IODINATING ABILITY OF VARIOUS LEUKOCYTES AND THEIR BACTERICIDAL ACTIVITY*

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A major function of mammalian phagocytes such as the tissue macrophage and circulating polymorphonuclear leukocyte (PMN)[1] is to ingest and kill invading microorganisms. Recently considerable data have been presented in the literature to clarify the linkage between the metabolism of the phagocyte and the killing of the ingested microorganism. The metabolic perturbations that accompany the process of phagocytosis in PMN include a cyanide-insensitive respiratory burst and an increased production of H_2O_2. Work in our laboratory (1) has indicated that a flavoenzyme, insensitive to cyanide and found in human and guinea pig PMN, might be responsible for the alterations in metabolism associated with phagocytosis, by promoting the oxidation of NADH by molecular oxygen to yield H_2O_2 (2). A seminal study linking leukocyte metabolism and killing is that of Klebanoff (3), who showed that human and guinea pig PMN are capable of fixing iodine (provided as iodide) covalently to ingested bacteria. This reaction employs H_2O_2 and is a function of the activity of the myeloperoxidase of the lysosomal granules of the PMN. The system has since been found to exhibit a marked toxic activity toward bacteria, fungi, and viruses (4–6), and is clearly linked to the respiratory changes in phagocytizing PMN noted above.

The mechanism of the bacterial killing per se is not yet known, but actual iodination is not necessarily involved, since killing in such systems occurs when bromide or chloride replace iodide. However, by including labeled iodide in the system one may obtain a useful measure of the peroxidase-peroxide reaction (i.e., ^{131}I-fixation). Recently, Pincus and Klebanoff (7) described a method for quantitatively measuring iodination of bacteria by PMN from various patients, including those suffering from chronic granulomatous disease, whose PMN are unable to generate significant H_2O_2 (8) [for review see (9)]. The method is based on the precipitation by trichloroacetic acid of iodine covalently bound to protein. The assay was not, however, correlated kinetically with the degree of ingestion of particles.

Work on the peroxide-mediated microbicidal phenomenon has been focussed predominantly on PMN. A purpose of our investigation was to draw a comparison among

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[1] *Abbreviations used in this paper:* AIK, alkaline isotonic KCl; AGH solution, 0.1% bovine serum albumin and 5.6 mM glucose in modified Hanks' solution; CGD, chronic granulomatous disease; CDG-PMN, PMN from children with chronic granulomatous disease; GP, guinea pig; KRP, Krebs-Ringer phosphate; M, mouse; MAC, peritoneal macrophages; MN, monocytes; PMN, polymorphonuclear leukocytes; PS, polystyrene; TB, tubercle bacilli.

various phagocytic cell types with regard to their ability to fix ^{131}I as a function of ingestion. Further, we wished to correlate iodination and killing in various cell types. We thus devised an assay using monolayers of phagocytes to assess these abilities quantitatively. The method provides a reliable, rapid, direct means of determining the uptake of bacteria by labeling them with [14]C. ^{131}I-fixation and killing can be followed simultaneously. Kinetic evaluation of each of these phenomena was thus made possible. Finally some enzymatic activities that underlie the iodination phenomenon were determined.

The cells examined included guinea pig, mouse, and human PMN, elicited guinea pig and mouse monocytes (mononuclear phagocytes) (MN), and mouse peritoneal macrophages (MAC). The metabolism of mouse MAC has been studied only in a limited fashion (10, 11) before this investigation, and certainly not with respect to iodination, even though they are a favorite cell in the host-parasite field. There are apparently no published reports of the elicitation of mouse PMN, and consequently PMN from mice have also not been studied extensively before.

Materials and Methods

Isolation of Cells.—Male and female guinea pigs, weighing 400–800 g each, were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass. The guinea pigs were fed chow pellets, cabbage, and water ad lib. Male "retired breeder" white mice, weighing 80–100 g were also obtained from the Charles River Laboratories and fed chow pellets and water ad lib.

PMN were elicited from guinea pigs, as described by Stähelin et al. (12) by injecting 30 ml of 12% sterilized sodium caseinate (Difco Laboratories, Inc., Detroit, Mich.) in normal saline (0.9% NaCl) intraperitoneally. After 16–18 h, the animals were killed with ether and the cells washed from the peritonea with two 30 ml volumes of ice-cold 0.9% NaCl, using a 25 ml volumetric pipet whose tip was drawn into a J. PMN were obtained from mice by anesthetizing the animals with ether and injecting 4–5 ml caseinate intraperitoneally. The animals were later killed with ether and the peritonea washed with two 2-ml samples of ice-cold 0.9% NaCl with siliconized Pasteur pipets. The yield of PMN, in terms of absolute numbers of cells and percentage of PMN present, was determined for a time-course of 1–16 h after injection. Optimal yields were obtained at 6½ h.

Human PMN were obtained from normal subjects with a high titer of PMN, and from children with chronic granulomatous disease (CGD-PMN). Venous blood was collected in 50-ml heparinized syringes. Plasmagel (Laboratoire Roger Bellon, France), was added (5 ml), and the red cells were allowed to sediment in the upright syringe for 30 min. The supernatant suspension of white cells was removed through a bent needle into a siliconized conical glass centrifuge tube to which three volumes of 0.87% NH_4Cl were added to lyse red cells. The tube was covered and shaken for 5 min. The cells were spun down at 100 $\times$ g for 5 min and the pellet, free of red cells, washed twice with Krebs-Ringer phosphate (KRP) pH 7.4 (13) and suspended in that medium.

MN were elicited from guinea pigs by intraperitoneal injection of 30 ml of sterile 1.2% sodium caseinate in normal saline, as described by Oren et al. (14). After 4 days, the animals were killed with ether and their cells harvested as above. Under the same conditions, mouse MN could be obtained after intraperitoneal injection of 4–5 ml of caseinate solution.

MAC were obtained from mice by washing the peritoneal cavity of ether-killed mice with ice-cold 0.9% NaCl. No eliciting agent was employed.

After they were harvested, cells were filtered through nylon gauze, and were spun down at 10°C, $<100 \times g$ for 10 min. When it appeared necessary, the pellet was suspended in 1–2 ml 0.9% NaCl and diluted to 20 ml with distilled water. After a few seconds, an appropriate amount of 9% NaCl was added to bring the solution to isotonicity. This osmotic shock served to lyse red blood cells without appreciable damage to leukocytes. However, preparations of white cells containing many erythrocytes were discarded. After a 10 min centrifugation at $<100 \times g$, the pellet was washed twice with KRP pH 7.4 (13). The final suspension of cells was in KRP. All glassware used in collection and centrifugation had been siliconized.

