer. Thin sections (60-80 nM) were cut and were stained with uranyl acetate and lead citrate.

Electron Microscopy of Whole Neutrophils

Tissue Preparation. Cells were fixed in 0.25% glutaraldehyde in 0.1 M PO_4, pH 7.4, at 4°C for 1 h (6). They were washed in 5% D-trehalose dihydrate (No. 18, 835-2; Aldrich Chemical Co., Milwaukee, WI) in 0.1 M PO_4, pH 7.4. The cells were incubated in 0.5 M trehalose in 0.1 M PO_4, pH 7.4, overnight at 4°C.

Freezing and Thin Sectioning. The tissue was frozen in propane cooled with liquid nitrogen and quickly transferred to liquid nitrogen. Thin sections (pink, blue, and green) were cut at a temperature of about −90°C on an Ultracut E (Reichert-Jung, Vienna, Austria) using an FC4E low temperature sectioning system with a glass knife. They were picked up on glycerol and placed on formvar- and carbon-coated 160 mesh hexagonal grids.

Labeling. All grids were floated on drops at room temperature throughout the labeling procedure. All grids were incubated in protein A at a concentration of 500 μg/ml in 0.1 M PO_4, pH 7.4, for 30 min. They were washed in a BSA buffer, consisting of 1% BSA plus 10 mM glycine in 0.1 M PO_4, pH 7.4, three times for 5 min. Grids were then incubated in either rabbit anti-human lactoferrin diluted 1:200 with BSA buffer or the BSA buffer alone for 1 h. All grids were washed three times for 5 min in BSA buffer. Then they were transferred to protein A-coupled 10-nm gold (Janssen Life Sciences Products, Piscataway, NJ) diluted 1:20 with BSA buffer for 30 min. The grids were washed in PO_4, pH 7.4, postfixed in 3% glutaraldehyde for 5 min, washed in water, and finally stained for 2 min in 7.7% uranyl acetate in water followed by 2% polyvinyl alcohol plus 0.1% uranyl acetate in water.

Grids were examined in an electron microscope (EM-300; Philips Electronic Instruments, Inc., Mahwah, NJ) at 60 KV.

Table I. Phagocytosis and Killing of S. typhimurium RG108 by Human Neutrophils

Ligand	Neutrophil pretreatment condition	Bacteria/PMN
None	Buffer	0.1
Adsorbed NHS	Buffer	17
Adsorbed NHS	NaN_3, NPGB, leupeptin	14
Adsorbed NHS	DFP, NPGB, leupeptin, pepstatin	13
IgG	Buffer	19
IgG	DFP, NPGB, leupeptin, pepstatin	13

The phagocytosis assay was done as described previously (22) and in Materials and Methods. Briefly, preopsonized bacteria and neutrophils pretreated as shown in the table were prewarmed, mixed at a ratio of 50:1, and rotated for 10 min at 37°C. Noningested bacteria were removed by washing, and the number of bacteria per cell was assessed by staining. Results represent the mean for two experiments in which 100 cells were counted for each condition. Greater than 95% of neutrophils had associated bacteria, and greater than 85% of bacteria were intracellular.

Results

Phagocytosis of S. typhimurium RG108 by Neutrophils

The RG108 strain of *S. typhimurium* used in these experiments was rapidly and extensively ingested by neutrophils after opsonization with adsorbed NHS or IgG. Ingestion of *S. typhimurium*, which under the growth conditions used was smooth and nonpiliated, was negligible in the absence of exogenous ligand (42, 60) (Table I). Since pretreatment of neutrophils with protease inhibitors decreased phagocytosis only marginally, phagolysosomes for our studies were obtained from neutrophils that were treated with protease inhibitors before mixing with opsonized bacteria.

Separation of Phagosomes on Sucrose Density Gradients

Distinct separation was obtained on sucrose density gradients between normal neutrophil constituents and phagosomes prepared with RG108 incubated in adsorbed NHS (Fig. 1, *a* and *b*). Compared with opsonized bacteria not phagocytosed by neutrophils, bacteria liberated from neutrophils by N_2 cavitation were shifted upward in the gradient (Fig. 1 *b*). A peak of MPO activity (27) was consistently found at tubes 28-32 in the presence (Fig. 1 *b*) but not in the absence (not shown) of *Salmonella* ingestion by neutrophils, suggesting that MPO was incorporated into the phagosome.

Electron Microscopy of Sucrose Density Gradient Fractions

To confirm the identity of the phagosome fraction, pools from the sucrose density gradient profile shown in Fig. 1 *a* were analyzed by transmission electron microscopy. Phagosome pools were heavily enriched for bacteria, surrounded partially or completely by an additional membrane (Fig. 2). Electron-dense material, possibly derived from granules, was observed in the phagosome space. Bacteria were visualized in various states of degradation.

Preparation and Characterization of Phagosomes from Neutrophils Ingesting S. typhimurium in the Presence of ^{125}INa

We next sought to identify components involved in phagosome formation. Neutrophils halogenate particulate targets during phagocytosis via the MPO-H_2O_2-halide system (26, 28) and, hence, will iodinate targets if supplied with the halide ^{125}INa. Phagosomes were prepared from neutrophils ingesting *S. typhimurium* in the presence of ^{125}INa. One-dimensional SDS-PAGE analysis was done on selected fractions from the sucrose density gradients (Fig. 3, adsorbed NHS lanes). A variety of labeled constituents (80, 60, 44, 24, 17, and 14 kD) are present within the cytosol and plasma membrane pools. Iodinated constituents of >10 kD are minimal in the portions of the gradient corresponding to either specific granules (SG lanes) or azurophil granules (not shown). In contrast, labeled constituents of >200, 150-170, 95, 75, 45, 37, and 28 kD and multiple components <17 kD are present in the phagosome band but not in corresponding tubes from neutrophils treated with PMA (not shown). Selected constituents were confined to either the cytosol and plasma

