RESPONSE OF CULTURED MACROPHAGES TO *MYCOBAC-TERIUM TUBERCULOSIS*, WITH OBSERVATIONS ON FUSION OF LYSOSOMES WITH PHAGOSOMES

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When large foreign bodies such as erythrocytes, polystyrene particles, or microorganisms have been ingested by cultured mouse macrophages, they are enclosed within endocytic vacuoles or phagosomes. The work of Cohn et al. (1–3) based on both light and electron microscopy has shown the presence, notably in high-serum media, of a large population of cytoplasmic "dense granules," which have been characterized as secondary lysosomes. These pre-existing granules are capable of fusing with and discharging their contents into phagosomes, so producing digestive vacuoles or phagolysosomes within which degradation of ingested materials takes place (4).

The case of microorganisms, some of which are well-adapted intracellular parasites, poses particular and interesting problems. For a bacterium that is rapidly inactivated in macrophages, lysosomal fusion followed by digestion is a common and effective means of disposal, as it is in polymorphonuclear leukocytes (5, 6). However, digestion is clearly incompatible with the survival and multiplication of a virulent bacterium. The organism might of course be resistant to the lysosomal hydrolases, as some investigators have proposed (7), perhaps even using them to its advantage (8); an alternative, it might be supposed, would be for the organism within its phagosomal compartment by some means to avoid contact with the lysosomal enzymes.

Employing cultures of mouse peritoneal macrophages, we have studied some aspects of these host-parasite relationships for the facultative intracellular parasite *Mycobacterium tuberculosis*. This bacterium is of special interest since its growth within these cells is relatively slow and primary cytotoxicity is low; consequently the cell monolayer remains intact until bacterial multiplication is well advanced. The growth of *M. tuberculosis* within macrophages varies considerably as an expression of the wide range of virulence possessed by different strains, but it can also be modified experimentally by the use of certain surfactant compounds which have been shown to act upon the host cell rather than directly upon the bacterium (9).

Materials and Methods

Tissue Culture Technique and Procedures.—Methods described previously (9, 10) were used. In brief, macrophages were obtained from the unstimulated peritoneal cavities of normal female mice of the albino P strain and were maintained as monolayers on glass cover slips in Leighton tubes containing 1 ml of medium suitable for long-term survival (NCTC 109 [Microbiological Associates, Bethesda, Md.] with additions, notably 40% horse serum [11]). The tubes were gassed with 5% CO_2 in air, closed tightly with silicone rubber bungs, and incubated at 37°C. After 2–7 days the medium was usually changed, with gassing; when a surfactant was to be used it was included in the fresh medium at this stage.

After 10–14 days of further incubation the medium was withdrawn. After a wash with Hanks' balanced salt solution (BSS),[1] and in some experiments exposure to ferritin as described later, the cell layers were exposed for 2 hr at 37°C to a suspension of tubercle bacilli within the Leighton tubes. Free bacilli were then removed by washing and fresh medium of the usual composition, plus streptomycin 5 units/ml to prevent extracellular bacterial multiplication, was introduced. The tubes were then gassed and reincubated, further changes of medium being made only if the experiment extended beyond a further 15 days. With medium changes at intervals of 12–15 days, the macrophage cultures can be maintiained (in the absence of progressive bacterial growth) for more than 4 wk.

Bacterial Strains.—These were *M. tuberculosis* virulent human strain H37Rv (from Trudeau Institute, Saranac Lake, N.Y.) and the attenuated bovine strain BCG, Moreau substrain (from State Serum Institute, Copenhagen, Denmark). Strain H37Rv was maintained by subcultivation on the surface of Proskauer and Beck liquid medium, and the BCG strain on Loewenstein and Jensen egg agar. Well-grown cultures (about 3-wk old) were glass-homogenized in water and filtered through paper; the suspension (consisting predominantly of single cells) was adjusted to give a ratio of bacteria to macrophages of from 20:1 to 40:1, resulting in a "heavy" infection with a mean of 3–6 bacilli/cell initially (10). In a few experiments not involving electron microscopy, a "light" infection, 0.5–1.0 bacillus/cell, was used.