Suspensions of PMN were ca. 95% pure with some MN and lymphocytes. The MN suspensions contained ca. 80% mononuclear phagocytes with PMN constituting the bulk of the impurity, and again some lymphocytes. The peritoneal macrophage preparations were 60–70% macrophages with many lymphocytes (11, 14). The final monolayers (see below) were always further enriched with respect to the relevant cell type in each case because lymphocytes did not stick to the plastic dishes, and PMN were less adherent than MN or MAC. Thus, these monolayers were at least 90% pure.

Preparation of Monolayers.—Monolayers were prepared by adding 1.0 ml of cell suspensions $(5–10 \times 10^6$ white cells per milliliter) to tissue culture dishes (Falcon Plastics, Div. of Bio-Quest, Oxnard, Calif., 3.5 cm diameter) cut down to fit into a Nuclear Chicago gas flow counter, Model D47 (Nuclear-Chicago Corp., Des Plaines, Ill.). They were incubated 1 h at 37°C in a Visidome incubator (Greiner Scientific Corp., New York). Monolayers and supernatant cells were swirled gently every 15 min to insure homogeneity. Supernatant suspensions were poured off and the monolayers rinsed in three washes of KRP (for killing studies) or of a special medium for iodination studies. The dishes were drained of the original fluid a few seconds before test media were added. The medium for studies of iodination consisted of 0.1% bovine serum albumin and 5.6 mM glucose in modified Hanks' solution (3), and will be referred to as AGH solution.

Uptake of ^{131}I and $[^{14}C]Tubercle Bacilli.$—The method of Michell et al. (15) for following ingestion of ^{14}C-labeled particles by cells in monolayers was adapted to examine, simultaneously, the uptake of ^{131}I as a function of time and load of microbial particles. To prepared monolayers were added 0.90 ml portions of serum solution, consisting of 10% guinea pig serum (Grand Island Biological Co., Grand Island, N. Y.) in the iodination medium above (AGH). After 6 min, 100 µl portions of 10^{-5} M NaI containing 4 µCi/ml $Na^{131}I$ (New England Nuclear, Boston, Mass.) were added to sets A and B of monolayers and 100 µl of 10^{-5} M Na ^{127}I to set C. 10 min later, 0.10-ml portions of a suspension of $[^{14}C]$tubercle bacilli were added to sets B and C, whereas 0.10-ml portions of AGH alone were added to set A. Dead tubercle bacilli, labeled with ^{14}C, had been prepared as described elsewhere (16) and suspended in the AGH. The monolayers were then covered and incubated for periods of 0–60 min at 37°C with occasional gentle swirling. After this incubation, the dishes were drained, washed in six beakers containing cold 0.9% NaCl and 10^{-6} M NaI (nonradioactive) and air dried. The set of dishes lacking radioisotope but exposed to ^{14}C-labeled particles (set C) was counted with a gas flow counter, to monitor ingestion. The ^{14}C and/or ^{131}I of sets A and B were similarly counted. Monolayers were digested with 0.5 N NaOH and their protein assayed (17). In all calculations for protein, allowance was made for the contribution of $[^{14}C]$tubercle bacilli.

Iodination of $[^{14}C]Tubercle Bacilli in the Absence of Phagocytes.$—The experiments proceeded essentially as described above, except that no phagocytes were present and dishes with particles were incubated for 20 min. At the end of this time the contents of the dishes were transferred quantitatively to centrifuge tubes, spun at $10,000 \times g$ for 10 min at 4°C, and the pellets washed three times with cold 0.9% NaCl-10^{-6} M NaI (unlabeled). The pellets were then transferred, in aqueous suspension (1 ml) to their original dishes, dried, and counted.

Uptake of ^{131}I and Inert Particles.—Monolayers of phagocytic cells were exposed to ^{131}I in the presence and absence of a noniodinatable particle, polystyrene (PS) (18). This control

monitored entry into the cell of iodide specifically attributable to the uptake of external medium during phagocytosis. The procedure was as described above, but 0.10 ml of a suspension of PS (0.81 μm diameter, Difco Laboratories) in AGH was substituted for [^{14}C]tubercle bacilli. The set of dishes C, lacking radioisotope but exposed to PS, was examined microscopically to confirm ingestion of particles by the phagocytes. The uptake of ^{131}I in the presence and absence of PS was determined by gas flow counting, as before.

Effect of Cyanide on Iodination by "Resting" Cells.—The effect of cyanide on nonphagocytizing cells was examined to determine whether the iodination observed in such cells could be a function of heme enzyme activity. Monolayers were exposed to 0.90 ml AGH solution containing or lacking 1.2 mM KCN pH 7.4 for 6 min, after which 100 μl 10^{-5} M NaI containing ^{131}I (0.4 μCi) were added. After 10 min, 0.10 ml of AGH was added. Dishes were drained, washed, and counted as before, after a 20 min incubation.

Measurement of Ingestion and Killing.—To monolayers of phagocytes were added 0.90 ml portions of KRP glucose (7.5 mM)—5% guinea pig serum heated at 56°C for 30 min, at 37°C. After 16 min, 0.10-ml portions of ice-cold live [^{14}C]*Escherichia coli* were added, and the covered dishes incubated at 37°C for 0–90 min. *E. coli* had been grown to stationary phase in the presence of D-[^{14}C]glucose (uniformly labeled) (New England Nuclear) and washed to a constant specific activity. The multiplicity of *E. coli* per leukocyte was determined. At the end of the incubations, monolayers were drained and washed six times in cold 0.9% NaCl, all under sterile conditions. Those dishes to be used for assaying total uptake of *E. coli* were dried and counted on the gas-flow counter, and their protein contents assayed. To the other monolayer dishes, placed on ice, were added ice-cold 1.0-ml portions of sterile 0.5% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) in 0.9% NaCl. The monolayers were then suspended and lysed by rapid pipetting with sterile Pasteur pipets and 0.10-ml samples were serially diluted, for plating, in sterile 0.9% NaCl. Other samples were taken for protein analysis. The effects of such a concentration of Triton X-100 on both the viability of *E. coli* and the intactness of the phagocytes examined had been determined previously. This substance at this concentration in no way impaired the viability of *E. coli*, whereas it promoted the release of phagocytic cells from dishes and lysis of those cells (>90%). In parallel with every killing experiment, a test was run for the viability of *E. coli* with time in the incubation medium, without monolayers. The experimental procedure is outlined in Fig. 1. After washing of all dishes had been completed, unexposed phagocytes in monolayer form were passed through the wash solutions to check for contamination by ^{14}C and by viable bacteria.