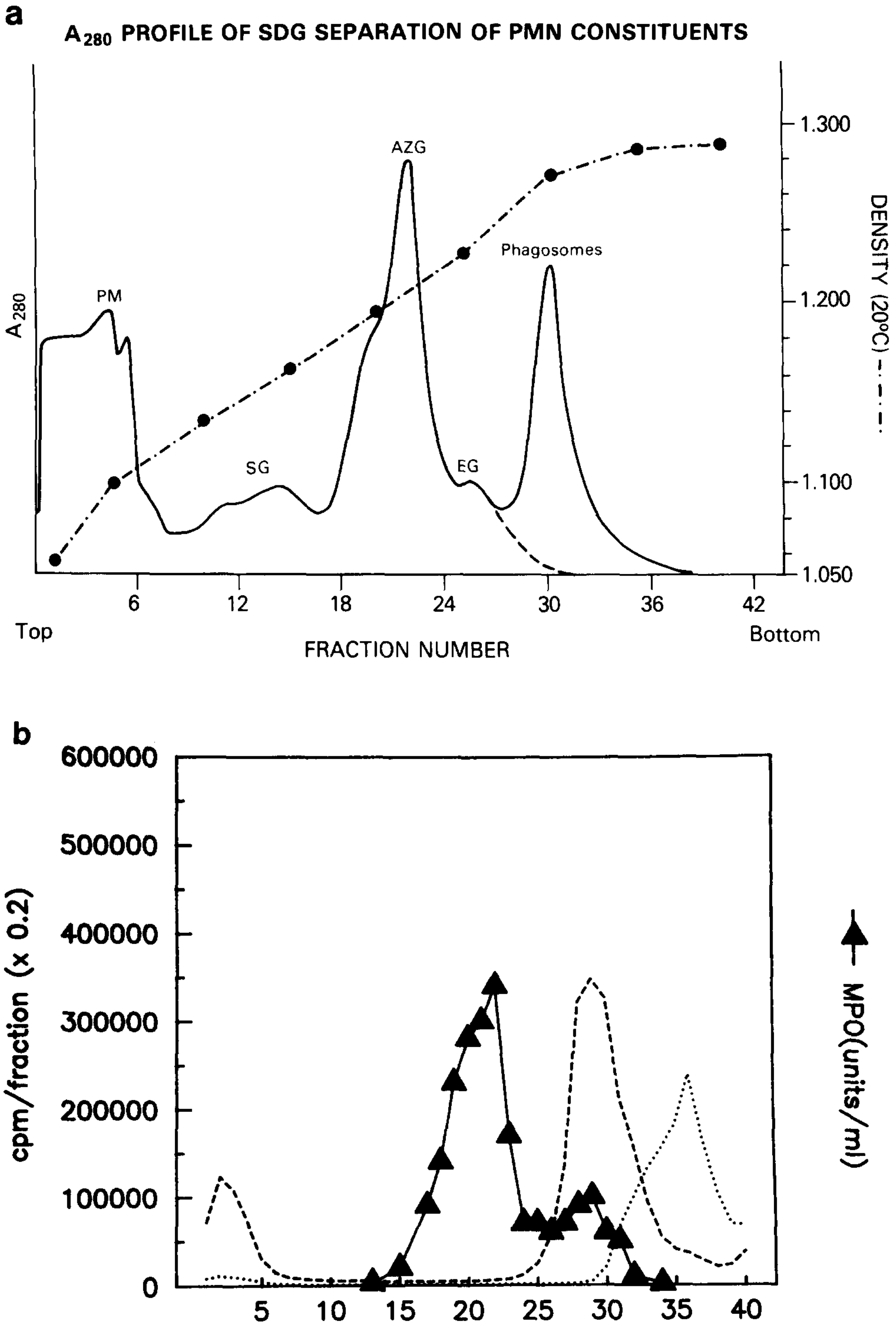

Figure 1. (*a*) A_{280} profile of sucrose density gradient separation of PMN constituents. Shown in the solid line is the A_{280} profile of the nitrogen cavitation supernatant from human neutrophils ingesting serum-incubated *S. typhimurium* after separation on a 28–75% linear sucrose density gradient (3-ml cushion of 75% sucrose). Peaks are labeled according to their standard migration position in this gradient system as well as identification using enzyme assay or by electron microscopy (Fig. 2). The dashed line shows the density profile of the gradient at 20°C. The phagosome peak is drawn as smooth, although the actual A_{280} tracing was irregular due to clumping of the constituents. *PM*, plasma membrane; *SG*, specific granules; *AZG*, azurophil granules; *EG*, eosinophil granules. (*b*) Sucrose density gradient profile of ^{35}S-*S. typhimurium* after ingestion by neutrophils (- - -). The position of ^{35}S-*S. typhimurium* RG108 after serum incubation and addition to nitrogen cavitation supernatant is shown (· · · · ·). Also shown are the results from the MPO assay (▲) (27) for selected fractions from ^{35}S-*S. typhimurium* after ingestion by neutrophils. Neutrophils were pretreated with 3 mM DFP, 50 μM NPGB, 20 μM pepstatin, and 20 μM leupeptin for 30 min before phagocytosis of ^{35}S-*S. typhimurium*. Phagocytosis was allowed to proceed for 10 min at 37°C, and the nitrogen cavitation supernatant was separated by sucrose density gradient using the gradient conditions described in *a*.

membrane fractions (80 and 22 kD) or to the phagosome fractions (>150, 75, 37, and 28 kD).

Two-dimensional gel electrophoresis was also used to analyze the iodinated constituents in the phagosome band (Fig. 4). Spots 1, 2, and 3 represent OMP F, OMP C, and OMP A (22; data not shown), the most prominent outer membrane proteins in *S. typhimurium* (for review see 32). Spot 7 has been identified as a fragment of C3 (22). Spots or bands 8–15 are unique to the phagosome fraction since they are not seen in two-dimensional gels of exogenously iodinated *S. typhimurium* (data not shown).

Phagosome fractions were analyzed for the presence of iodinated granule components. The species that make up most of the PMN granule protein (lactoferrin, MPO, elastase, cathepsin G, lysozome, and defensins) are readily acid soluble. Accordingly, their relative role as iodination targets can be estimated by analyzing acid extracts of homogenate fractions. Analysis by 10–20% gradient SDS-PAGE (Fig. 5)