Staining for Light Microscopy.—Cover slips were fixed in methanol and stained by a Ziehl-Neelsen method; macrophages shed into the culture medium were deposited on slides after centrifuging lightly, fixed by drying in air, and stained by a similar method (10). For acid phosphatase activity the cell layers were stained, with or without prior fixation in cold 2.5% glutaraldehyde buffered with 0.03 M sodium cacodylate, pH 7, by the Gomori method modified to combine with the Ziehl-Neelsen stain for acid-fast bacilli (12).

Assessment of Intracellular Bacterial Multiplication.—At appropriate intervals the frequency distribution of acid-fast bacilli in the cells on the cover slips was determined, together with a parallel enumeration of the proportion of cells shed into the medium that were heavily infected (10). Since for the most part the cell layers were substantially intact at the times of assessment, these counts agreed with others obtained by total recovery of bacteria from the whole cell culture (13). The mean number of acid-fast bacilli per macrophage on the cover slips was recorded graphically.

Counts of viable bacterial units per monolayer were made by withdrawing a cover slip from its Leighton tube and, after washing in several changes of BSS, crushing it manually with a glass pestle in a dry test tube. The fragments were covered with 1.0 ml of 0.9% NaCl, cooled to 4°C, exposed for 30 sec to ultrasonic vibration (400 kHz supplied by a 200-watt generator), and cooled again; this procedure disrupted the cells. The homogenate was diluted as required and drops were inoculated on oleic-albumin agar; colonies were counted after 2½–3 wk (14), and the mean number of viable units per monolayer was recorded.

Surfactant.—One of the polyethylene glycol ethers prepared by Dr. J. W. Cornforth and

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[1] *Abbreviation used in this paper:* BSS, balanced salt solution.

his colleagues (15) was used, namely, the antituberculous Macrocyclon (HOC-12.5, mean mol wt about 3100), having an average of 12.5 ethylene oxide units/phenolic group (9). A solution containing 12.5% (w/v) in 0.45% (w/v) NaCl was prepared, adjusted to pH 7, and autoclaved. When required, this was added to the macrophage culture medium in a volume of 0.05 ml per Leighton tube (6 mg/ml).

Electron Microscopy.—

Standard procedure: After preliminary tests with conventional methods employing single or sequential fixation of the macrophages with glutaraldehyde and osmium tetroxide, we finally adopted the technique of simultaneous fixation with a glutaraldehyde-osmium tetroxide mixture, essentially as described by Hirsch and Fedorko (16). This gave consistently good preservation of lysosomal and other unit membrane systems of the host cell, as well as an adequate standard of fixation of the tubercle bacillus. Aseptic precautions were observed, and the procedure modified slightly in order to ensure safety from accidental laboratory infection.

After cooling 5 or 6 Leighton tubes to room temperature, their cover slips were withdrawn, drained, rinsed briefly in 0.9% NaCl, drained once more, and totally immersed in tubes containing freshly mixed ice-cold fixative solution. This was a 2:1 (v/v) mixture of 1.0% (w/v) OsO_4 and 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. After 1 min the cover slips were transferred to capped tubes containing fresh fixative solution, and left on ice for 45–60 min; preliminary tests had established that no viable bacilli survived after 30 min of this treatment. The cover slips were rinsed and then submerged in ice-cold Ringer's solution, and the cells pushed off gently with a rubber policeman. The pooled cells were centrifuged gently (less than 500 g for 1 min), resuspended for 15 min in 0.25% uranyl acetate in 0.1 M Veronal-acetate buffer, pH 6.3, washed again in Ringer's solution, and transferred to 25% ethanol for about 10 min (all solutions were ice cold). Dehydration was completed in the usual manner with graded concentrations of ethanol at room temperature, followed by dry acetone. The pelleted cells were infiltrated with a 1:1 (v/v) mixture of acetone and complete epoxy resin mixture (Epikote 812, Shell Chemical Company Ltd., London) for 1–3 days at room temperature, and infiltration was completed in gelatin capsules by allowing the cells to settle through complete resin mixture in a dessicator *in vacuo* overnight at room temperature. After a final centrifugation the capsules were transferred to an incubator at 60°C and the blocks cured for 7 days.

Thin sections were cut in widely spaced interrupted series, so as to promote the sampling of as many different cells as possible from each block, and were mounted on bare copper grids. After staining with uranyl acetate and lead citrate (the latter omitted in experiments with ferritin), the sections were examined in a Philips EM 300 electron microscope (Pye Unicam Ltd., Cambridge, England).