After the monolayers were lysed as above and diluted, the samples were plated on "Certa Plate Antibiotic No. 3" (Hospital Service Tech. Corp., North Andover, Mass.). After a 16 h overnight incubation at 37°C, the bacterial plates were visually counted.

Enzymatic Activities of Phagocytes.—

NADH and NADPH oxidases: The assay was based on the method described by Cagan (1, 19). Washed suspensions of phagocytes were resuspended in alkaline isotonic KCl (AIK: 0.32 ml of 10 mg/ml KHCO$_3$ in 100 ml of 1.15% KCl), 30% by volume. The suspensions of cells were then homogenized on ice in a Potter-Elvehjem homogenizer with a mechanically driven Teflon pestle (Arthur H. Thomas Co., Philadelphia, Pa.) to give >90% breakage as determined by microscopic observation. The homogenates were spun at 15,000 $\times$ g for 30 min at 4°C and the resulting supernatants were carefully removed and assayed. A sample of the homogenate, before centrifugation was saved for protein determination.

NADH and NADPH oxidase assays (for both cyanide-sensitive and cyanide-insensitive activity) were performed at 37°C in a Perkin-Elmer Model 202 spectrophotometer (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.), monitoring the optical density at 340 nm. The cuvettes (1 cm path length) contained a total volume of 1 ml. NADH and NADPH (Sigma Chemical Co., St. Louis, Mo.) each dissolved in 0.10 M potassium phosphate
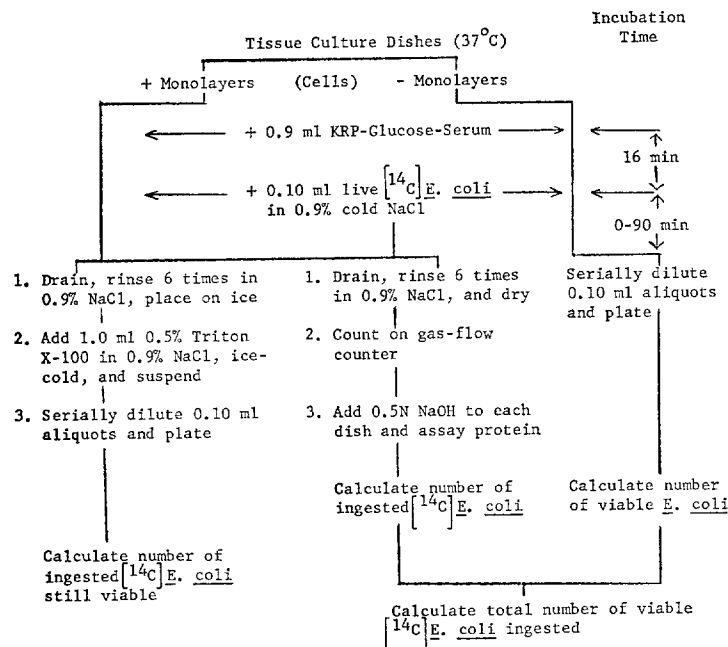
Tissue Culture Dishes (37°C)

Incubation Time

+ Monolayers   (Cells)   - Monolayers

⟵———— + 0.9 ml KRP-Glucose-Serum ————⟶   ⟵———
16 min

⟵———— + 0.10 ml live [^{14}C] E. coli ———⟶   ⟵———
in 0.9% cold NaCl
0-90 min

1. Drain, rinse 6 times in 0.9% NaCl, place on ice

2. Add 1.0 ml 0.5% Triton X-100 in 0.9% NaCl, ice-cold, and suspend

3. Serially dilute 0.10 ml aliquots and plate

Calculate number of ingested [^{14}C] E. coli still viable

1. Drain, rinse 6 times in 0.9% NaCl, and dry

2. Count on gas-flow counter

3. Add 0.5N NaOH to each dish and assay protein

Calculate number of ingested [^{14}C] E. coli

Serially dilute 0.10 ml aliquots and plate

Calculate number of viable E. coli

Calculate total number of viable [^{14}C] E. coli ingested

FIG. 1. Flow sheet of the kinetic assay of bactericidal activity. (See text for determination of killing efficiency.)

buffer pH 7.0 to give a concentration of 1.6×10^{-3} M. The pyridine nucleotide concentrations were calculated from the value of $E_{340}^{1cm} = 6.22 \times 10^3$ liters-mol^{-1}. Assay mixtures contained 0.10 ml 10^{-2} M KCN, pH 7.0 (or 0.10 ml 0.10 M potassium phosphate pH 7.0), 0.30 ml supernatant preparation (1 mg supernatant protein, or 0.30 ml AIK), 0.50 ml 0.10 M potassium phosphate buffer, pH 7.0, and 0.10 ml (1.6×10^{-4} mmol) of the above solution of NADH or NADPH (or 0.10 ml of buffer). The reaction was initiated by the addition of pyridine nucleotide, and the decrease in optical density was followed with time. Activity, based on extrapolation of an initial linear decrease, was expressed as nanomoles of nucleotide min^{-1} mg^{-1} homogenate protein or for 10^7 cells.

Peroxidase: The peroxidase method was based on that used by Michell et al. (20), a modification of that of Maehly (21). Suspensions of cells were homogenized as a 30% suspension in AIK, as described above, and samples taken for protein analysis. The assay mixtures were incubated at 37°C in a Perkin-Elmer spectrophotometer monitoring the appearance of color at 470 nm. To the cuvettes (1 cm path length) were added 0.2 ml of 0.1 M sodium phosphate buffer, pH 7.0, 1.0 ml of 20 mM guaiacol (0.22 ml liquid diluted to 100 ml with water), 0.2 ml of 1.6% Triton X-100, 0.1-0.3 mg homogenate protein in AIK, and water to a total volume of 3 ml. The reaction was started by the addition of 10 μl H$_2$O$_2$ solution (final concentration ~0.6 mM). The initial reaction rate was calculated by using $E_{470}^{1cm} = 26.6 \times 10^3$ liters-mol^{-1} for tetraguaiacol, as nanomoles tetraguaiacol evolved min^{-1} mg^{-1} homogenate protein or for 10^7 cells.

Catalase: The catalase assay, run at 25°C, was essentially that of Baudhuin et al. (22) adapted by Michell et al. (20). Washed phagocytes were resuspended in 0.34 M sucrose solution (pH adjusted to 7.2 with NaHCO$_3$) to give a 30% suspension. Cells were then homogenized on ice to give maximal cell breakage and minimal lysosomal breakage as determined

by Michell et al. (20). A sample of homogenate was taken for determination of protein, and the remainder spun at 15,000 $\times$ g for 60 min at 4°C. The supernatant was carefully pipetted off and used for assay. By definition, one unit of catalase activity destroys 90% of the H_2O_2 min^{-1} for a 50 ml assay volume at 25°C.