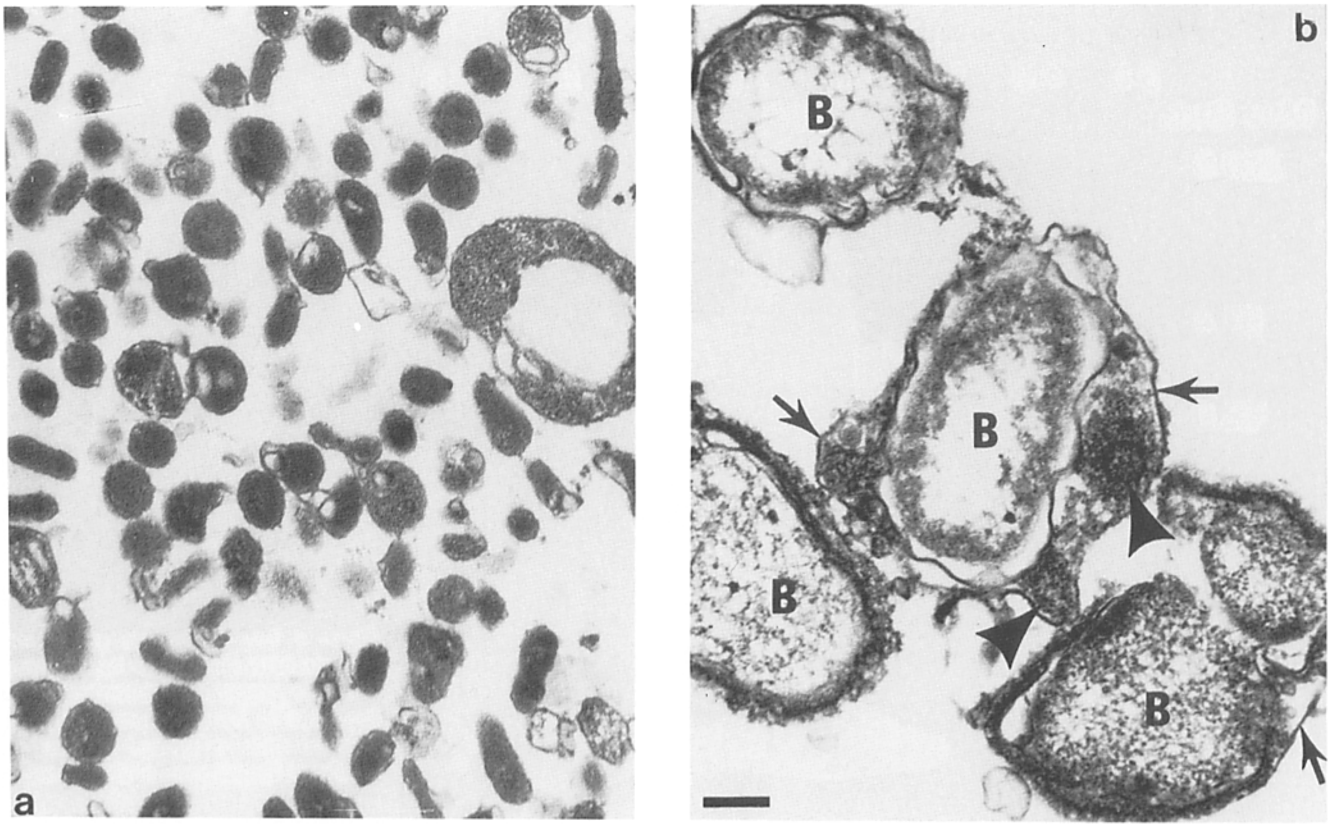

Figure 2. Transmission electron micrographs of PMN containing *S. typhimurium* RG108: azurophil granule fraction (*a*) or phagosome fraction (*b*). In the phagosome fraction, the presence of bacteria (*B*) enclosed in phagosome membrane (*arrows*) and in various states of degradation are shown. Electron-dense material, possibly derived from granules, is illustrated in the phagosome space (*arrowheads*). Bar, 2 μm.

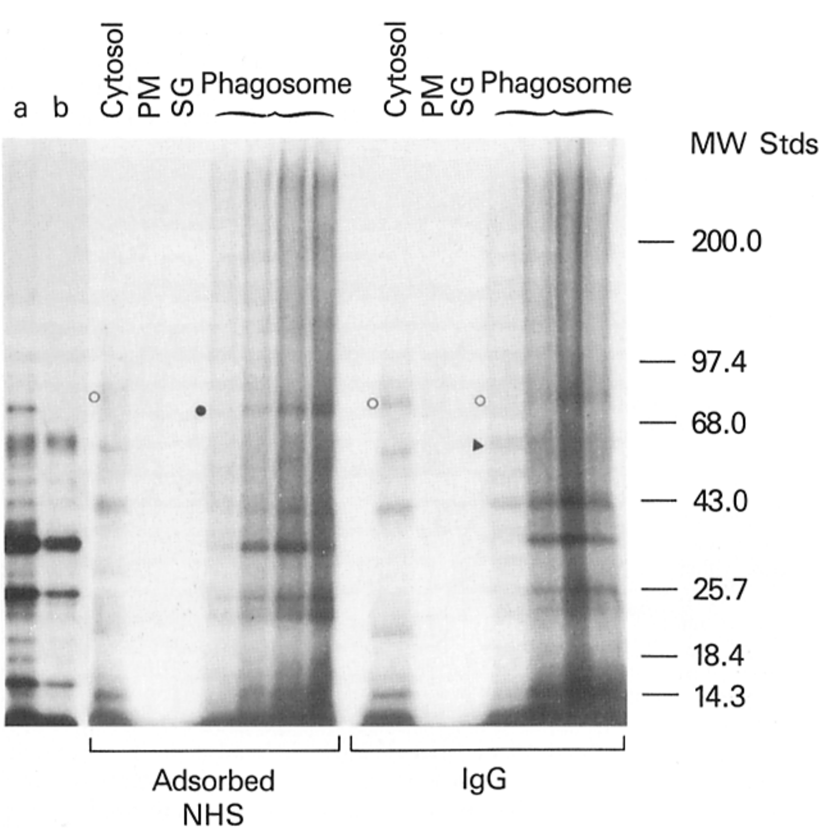

Figure 3. One-dimensional SDS-PAGE pattern of constituents endogenously iodinated by neutrophils during ingestion of *S. typhimurium* opsonized with adsorbed NHS or IgG. SDS-PAGE (5–15%) profiles of selected fractions from the sucrose density gradient separation are illustrated. Constant cell equivalents were loaded in each lane. The fractions are labeled according to composition based on the profile in Fig. 1 *a*. *PM*, plasma membrane; *SG*, specific granules. Also shown are the one-dimensional SDS-PAGE profiles of exogenously iodinated *S. typhimurium* after presensitization with adsorbed NHS (lane *a*) or IgG (lane *b*). The 75-kD β chain of C3 is marked (●). Molecules of 80 (○) and 64 kD (▲) are described in the text. On SDS-PAGE gels allowing resolution of components <10 kD, a major band of ∼6–7 kD was seen in cytosol and phagosome fractions under both opsonization conditions.