Acid phosphatase activity: After cooling the Leighton tubes to room temperature the cover slips were withdrawn, rinsed briefly in 0.9% NaCl, drained, and immersed for 20 min at 4°C in fixative solution (2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7) in half-test tubes. The cover slips were washed in three changes of 0.9% NaCl (total 15 min), followed by 0.05 M sodium acetate buffer saline, pH 5.0, for 5 min. They were then transferred to Gomori substrate, pH 5.0 (12), for 20 min at 37°C, washed in 0.9% NaCl, and postfixed with ice-cold 1.0% OsO_4 in phosphate buffer (17) followed by dehydration, embedding, and thin sectioning as above.

Ferritin labeling: The macrophage monolayers were established for 2 wk, with a medium change at 2–4 days, in order to induce an abundance of lysosomes. The cultures were then exposed to ferritin, with or without subsequent ingestion of tubercle bacilli. Culture medium was withdrawn from the Leighton tubes and temporarily stored, and the cover slips were washed with BSS; this was in turn replaced with 0.5 ml of BSS containing 2.5% human cord serum and ferritin at a final concentration of 20 mg/ml. The tubes were then incubated for

3½ hr at 37°C. After washing with BSS twice to remove free ferritin from the cell layers, the original culture medium was restored and the tubes incubated for a further 3 hr, so that ingestion of ferritin adsorbed on the cell surface could be completed. Monolayers to be infected were washed again and the usual procedure for ingestion of bacilli was followed (i.e. exposure for 2 hr at 37°C); finally 1 ml of fresh culture medium (including streptomycin) was introduced and the tubes gassed and cultivation resumed at 37°C. The ferritin (Calbiochem, Los Angeles, Calif., B grade, twice crystallized, "cadmium-free," 100 mg/ml) had been prepared from horse spleen. Some batches showed immediate toxicity for the macrophages, for reasons unidentified; suitable batches showing no such toxicity were selected after trial.
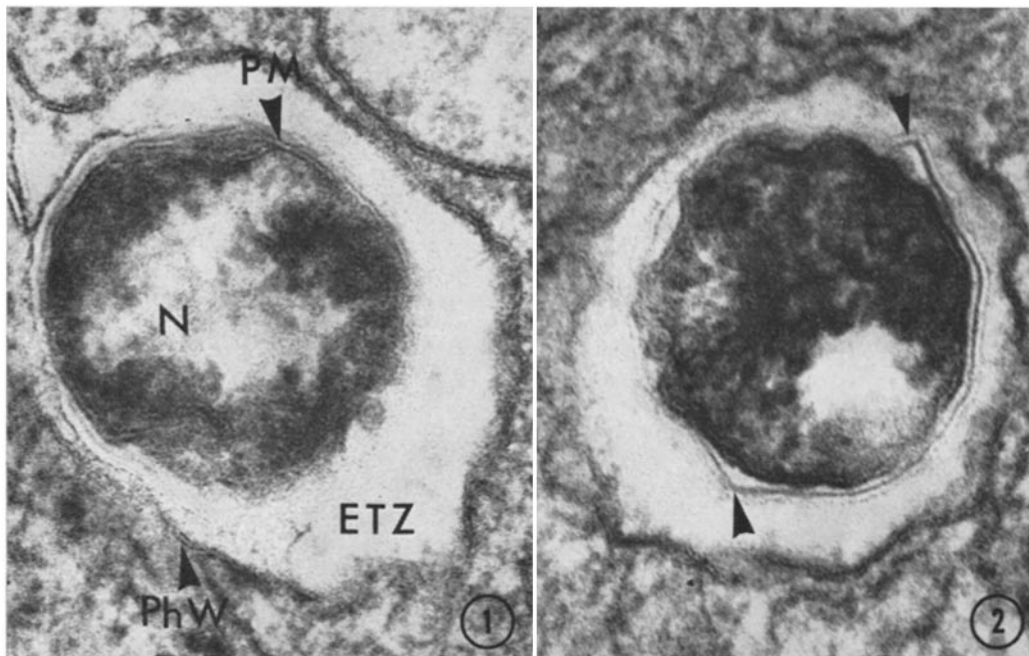
FIGS. 1 and 2. Intracytoplasmic phagosomes containing sectioned intact tubercle bacilli (strain H37Rv) 4 days after ingestion, illustrating some details of the bacterial morphology. A central nuclear region (N) is surrounded by dense bacterial cytoplasm; and the organism is bounded by a typical plasma membrane (PM) and a morphological cell wall (arrowed at two points in Fig. 2). An irregular electron-transparent zone (ETZ) separates each organism from the phagosome wall (PhW). × 160,000.