RESULTS

Ingestion and Iodination.—

Ingestion of [¹⁴C]*tubercle bacilli:* Uptake of [¹⁴C]tubercle bacilli ([¹⁴C]TB), determined as a function of time, is represented in Fig. 2. Classical saturation curves for uptake with time (Fig. 2) were noted for all five cell types. For an average load per monolayer dish of 2.2–2.5 mg dry weight [¹⁴C]TB (adequate to permit maximal rates) ingestion was linear for 10 min.

Fixation of iodide: The uptake of [¹⁴C]TB in the presence of ¹³¹I is represented by Fig. 3. There is a 5 to 10 min lag in the fixation of ¹³¹I compared to the ingestion of [¹⁴C]TB (particles). The ratio of fixed ¹³¹I to ingested [¹⁴C]TB is not a constant with time but reaches a constant maximum 25–30 min after the initiation of particle uptake under the conditions used. Fixation of ¹³¹I to [¹⁴C]TB after in-
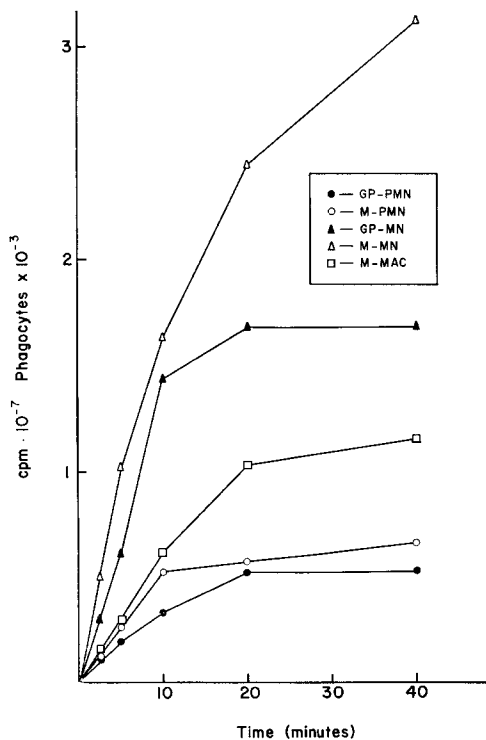

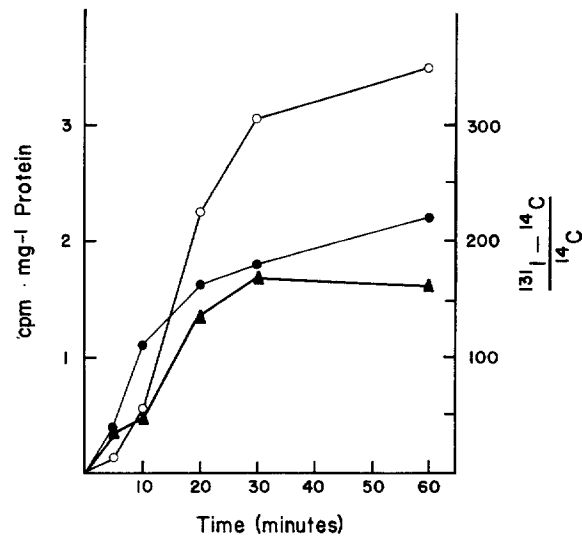FIG. 2. The rate of uptake of [¹⁴C]tubercle bacilli by five types of phagocytes (2.2–2.5 mg dried tubercle bacilli present).

Fig. 3. Ingestion and iodination of dead tubercle bacilli. The rate of uptake of [^{14}C]tubercle bacilli (solid circles: $\times$ 10^{-3}) and [^{14}C]tubercle bacilli plus ^{131}I (open circles: $\times$ 10^{-5}) are given for guinea pig PMN. The counts due to ^{131}I fixed are approximately two orders of magnitude greater than those for ^{14}C. All data expressed per milligram of phagocyte protein (left hand ordinate scale). The ratios of fixation of ^{131}I to ingestion of [^{14}C]tubercle bacilli at each time are given as solid triangles (right hand ordinate scale).

cubation for 20 min in the absence of monolayers of phagocytic cells was found to be insignificant.

An experiment was designed to show whether or not ^{131}I uptake during phagocytosis was simply an artifact of "leakage" of iodide during the ingestion of solids. Polystyrene (PS) was used as the particle. Fixation of ^{131}I was approximately the same, with or without PS (which, as assessed by microscopic observation, was readily phagocytized) and was virtually linear to 60 min. Such fixation in the absence and presence of inert (noniodinatable) (18) particles varied from one cell type to another, and in the cases of PMN and MN was always minimal compared with the iodination observed in the presence of [^{14}C]TB or *E. coli*, i.e., about 1%.

It was desirable to determine whether or not the concentration of iodide usually used in these studies, 10^{-6} M, was limiting for iodide fixation at the times and particle loads employed. This concentration of iodide was used by Klebanoff in his original paper (3). During the experiments to measure uptake of iodide as a function of the concentration of NaI and with standard loads of [^{14}C]TB, it had been observed that phagocytes, especially PMN, stuck to their dishes very poorly at iodide concentrations $\geq$ 10^{-3} M. However, when counts due to iodine fixation were adjusted for loss of protein from the dishes, the uptake of ^{131}I per milligram protein was found to increase with increasing iodide concentration. Uptake of ^{131}I had still not reached saturation at applied

iodide concentrations of 10^{-2} M, whereas cellular protein attached to dishes had decreased to 10% of the protein present on dishes with 10^{-6} M NaI. It was thus impossible to saturate the ^{131}I fixation mechanism if reasonably intact mono-layers were to be preserved. Thereafter, 10^{-6} M NaI and 0.4 μCi/ml ^{131}I per dish were used for most experiments to obtain ^{131}I counts in a manageable range for counting and to minimize toxic effects.