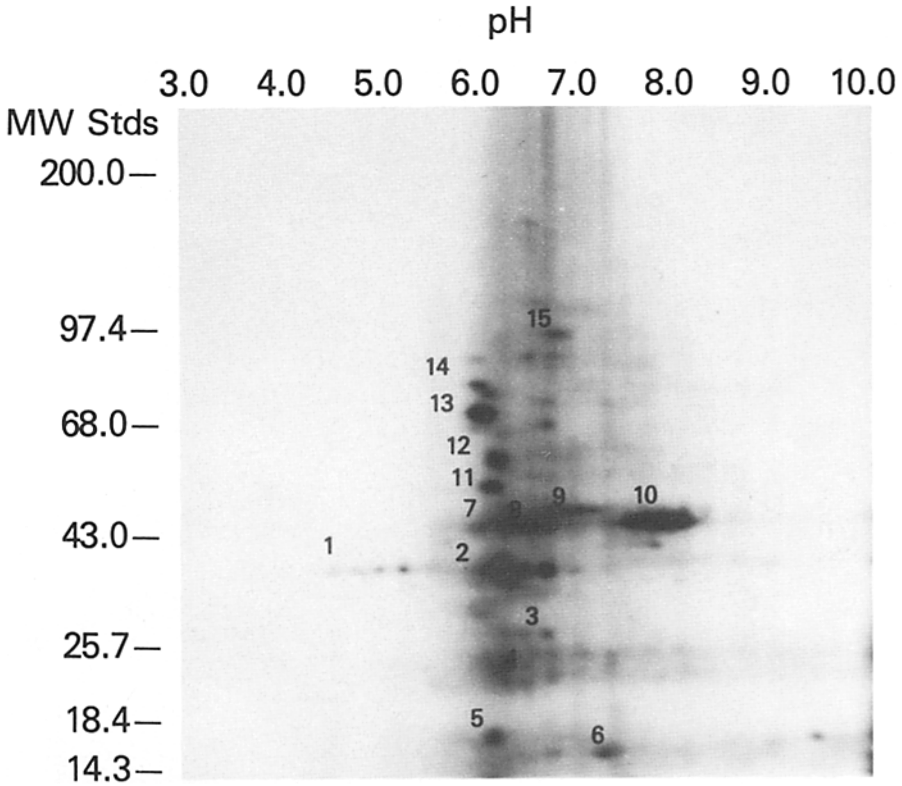

Figure 4. Two-dimensional gel electrophoresis of the phagosome fraction from a sample prepared as described in the legend to Fig. 3 for adsorbed NHS lanes. Selected spots or groups of spots are numbered for purposes of identification.

showed that proteins of molecular weight ∼4,000, comigrating with purified defensin standards (13), constituted the predominant acid-soluble iodinated species in the *S. typhimurium* phagosome. Identification of the 4-kD proteins as defensins was confirmed by their comigration with purified defensin standards in Au-PAGE (Fig. 6 *a*), an electrophoretic system in which the migration of proteins is both charge and size dependent. Defensin iodination yielding the same electrophoretic patterns was readily observed when purified defensins were subjected to the action of the MPO-H_2O_2-^{125}I system or when H_2O_2 was added to granule-enriched PMN fractions (Fig. 6 *b*). In both SDS-PAGE and Au-PAGE, several less prominent iodinated bands were seen on overexposed autoradiograms. One of the bands comigrated in both systems with purified elastase. Prominent iodinated bands comigrating with lactoferrin were identified only in preparations from neutrophils that ingested antibody-coated bacteria.

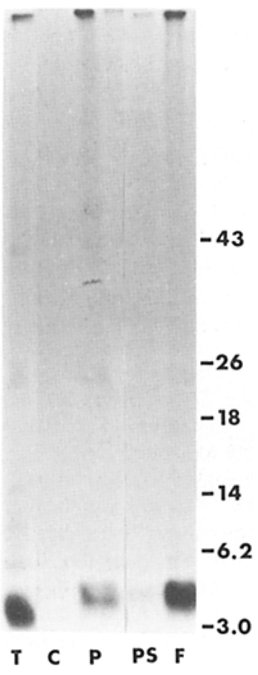

Figure 5. Identification by one-dimensional gel electrophoresis of acid-soluble granule components within phagosomes. SDS-PAGE (10–20%) profile of acid-soluble components within fractions from a sample prepared as described in the legend to Fig. 3 and in Materials and Methods. (Lanes *T*, *C*, *P*, *PS*, and *F*) total, cytosol, plasma membrane, plasma membrane and specific granules, and phagolysosome, respectively.

Comparison of Phagosomes from Neutrophils Ingesting S. typhimurium Bearing C3 or IgG

The iodination profile of phagosomes containing *S. typhimurium* sensitized with either adsorbed NHS or IgG was compared. The extent of total iodination within the phagosomes was equivalent under the two opsonization conditions (Table II). Furthermore, the percentage of ^{125}I counts per minute either precipitated by TCA, pelleted by ultracentrifugation, or remaining detergent insoluble were equivalent. Several major differences were noted when phagosomes were compared by one- (Fig. 3) and two-dimensional (not shown) gel electrophoresis. A major band at 75 kD, representing the β chain of C3, was present in phagosomes only when adsorbed NHS was the ligand (Fig. 3, adsorbed NHS lanes) (22). On the other hand, bands at 80 and 64 kD were present in the phagosomes when IgG was the ligand (Fig. 3, IgG lanes) but were much less prominent when organisms were opsonized with adsorbed NHS. The 80-kD band includes lactoferrin, and the 64-kD band is vitamin B_{12} binding protein (TCl/TC3) as shown below. Additional categorization of bands by mo-