The uptake of ^{131}I per milligram of phagocyte protein was a function of the load of particles presented to the five different types of cells. Uptake was linear with load for all cells only up to 0.5 mg of [^{14}C]TB. Total uptake of iodide peaked at loads of [^{14}C]TB between 1 and 2 mg. Fixation of iodide by PMN was almost an order of magnitude greater than that by MN; iodination by MAC was virtually negligible. Perhaps the optimal way in which to compare different types of phagocytes for their ability to iodinate is to examine the "maximal iodination ratio", i.e., the fixation of ^{131}I per unit of ^{14}C particles ingested. The maximum occurs at a load of 0.25–0.50 mg [^{14}C]TB per dish (Fig. 4), and cannot keep
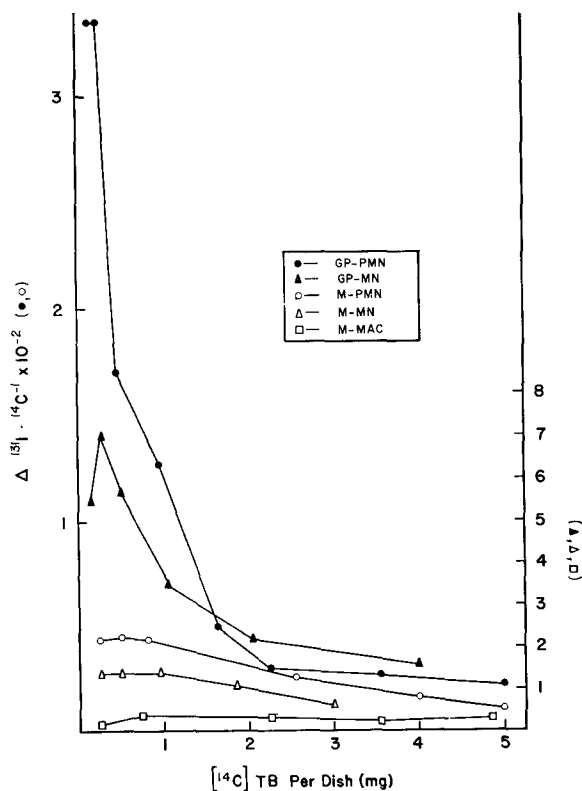

Fig. 4. The iodination ratios (^{131}I/^{14}C, i.e., iodine fixed per unit of bacteria ingested) derived for five types of phagocytes exposed to various loads of [^{14}C]tubercle bacilli. (See Materials and Methods for details.)

pace with the uptake (phagocytosis) of particles beyond that load. This situation could be due to a number of factors, including limitation in the available H_2O_2 and/or iodide, or perhaps a limiting rate of degranulation of the cells after a certain rate of ingestion is achieved, which would then control the activation of peroxidase. It was impossible to get reliable measurements of ingestion below a load of 0.25 mg [^{14}C]TB per dish because of the low counts of ^{14}C to be observed, and a particle with higher specific activity would therefore have been preferable. However, it is still reasonable to make comparisons among cell types with respect to their maximum iodination ratios under the conditions specified. This comparison is made in Table I, where the superiority of PMN, particularly guinea pig PMN, in iodination during phagocytosis is evident.

TABLE I

Optimal Iodination Ratios for Monolayers of Various Cells

Cells	Species	
	Guinea pig	Mouse
PMN	335	45
MN	7.0	1.4
MAC	—	0.35

* Expressed as the ratio of ^{131}I fixed (cpm·mg^{-1} phagocyte protein) to the ^{14}C ingested (cpm·mg^{-1} phagocyte protein). The phagocytes were permitted to ingest ^{14}C-labeled tubercle bacilli in a medium containing ^{131}I$^-$. Values are computed for 3×10^5 cpm I$^-$ applied; the specific activity of the bacteria was 2.5×10^4 cpm·mg^{-1} protein. Data are corrected for fixation in the absence of ingestible particles, (See Materials and Methods). The numbers given are averages from two experiments each performed in duplicate or triplicate, and are for loads of particles and a period of incubation yielding an uptake of ^{131}I linear with ingestion of [^{14}C]tubercle bacilli.

A dramatic case of impaired iodination in cells normally extremely capable in that function is that of the PMN from patients with chronic granulomatous disease (CGD). A comparison of such cells with normal human PMN is seen in Table II. The inability of CGD-PMN to generate peroxide, due probably to a deficiency of NADH oxidase (8, 9), has thus resulted in marked diminution of the "iodination ratio" (i.e., the fixation of iodine per unit of ingested particles). Uptake of particles by CGD-PMN was about half of that by normal human PMN in this particular case. A depression in particle uptake by CGD vs. normal PMN has been denied previously, but ingestion in such published cases has been monitored microscopically rather than with radioactively-labeled particles in monolayers. However, using the monolayer method, Michell (unpublished data) found an uptake of [^{14}C]starch by monolayers of PMN from two patients that was normal. The depression observed here may be within limits of biological variability, or conceivably there may be differences between normal and CGD human PMN with respect to their ingestion of different kinds of par-

ticle. The decrease in iodination is, however, two orders of magnitude, much greater than could be explained by decreased ingestion.

It is also worth noting that "resting" iodination was inhibited in CGD cells by an order of magnitude, which would implicate H_2O_2 in resting as well as phagocytic iodination. In order to obtain some clue to the nature of the fixation of iodide observed for nonphagocytizing cells, we attempted to inhibit such iodination. Myeloperoxidase and possibly catalase have been implicated in the process (4), and both are inhibited by 1 mM cyanide (23). Iodination by resting cells in the presence of 1 mM cyanide was observed to be maximally inhibited in the case of PMN (75–80%), and minimally inhibited in mouse MAC (22%). Apparently resting iodination in MAC is largely independent of a heme enzyme. The data are presented in Table III.

Ingestion and Killing.— [14C]*E. coli* were used for monitoring ingestion and the fate of the organism. Live *E. coli* did not replicate in the medium employed for the studies of ingestion, as assessed by optical density measurements made over a 90 min period. This removed a possible complication.

TABLE II

Uptake of ^{131}I *and* [14C]*TB By Human PMN Monolayers**

| Cells | Resting | Phagocytizing | | Ratio |
	(^{131}I)	(^{131}I + 14C)	(14C)	$^{131}I/^{14}C$‡
Normal PMN	8,150	272,000	3,880	67
CGD-PMN§	870	3,530	1,810	0.47

* Uptake expressed as $cpm \cdot mg^{-1}$ phagocyte protein. [I⁻] = 10^{-6} M. The load of particles applied per dish, where appropriate, was 2.5 mg [14C]TB (dry weight), and the time of exposure to particles was 20 min. The radioactivity was normalized to 3×10^5 cpm ^{131}I⁻ applied. Other conditions as in Table I. Experiment was performed in triplicate.

‡ Expressed as the ratio of ^{131}I ($cpm \cdot mg^{-1}$ phagocyte protein) associated with the ingestion of particles, to the 14C ($cpm \cdot mg^{-1}$ phagocyte protein) of the TB particles ingested.

§ Cells obtained by Dr. Robert Baehner from a patient with chronic granulomatous disease (CGD).

TABLE III

*Influence of Cyanide on Iodination by Monolayers of "Resting" Cells**

| Cells | PMN | | MN | | MAC |
Species	Guinea pig	Mouse	Guinea pig	Mouse	Mouse
− Cyanide	9,000	4,600	2,500	2,000	1,000
+ Cyanide	1,300	1,150	1,200	1,100	800
% Inhibition by CN⁻	85	75	52	45	20

* Data as $cpm \cdot mg^{-1}$ cell protein, normalized to 3×10^5 cpm ^{131}I applied; final [I⁻] = 10^{-6} M. Experiments were performed in triplicate, and incubation time with iodide was 30 min. Where present, KCN was added to dishes to give a final concentration of 1 mM; monolayers were then incubated for 10 min before addition of iodide.

Guinea pig serum, heated at 56°C before use, was employed in the medium to promote ingestion. In order to make possible determinations of killing efficiency of each phagocyte in different experiments, we included in each trial a kinetic study of the bactericidal activity of the medium itself. Given the change in the ingestion of *E. coli* by a monolayer of phagocytes for an interval of time, one could perform a stepwise integration to arrive cumulatively at the percentage of viable ingested [14C]*E. coli* in a monolayer 5–90 min after ingestion had commenced, if there were no killing by the phagocyte and no internal bacterial replication. This value is represented by the expression

$$Vm_t = \sum_{i=o}^{i=t} \frac{fm_i \, \Delta U_i}{U_t} \times 100.$$

In this expression one makes use of the total number of bacteria that have been ingested after t min (U_t), the increment in the number of particles ingested over a limited interval of time (ΔU_i), and the average viability of the *E. coli* present in the medium (in the absence of phagocytes) after a given interval of time, as the fraction of the bacteria which were originally viable at t_o (fm_i). The last function thus allows for any toxic effects of the medium.

The assumption is made that viable bacteria and those that may have died in the external medium as the experiment proceeds are phagocytized equally. That this is an approximation is indicated by the fact that bacteria killed by extreme means that might affect their surface (heat or UV) are not ingested as rapidly as live *E. coli* under our conditions. For this reason it is well to choose sera and other conditions that are minimally damaging to the bacteria.

One can compare the curve resulting from all the values of Vm_t plotted against time with the curve resulting from the experimental determination of the percentage of viable intracellular *E. coli*, $Vk_t = 100$ (number of live *E. coli*)/ (number of ingested *E. coli*). Knowing these two values for each point in time, one can determine the cumulative killing efficiency for each point in time as $100(Vm_t-Vk_t)\cdot(Vm_t)^{-1}$. Of course, much simplification results from the use of a medium or serum completely nontoxic to *E. coli*. In that case the assumption made above is eliminated and further, the expression for the killing efficiency reduces to $100-Vk_t$ (since Vm_t would be 100). Occasionally, negative values were obtained for the "killing efficiency", due to replication of bacteria within phagocytes that killed poorly.[2]

Killing by phagocytic cells was studied for loads of *E. coli* that covered two ranges: a low multiplicity of about 5:1 and a high multiplicity of about 100:1. At low loads a linear uptake of bacteria continuing for 90 min was apparent for all cell types examined. Similar studies at much higher loads (ca. 100:1) revealed saturation of the ingestion process in the case of elicited monocytes, whereas PMN and MAC ingested [14C]*E. coli* at a constant rate for 90 min (cf.

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[2] A simple computer program has been developed in this laboratory to plot the time-course of ingestion and the killing efficiency.

plateau effect in Fig. 2 for a saturating load of particles). The various cells ingested *E. coli* in a relation to each other comparable to that for the tubercle bacillus (Fig. 2). The killing efficiencies at low loads (Fig. 5) are seen to be the greatest for PMN. However they are followed very closely by the values obtained for MN and MAC. At such loads the bactericidal activity of the cells is not being stressed too severely. Data for three cell types (GP-PMN, M-MN, and M-MAC) at high loads, presented in Fig. 6, reveal a much greater disparity under such a stress. Mouse MN deal the least efficiently with ingested [^{14}C]*E. coli*, guinea pig PMN exert the most efficient bactericidal activity, and mouse
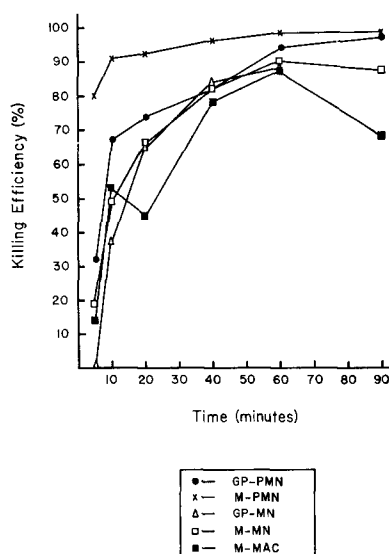

FIG. 5. The "killing efficiency," or percentage of ingested viable [^{14}C]*E. coli* that were killed by monolayers of five types of phagocytes. The abscissa refers to the period over which low loads of *E. coli* were presented to the phagocytes (multiplicity ca. 5:1).

MAC fall between, behaving more like PMN than MN. Other studies at high loads with human and mouse PMN and guinea pig MN have indicated that PMN from various species behave similarly to one another and are consistently superior MN in their killing of *E. coli*.

The data would thus indicate that the bactericidal capacity of these cells is being stressed earlier at higher loads, whether because of limiting rates of generation of H_2O_2, degranulation, digestion by lysosomal enzymes, or other factors is uncertain.

NADH Oxidase, Peroxidase, and Catalase.—Activities of enzymes were calculated with respect to numbers of phagocytes. The results for NADH oxidase, peroxidase, and catalase, are presented in Table IV. NADH oxidase was assayed by the disappearance of NADH, on the hypothesis that it would prove to
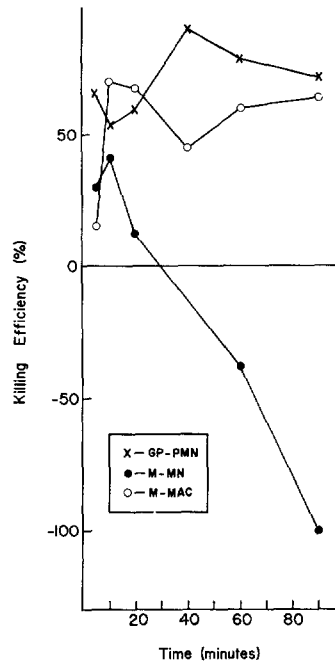
Fig. 6. The "killing efficiency," or percentage of ingested viable [^{14}C]$E.$ $coli$ that were killed by three types of phagocytes. The abscissa refers to the period over which high loads of $E.$ $coli$ were presented to the phagocytes (multiplicity ca. 100:1). Negative values indicate proliferation of bacteria intracellularly.