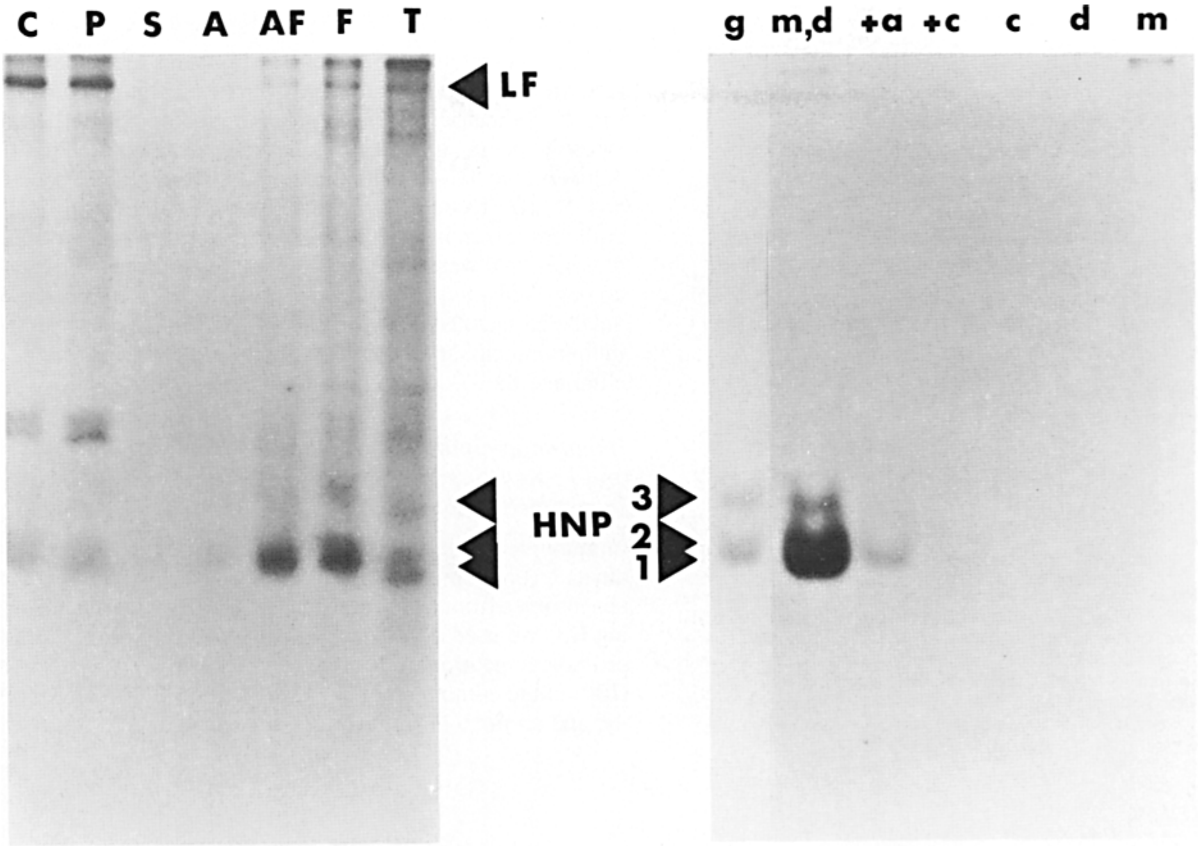

Figure 6. Identification of low molecular weight, acid-soluble proteins as defensins based on comigration with purified defensin standards in Au-PAGE. The left-hand panel shows cytosol, plasma membrane, specific granules, azurophil granules, azurophils and phagolysosomes, phagolysosomes, and total (lanes *C, P, S, A, AF, F,* and *T,* respectively). The positions of the three defensins are labeled HNP-1,2,3. Lactoferrin is labeled LF. The right-hand panel shows in vitro iodinations with mixtures including 0.5 mM hydrogen peroxide, 25 μCi Na^{125}I, and the following: (lane *g*) PMN granules; (lane *m,d*) defensins and MPO; (lane *+a*) same as lane *m,d* with 1 mM Na azide; (lane *+c*) same as lane *m,d* with 40 μg/ml catalase; (lane *c*) catalase only; (lane *d*) defensins only; (lane *m*) MPO only. Lane *m,d* was exposed for 30 min, and the other lanes were exposed for 180 min (amounts as described under Materials and Methods). Since similar amounts of defensins and MPO are present on the gel in the first two lanes, it appears that the system is partially latent in (intact) granules.

lecular weight, location, and identity, when the two opsonization conditions are compared, based on results presented above and below, is given in Table III.

Table II. Incorporation of ^{125}I into Phagosome Fractions of Neutrophils Ingesting S. typhimurium

	Opsonizing ligand			
Experiment	Adsorbed NHS		IgG	
	n	%	*n*	%
1	7.4 × 10^{6}*	67.1‡	7.8 × 10^{6}*	65.7‡
2	5.0 × 10^{6}	93.3§	6.12 × 10^{6}	90.4§
3	2.8 × 10^{6}	33.9‖	2.3 × 10^{6}	29.8‖
4	3.8 × 10^{6}		5.0 × 10^{6}	

* Total counts in peak tube from phagosome fraction.

‡ Percent of total counts precipitated by 10% TCA.

§ Percent of total counts pelleted by centrifugation. The phagosome sample from the initial sucrose density gradient was diluted 1:1 in relaxation buffer and centrifuged for 1 h at 68,000 *g*.

‖ Percent of total counts remaining detergent insoluble. The phagosome sample was mixed 1 vol sample to 2 vol lysis buffer and rotated overnight at 4°C. The percent of ^{125}I counts per minute pelleted at 15,000 *g* for 30 min, representing predominantly bacterial outer membrane proteins (Joiner, K. A., unpublished observation), is shown.

Identification of an 80-kD Band as Lactoferrin

Iodinated lactoferrin was immunoprecipitated from cytosol but not from specific granules under both opsonization conditions (Fig. 7). Cytosol, plasma membrane, specific granule, and phagosome fractions from neutrophils ingesting *S. typhimurium* bearing C3 or IgG were probed by immunoprecipitation and immunoblot for the presence of lactoferrin. Iodinated lactoferrin was identified in the phagosome with IgG as a ligand but was barely detectable (fivefold less intense by densitometric scanning) in the phagosome fraction from neutrophils ingesting *S. typhimurium* bearing C3. This result was confirmed by immunoblot (not shown). Iodinated lactoferrin was present to the same extent in phagosomes when IgG alone and C3 plus IgG were compared as ligands (not shown).

Incorporation of lactoferrin within phagosomes was examined by electron microscopy of protein A–gold–stained frozen thin sections of whole neutrophils (Fig. 8). There were threefold more gold particles specific for lactoferrin within phagosomes containing IgG-coated *S. typhimurium* than within phagosomes containing serum-opsonized *S. typhimurium* (Table IV).

Table III. Categorization of Bands by Molecular Weight, Location, TX 114 Partition, and Identity when Two Opsonization Conditions Are Compared

Location	Adsorbed NHS	IgG	Identity
Cytosol/plasma membrane	22	22	
	64, 80	–	TC1/TC3
	80	–	Lactoferrin
Phagosome	28*	28*	OMP A
	37*	37*	OMP F, C
	75	–	β Chain C3
	<10	<10	Defensins
Cytosol/plasma membrane and phagosome	14	14	
	24	24	Elastase‡
	44	44	
	–	64, 80	TC1/TC3
	–	80	Lactoferrin
	95*	95*	β Chain Cd18/Cd11 Complex

The location and molecular weight of bands is based on the gels from seven experiments with adsorbed NHS and three experiments with IgG. Additional bands are apparent in Fig. 3, but only those molecules demonstrating a consistent migration pattern in all experiments are listed. The identification of bands is based on experiments present elsewhere in this manuscript.

* Partition in detergent phase in TX 114.

‡ Presumptive, based on relative molecular mass and acid solubility.

Identification of 64- and 80-kD Bands as TC1/TC3

To confirm that specific granule components other than lactoferrin were differentially incorporated as the ligand was varied, iodinated TC1/TC3 was immunoprecipitated from cytosol, specific granule, and phagosome fractions (Fig. 9). Antiserum to TC1/TC3 immunoprecipitated bands of 80, 64, and 42 kD. These components were seen in cytosol under both opsonization conditions, were absent from specific granules, and were sixfold more intense in phagosomes containing *Salmonella* opsonized with IgG. Addition of excess unlabeled lactoferrin to the anti-TC1/TC3 antiserum before immunoprecipitation decreased the intensity but did not eliminate the 80-kD band (not shown).

Immunoprecipitation of Iodinated Complement and Fc Receptors in Phagosomes from Neutrophils Ingesting Salmonellae Bearing C3

Immunoprecipitation experiments were done to identify iodinated complement and Fc receptors within the purified phagosomes from neutrophils ingesting *S. typhimurium* bearing C3. We used a monoclonal antibody (3G8) to the most prominent neutrophil Fc receptor, FcγRIII (11), an mAb (IB4) to the common β chain (Cd18) of the Cd18/Cd11 family, and an mAb (44D) directed against CR1 (Fig. 10, *left*).