TABLE IV

*Enzyme Activities of Phagocytic Cells**

Cells	PMN			MN		MAC
Species	Guinea pig	Mouse	Human	Guinea pig	Mouse	Mouse
NADH oxidase‡						
+CN⁻	1.9 ± 0.4 (3)	0.89 ± 0.05 (3)	0.46§	3.2 ± 0.4 (3)	2.4 ± 0.1 (2)	2.7 ± 0.4 (2)
−CN⁻	2.0 ± 0.2 (2)	0.89 ± 0.05 (3)		3.2 ± 0.4 (3)	2.4 ± 0.1 (2)	2.7 ± 0.4 (2)
Peroxidase‖	340 ± 10 (2)	470 ± 50 (2)	1,400¶	170 ± 60 (2)	110 ± 30 (2)	5.3 ± 2 (2)
Catalase**	110 (1)	0.5 (1)	80 (1)	81 (1)	2.7 (1)	6.2 (1)

* Means for different batches of cells and average errors are given where possible; values in parentheses refer to the number of batches of cells examined. Where present, cyanide was at 1 mM concentrations. Values are calculated for cell preparations which are 100% of the type designated, e.g., 100% MN.

‡ Data as nanomoles O₂ (or NADH). 10⁻⁷ cells·min⁻¹.

§ Results from Baehner and Karnovsky (8).

‖ Data as nanomoles tetraguaiacol. 10⁻⁷ cells·min⁻¹.

¶ Performed, in sucrose medium, by R. L. Baehner.

** Data as milliunits·10⁻⁷ cells (see Materials and Methods).

be the most likely source of H_2O_2 in conjunction with enhanced O_2 consumption, judging from data for guinea pig PMN (9). The enzyme itself has been partially characterized only for guinea pig PMN by Cagan (19), and some effort has been spent in studying it in human PMN by Baehner et al. (2). The NADH oxidase ($K_m = 1 \times 10^{-3}$ M) of guinea pig PMN was observed, for example, to have a K_m two and a half times that of the enzyme from human PMN at the optimum pH of 4.5 (2). The activities were largely cyanide-insensitive, which would rule out a participation by peroxidase. In parallel with these experiments, assays for soluble cyanide-insensitive NADPH oxidase activity were under-taken. The only case for which some such activity was significant was that of guinea pig MN, whose oxidase activity with NADPH was 50% of that with NADH, in the presence of cyanide.

It was discovered here, in the course of peroxidase assays on cell homogenates in AIK and sucrose that sucrose (at about 0.25 M) could cause up to 50% inhibition of peroxidase assayed by the guaiacol method (20, 21). Therefore, all peroxidase assays were performed on AIK homogenates, with the exception of that performed on a human PMN homogenate. It is probable that human PMN peroxidase activity is substantially higher than that listed. Nevertheless, it can be seen that human PMN contain notably more peroxidase than do the other cells examined. Mouse MAC, as has been confirmed by other investigators on macrophages (24), contain negligible amounts of peroxidase, amounts that could easily be accounted for by very slight contamination by MN or PMN.

Catalase activities summarized in Table IV are seen to be dramatically less for mouse cells than for guinea pig and human cells.

DISCUSSION

The introduction of the monolayer technique to iodination studies has provided a finer measure of the correlation between particle ingestion and iodine fixation. Iodination has been seen to be virtually a linear function of particle uptake within certain limits of time and iodinatable particle load, an observation that one would not expect if "iodination" were simply an artifact of, or limited by, diffusion of I^-. This function is an important consideration when dealing with quantitative differences in iodination among cell types. If, for example, a cell's ability to iodinate during phagocytosis were to be used as a tool in the detection of a metabolic defect, such as that of CGD, it would have to be considered in the light of quantitative measures of ingestion per se. In the particular example studied here, however, the defect in iodination by CGD-PMN is dramatic. The discrepancies observed in ingestion of particles revealed in experiments using monolayers of PMN from a CGD patient are negligible in comparison with the differences in ability to iodinate. Pincus and Klebanoff (7) previously noted a great depression in iodination by CGD-PMN, but did not simultaneously determine ingestion on a comparably accurate scale.

It is also worthy of notice that iodination by resting cells is a variable phe-

nomenon from one cell type to the next. Susceptibility of this function to inhibition by cyanide was greatest for PMN, less for MN, and least for mouse MAC. The cyanide-insensitive residual activity appeared about the same for all cells, but it is not known if it represents simply a "blank" value. The inhibition observed with MN and PMN indicates that in these cell types iodide fixation is not due simply to nonenzymatic fixation of iodide, but is probably dependent on a heme enzyme and/or on an active transport of the iodide into the cells. If resting iodination is at all comparable to the phenomenon observed in phagocytizing PMN, then one would certainly expect peroxidase to play a major role, and peroxidase is markedly inhibited by cyanide. If iodide enters the cell by an active process, the situation might be comparable to that observed in the thyroid gland. There, active transport of iodide is inhibited by cyanide and DNP, in vitro (25). As has been noted in the literature (26–28) myeloperoxidase, where present, is latent and granule-bound in phagocytes. Such latency might be expected to limit catalysis by myeloperoxidase in resting cells. One could, alternatively, invoke the action of soluble catalase (also inhibited by cyanide), but, given its small ability to catalyze iodination and its very low activity in mouse phagocytes, the function of catalase in promoting iodination by resting cells seems dubious. Some degranulation and release of peroxidase might be occurring in so-called resting cells that might then account for the fixation of ^{131}I observed when phagocytosis was not occurring.

In view of the possible role of pinocytosis in fixing iodine, one might speculate on whether ingestion by pinocytosis is competitive with that by phagocytosis. One might question if it is valid to subtract resting level iodination (possibly attributable to pinocytosis) from phagocytically-associated iodination for calculations of iodination ratios as a measure of a cell's iodinating capability. The answer is not critical in cases where the level of iodination during phagocytosis far exceeds that at "rest" (as for PMN), but in less extreme instances (as for mouse MN), the correction could diminish a measure of the cell's iodinating activity, and in turn, the estimation of available H_2O_2 during phagocytosis. Perhaps the best evidence that fixation and/or facilitated entry of iodide in resting cells is not inhibited by phagocytosis was provided by an experiment in which fixation of iodide by resting guinea pig PMN and by those phagocytizing a noniodinatable particle, PS, was measured with time. The two curves were virtually superimposable over 60 min. Therefore, whatever mechanism was responsible for the resting iodination was still apparently active during ingestion of PS. The same hierarchical arrangement of phagocytes with respect to iodination, indicated by Table I, pertains, however, if one does not subtract the resting level of iodination. This ranking seems to reflect, grossly, the cellular levels of peroxidase activity.

A quantitative determination of the various enzymes in the different cell types examined here is essential to an understanding of the varying degrees of iodination and killing in these cells. However, it does not necessarily furnish a

complete explanation of the process. One complication whose role is difficult to evaluate is the issue of enzyme localization. Peroxidase, as mentioned above, is localized in the leukocyte's granule fraction and is latent until the granule merges in vivo with a phagocytic (or pinocytic) vacuole or is lysed in vitro with an agent such as Triton X-100. After release in vivo the activity would be manifest in the vacuole. Catalase is present in the soluble cytoplasmic fraction, at least in PMN (20). NADH oxidase of guinea pig and human PMN is either granule-associated or soluble, depending on whether the leukocytes are homogenized in sucrose or AIK (2). It thus becomes a problem to assess how much of the NADH oxidase-generated H_2O_2 is available to peroxidase or to cytoplasmic catalase in vivo. Further complications could arise if degranulation occurred with varying degrees of delay for different types of phagocytes relative to the production of H_2O_2 during phagocytosis (29). For example, an initial rapid generation of H_2O_2 relative to a slow degranulation could favor catalatic decomposition, rendering the peroxide less useful for iodination than were H_2O_2 generation to be more gradual, favoring peroxidatic activity of myeloperoxidase released from the granules.

With such variables still undefined, it is impossible to arrive at a realistic equation correlating iodination in the phagocytes and the activities of NADH oxidase, catalase, and peroxidase, even if the H_2O_2 stemmed only from the action of NADH oxidase, a matter of some disagreement in the literature (9, 30).

The absence of myeloperoxidase in the mouse macrophage, and in most macrophages (31), provides an interesting subject for conjecture. It is generally thought that tissue macrophages are the result of the maturation of monocytes (32), and maturation in vitro of circulating horse blood monocytes to a cell species very similar to the macrophage has been achieved by Bennett and Cohn (33). These workers noted that the cultivation of monocytes resulted in an increase in cell size, acid hydrolases, the number of mitochondria and of granules, all observations consistent with maturation to macrophages. Peroxidase has been observed in circulating MN (31), and it is reported above that mouse MN do contain peroxidase. If mouse peritoneal MAC are the products of maturation of mouse MN the peroxidase would have to have been inactivated, presumably leading to a change in the phagocyte's microbicidal arsenal. Yet in the present studies, the disappearance of peroxidase, and the absence of a significant iodination potential do not appear to have radically impaired the ability of MAC to kill *E. coli*. On the contrary, they were more efficient killers than were mouse or guinea pig MN. A general parallel between efficiencies of iodination and killing can be arrived at for PMN vs. MN, but mouse MAC present a paradox in the context of this study. PMN possess multiple microbially lethal agents, including cationic proteins and lysozyme (26) as well as a superior iodinating potential. Elicited MN are less impressive in their potential, and certainly their performance in killing *E. coli* was inferior to that of PMN,

especially at high loads. Mouse MAC, predicted to be the least effective on the basis of the criteria just mentioned, compared favorably with PMN in microbicidal activity toward *E. coli*.

Perhaps the most valid comparison employing the criteria of iodinating and killing capabilities is that between normal human- and CGD-PMN. For these cells, only the generation of H_2O_2 (presumably arising from the levels of cyanide-insensitive NADH oxidase) (8, 9) appears to differ, and here, certainly, the disparity in iodinating potentials, correlated with the radically differing microbicidal activities noted in other laboratories, (34, 35) is most dramatic.

It can be seen from the killing studies performed with monolayers that killing efficiency varies with time and the ratio of microbes to phagocytes (and therefore, with the rate of ingestion). It is interesting that PMN displayed their most notable superiority to MN against high titers of bacteria, that is, under a stressful condition not employed in the killing studies with suspensions of phagocytes described in the literature. Low multiplicities are employed by most workers who study killing of bacteria by suspensions of phagocytes primarily because larger changes can be measured in total bacterial viability compared to the original number of viable bacteria. However, the use of high multiplicities does offer an opportunity, in comparative studies, to "exaggerate" differences in levels of bactericidal activity between different cell types. Since PMN are the first phagocytes to arrive at a site of infection, to be replaced somewhat later by mononuclear phagocytes, there may be a correlation between cells which seemingly (as observed here) can handle high bacterial loads most efficiently in vitro and cells which must do so in vivo. The monolayer technique for simultaneously following the kinetics of ingestion and of killing under a wide range of conditions provides a tool for investigating some of these complex questions.

SUMMARY

A rapid method that employs monolayers of different phagocytic cells, primarily from guinea pigs and mice, has allowed a kinetic determination of (*a*) ingestion by these cells of labeled particles, (*b*) fixation of ^{131}I and (*c*) microbicidal activity in the cells after periods as short as 5' of exposure of bacteria to phagocytes. Phagocytes so examined included polymorphonuclear leukocytes (PMN) elicited into the peritoneal cavity, elicited peritoneal mononuclear cells (monocytes) (MN), and peritoneal macrophages (MAC) obtained simply by lavage. Circulating PMN from normal human subjects and from children afflicted with chronic granulomatous disease were also studied.

The potential for generation of H_2O_2 (a key component of the iodinating system) of all the normal cells studied, gauged by their content of cyanide-insensitive NADH oxidase, seemed comparable. Peroxidase levels varied widely, and were highest in PMN and almost undetectable in MAC. Catalase was at negligible levels in all the cell types obtained from mice. The fixation of

[131]I by phagocytes ingesting ^{14}C-labeled dead tubercle bacilli appeared to be primarily a function of the cellular peroxidase content. Thus, mouse macrophages, with virtually no peroxidase, displayed no fixation of iodide. PMN proved far more able to fix ^{131}I during phagocytosis than did MN. In experiments comparing PMN from normal human subjects and from children with chronic granulomatous disease (CGD), a sex-linked condition characterized by a deficiency of H_2O_2 production during phagocytosis and low microbicidal activity, the iodination ratio of CGD cells was dramatically less than that of normal PMN (by about two orders of magnitude). Capacity for iodination was correlated with bactericidal activity toward E. coli.

At low bacterial loads (ca. 5:1), phagocytes killed efficiently, and little discrepancy in ability among cell types was apparent. Under the stress of higher loads of ^{14}C-labeled E. coli (ca. 100:1), differences in bactericidal activity were exaggerated, and a substantial disparity between MN and PMN was observed in favor of the latter. The hierarchy for killing efficiencies therefore agreed with that for iodination, with one notable exception: mouse MAC were consistently competent in their killing activity, more so than MN, even though they virtually lack peroxidase and the ability to iodinate ingested bacteria.

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