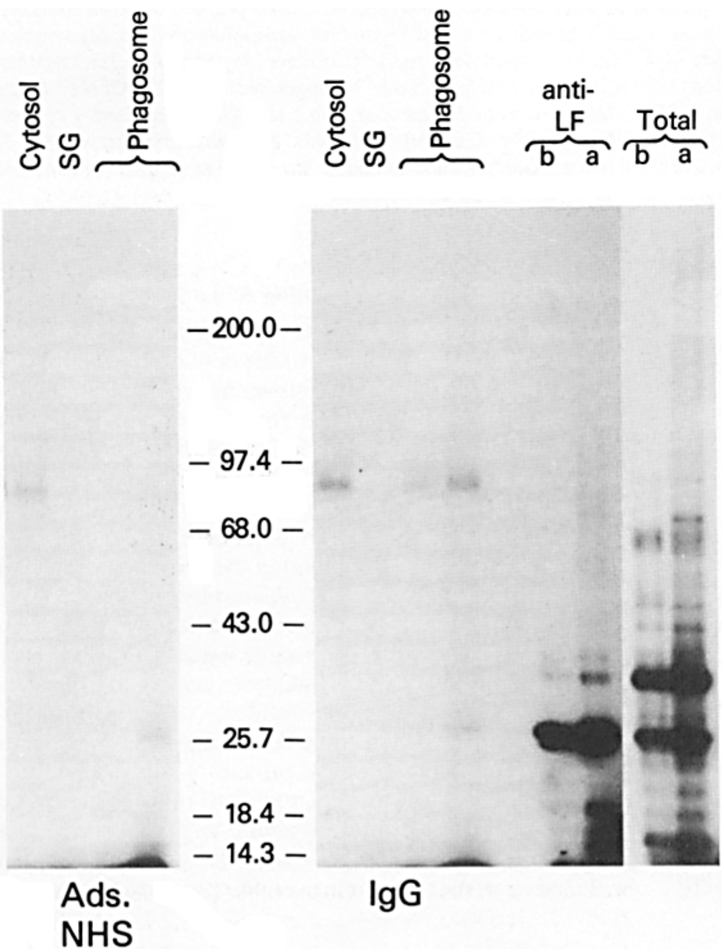

Figure 7. Immunoprecipitation of iodinated lactoferrin from cytosol, specific granule, and phagosome fractions of PMN ingesting *S. typhimurium*. Samples prepared as described in the legend to Fig. 3 were immunoprecipitated with antibody to lactoferrin as described in Materials and Methods and analyzed by 5–15% SDS-PAGE. Also shown are the SDS-PAGE profiles of total lysates (Total lanes) of exogenously labeled *S. typhimurium* after presensitization with adsorbed NHS (lanes *a*) or IgG (lanes *b*), and the results when these two samples were immunoprecipitated with antilactoferrin (anti-LF lanes). Results of total lysates from lanes *a* and *b* show that no iodinated constituent from the bacteria comigrates with lactoferrin. Results from the immunoprecipitation of samples in lanes *a* and *b* with antilactoferrin show that the lower molecular mass bands (<26 kD) seen in the phagosome fractions are derived from the bacteria rather than representing degradation fragments of lactoferrin.

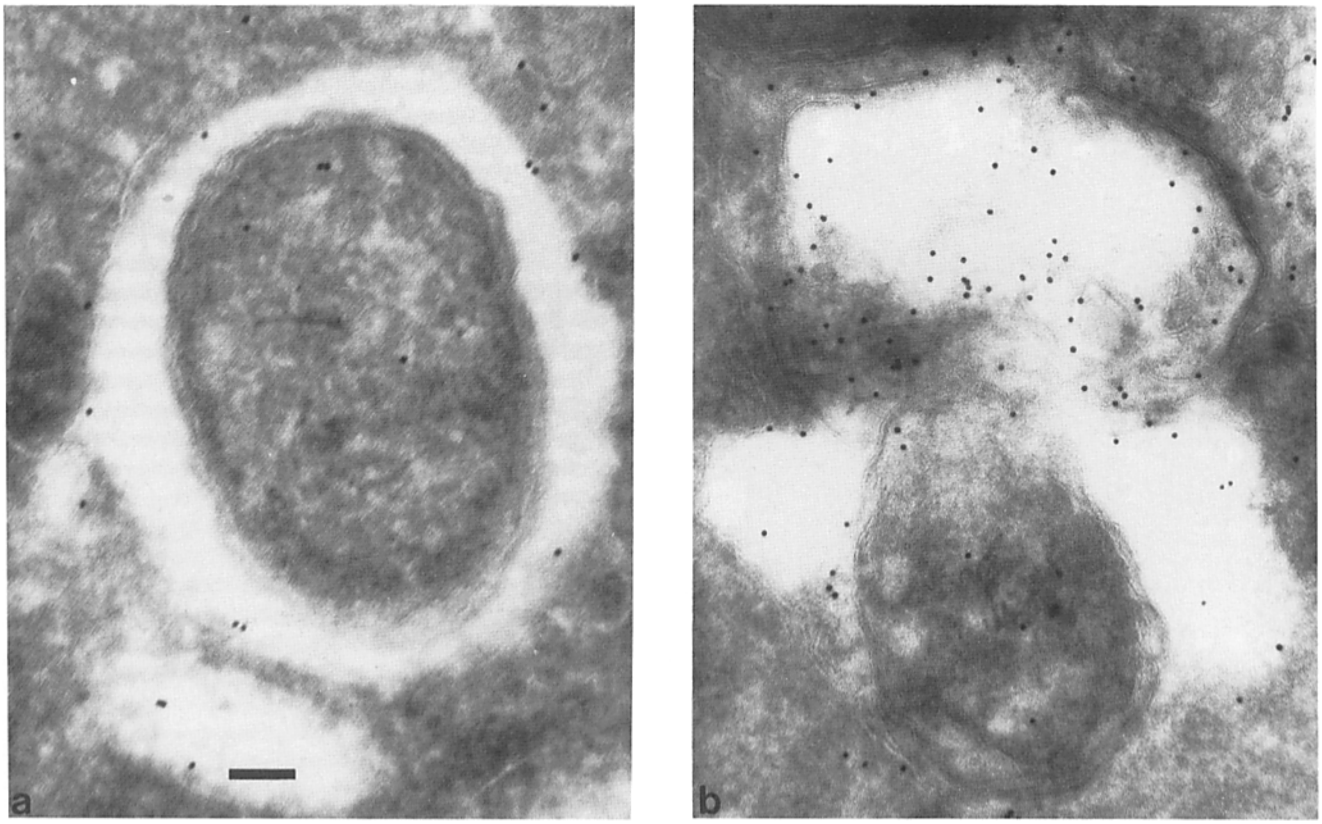

Figure 8. Antilactoferrin/protein A-gold staining of frozen thin sections of whole neutrophils injesting *S. typhimurium*. (*a*) Organisms opsonized with adsorbed NHS; (*b*) organisms opsonized with IgG. Bar, 1 μm.

A diffuse band, migrating between 50 and 70 kD and consistent with FcγRIII (11, 21, 47, 48), was visible when mAb 3G8 was used with phagosome fractions. Bands at 150 and 165 kD, consistent with the α chains of p150,95 and CR3, respectively, along with the common 95-kD β chain were identified using IB4. The identity of these molecules was confirmed in separate experiments using monoclonals specific for the α chains of p150,95 and CR3 (not shown). A faint band at 240 kD was immunoprecipitated with the anti-CR1 mAb 44D.

Table IV. Protein A-Gold Staining for Lactoferrin in Frozen Thin Sections of Whole Neutrophils Ingesting Salmonella

Opsonization condition	*n*	Gold particles/phagosome
IgG	28*	38.4 ± 11‡
IgG control	23	6 ± 5
Adsorbed NHS	36	16 ± 12
Adsorbed NHS control	18	6 ± 3

Thin frozen sections of whole neutrophils containing *Salmonella* opsonized with IgG or adsorbed NHS were stained with antilactoferrin followed by protein A-gold as described in Materials and Methods. In control samples, the antilactoferrin antibody was omitted. Phagosomes of equivalent size were counted for the two opsonization conditions. Gold particles overlying bacteria and within the phagosome space were counted.

* Number of phagosomes assessed in two separate experiments.

‡ Mean ± SD.

Immunoprecipitation of Iodinated Complement and Fc Receptors in Plasma Membranes and Phagosomes from Neutrophils Ingesting S. typhimurium Bearing IgG or C3 and IgG

Iodinated CR1 molecules, p150,95, CR3, and FcγRIII were identified by immunoprecipitation from plasma membrane and phagosome fractions of neutrophils ingesting *S. typhimurium* presensitized with IgG alone (Fig. 10, *right*) or IgG and C3 (not shown). These results extend the observations made above with adsorbed NHS as the ligand by indicating that cell surface phagocytic receptors are indiscriminately iodinated and incorporated into the phagosome regardless of whether C3 fragments, IgG, or a combination of the two ligands mediates phagocytosis.

Discussion

We have purified and characterized phagosomes from human neutrophils ingesting *S. typhimurium*. As demonstrated by one- and two-dimensional electrophoresis and immunoprecipitation, bacterial outer membrane proteins, humoral ligands, azurophil granule components, and cell surface complement and Fc receptors are iodinated and incorporated to a similar extent within the phagosome, regardless of whether complement alone, antibody alone, or a combination of both, is used as the ligand. On the other hand, lactoferrin and TC1/TC3, components of the neutrophil-specific granule, are 3.5- to 5-fold more prominent in phagosomes containing

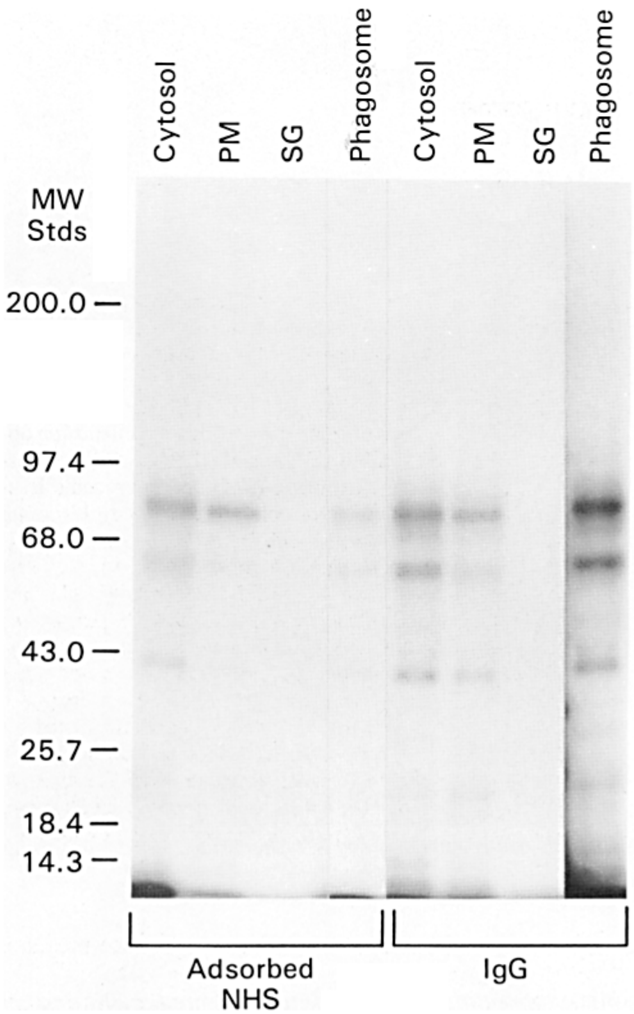

Figure 9. Immunoprecipitation of iodinated TC1/TC3 from cytosol, specific granule, and phagosome fractions of PMN ingesting *S. typhimurium.* Samples prepared as described in the legend to Fig. 3 were immunoprecipitated with an antibody to TC1/TC3, as described in Materials and Methods, and analyzed by 5–15% SDS-PAGE. The phagosome lanes were from the same gel and were exposed for the same period as the remainder of the autoradiogram.

antibody-coated *S. typhimurium* than complement-opsonized *Salmonella*. This result suggests that incorporation of specific granule constituents into neutrophil phagosomes is influenced by the ligand–receptor interactions mediating the process of phagocytosis.

Massive discharge of azurophil granules into phagosomes is the sine qua non of phagolysosome formation in neutrophils (for review see 4, 16). In our experiments, several iodinated azurophil granule components were found in phagosomes, and the azurophil granule marker MPO was identified by enzymatic assay (Fig. 1 *b*) within the phagosome-enriched subcellular fractions. More importantly, the process of endogenous iodination, which we used to identify constituents involved in phagolysosome formation, depends upon the presence of MPO within the phagocytic vacuole. In contrast, the extent to which specific granules fuse with and discharge contents into phagosomes is unclear. Bainton (5) suggested that fusion of specific and azurophilic granules from rabbit peritoneal exudate neutrophils with phagocytic vacuoles was sequential, with specific granule fusion occurring early in the process of phagosome formation. Subsequently, Pryzwansky et al. (40) demonstrated that both MPO and lactoferrin appeared on the cell surface within 5 s after neutrophils were challenged with serum-incubated *E. coli*. Earlier, Leffell and Spitznagel (30, 31) exposed human neutrophils to Latex beads covered with either immune complexes or immunoglobulin. The majority of lactoferrin released by the neutrophils was recovered in the medium, while MPO, an azurophil granule marker, was preferentially located in the phagosomes. Specific granule contents are readily and, in many cases, uniquely discharged from the neutrophil by soluble stimuli or during "frustrated phagocytosis" (for review see 4, 16). Taken as a whole, these results have suggested that specific granules function predominantly as secretory vesicles (59). Nonetheless, the mechanism responsible for the ready fusion of specific granules with the plasma membrane is unknown. One interpretation of our results is that aggregated Fc receptors, induced by immunoglobulin on the phagocytic particle, may be a sufficient signal to induce fusion of specific granules with the phagosome membrane. The capacity of aggregated immunoglobulin within immune complexes or on phagocytic particles to direct these ingested constituents to lysosomes is well established in macrophages (33, 35, 55). Alternatively, the rate of phagosome closure may be slower for complement-opsonized than for antibody-opsonized *S. typhimurium*, leading to more complete discharge of specific granule components outside the cell in the former case.

Antibody presensitization of *Toxoplasma gondii* (25), *Legionella pneumophila* (19), *Chlamydia psittaci* (12), and *Mycobacterium tuberculosis* (3) before entry of these organisms into macrophages overcomes the block in phagosome–lysosome fusion associated with entry of native organisms into the cells. We have recently shown that entry of antibody-coated *T. gondii* into fibroblasts stably transfected with murine FcγRII also leads to fusion of parasitophorous vacuoles with lysosomes (Joiner, K. A., S. A. Fuhrman, H. Miettinen, L. H. Kasper, and I. S. Mellman, manuscript submitted for publication). Although rapid and efficient fusion of neutrophil azurophil granules with phagosomes does not require antibody coating of the particle, one interpretation of our results is that fusion of phagosomes with specific granules may be enhanced by phagocytosis via Fc receptors.

Components involved in phagosome formation were identified by endogenous iodination. The major advantages of this approach are its sensitivity and the information it provides about the major components of the phagocyte and the phagocytic target which are exposed to the MPO-H$_2$O$_2$-halide system. Despite the demonstration by Klebanoff (26) over 20 years ago that bacteria are iodinated during phagocytosis, this system has not been used to identify specific microbial constituents exposed to halogenating substances. Segal et al. (44) performed subcellular fractionation studies on neutrophils ingesting IgG-coated *Staphylococcus aureus* in the presence of ^{131}INa and compared their results with neutrophils treated with PMA. These workers concluded that the subcellular distribution of ^{131}I and the SDS-PAGE profile of iodinated constituents was not substantially different in the presence or absence of bacteria and that no iodination of bacterial proteins or opsonins could be detected. These studies

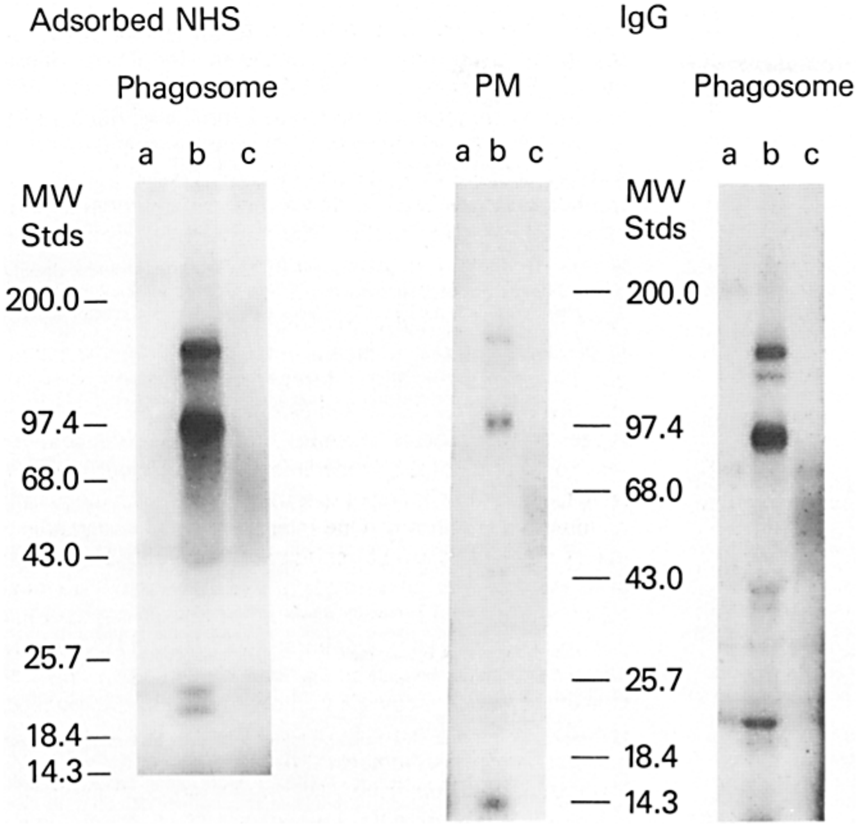

Figure 10. Immunoprecipitation of CR1, Cd18/Cd11 molecules, and FcγRIII from sucrose gradient fractions of neutrophils ingesting *S. typhimurium*, prepared as described in Fig. 3. (*Left*) Phagosome fraction of neutrophils ingesting *S. typhimurium* opsonized with adsorbed NHS. (*Right*) Plasma membrane (*PM*) and phagosome fractions of neutrophils ingesting *S. typhimurium* opsonized with IgG. (Lanes *a*) 1B4 (anti-CR1); (lanes *b*) IB4 (anti-Cd18); (lanes *c*) 3G8 (anti-FcγRIII). No bands >35 kD were immunoprecipitated from the phagosome fraction with protein A–Sepharose alone (not shown). The majority of iodinated receptors within the pools were immunoprecipitated under the conditions used (as described in Materials and Methods): no specific bands were seen when the supernatant from the first immunoprecipitation was subjected to repeat immunoprecipitations.

were done in the absence of added protease inhibitors during phagocytosis and without the application of immunoprecipitation to identify iodinated constituents. Our results indicate that identification of iodinated constituents is critically dependent upon inclusion of protease inhibitors and, in particular, the lipid soluble inhibitor, DFP, before phagocytosis (2, 22). Of most interest, bands of >60 kD were only faintly iodinated in the absence of protease inhibitors (22; data not shown). Thus, cell surface receptors and microbial ligands, in particular C3, were especially susceptible to proteolysis.

Defensins are a family of peptides abundant in the dense granules of mammalian granulocytes and certain macrophages (14, 46). They are broadly microbicidal and cytotoxic in vitro (for review see 14, 45). It has recently been suggested that interactions between defensins and *S. typhimurium* may play a critical role in the pathogenesis of salmonellosis (10). Our finding that defensins are prominent components of phagolysosomes that contain *S. typhimurium* is consistent with this hypothesis. More generally, the recovery of iodinated defensins from phagolysosomes provides further circumstantial evidence for the participation of these peptides in microbicidal events. The prominence of defensins among the iodinated species may be due to their relative abundance in the azurophil granules of PMN, where they constitute ∼25% of total protein (41), and/or a result of their high content (10%) of tyrosine (46), the preferred amino acid for stable iodination. The physiologic role of phagolysosomal defensin halogenation remains to be established. Defensin-halogen adducts less stable than the iodopeptides noted here could function as potent cytotoxins or microbicides. Alternatively, the presence of defensins could protect more sensitive PMN components from damage by reactive halogen intermediates (52). These possibilities are under investigation.

In conclusion, these results indicate that azurophil granule constituents and cell surface phagocytic receptors of neutrophils are indiscriminantly iodinated and incorporated into phagosomes containing *S. typhimurium* regardless of the ligand on the organism, but that inclusion of secondary granule constituents into the phagosome is influenced by the ligand on the particle being ingested.

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