RESULTS

Ultrastructure of Tubercle Bacilli within Macrophages.—The following basic features characterize structurally intact tubercle bacilli as encountered in random thin sections of macrophages infected with either strain H37Rv or BCG (Figs. 1 and 2): (a) an innermost fibrillar nuclear region of overall low electron opacity, surrounded by (b) a high-density bacterial cytoplasm rich in

ribosomes; this is bounded externally by (c) the bacterial plasma membrane, outside which is (d) the cell wall complex, the outer aspect of which appears to
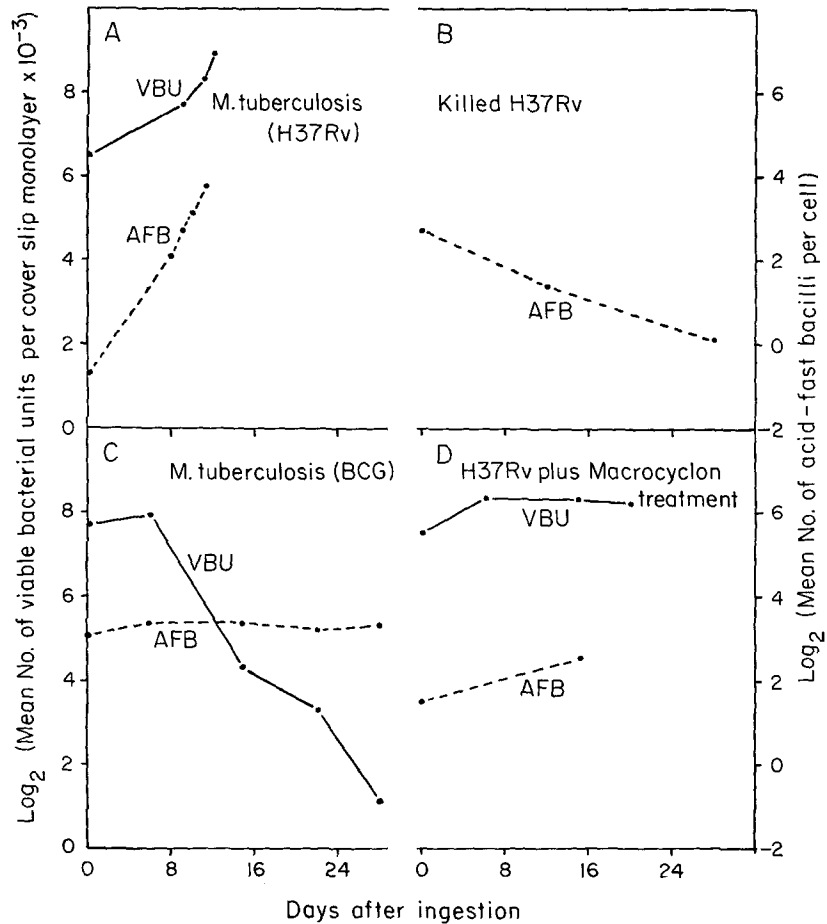

FIG. 3. The growth of *M. tuberculosis* in cultured mouse peritoneal macrophages, assessed by counts of viable bacterial units and of intracellular acid-fast bacilli in the monolayers, at different periods after ingestion of (A) virulent tubercle bacilli (strain H37Rv); (B) inactivated (irradiated) virulent bacilli (H37Rv); (C) attenuated tubercle bacilli (strain BCG); and (D) virulent bacilli (H37Rv) in Macrocyclon-treated macrophages. ●——● (VBU), viable bacillary units per macrophage monolayer, assessed by culture. ●----● (AFB), acid-fast bacilli per macrophage in monolayer, stained by Ziehl-Neelsen method.

be continuous with (e) a peculiar capsule-like electron-transparent zone or "clear substance" (18). The latter is a constant feature but very variable in thickness; its outer surface (presenting as a single dense line in favorable planes

of section) is in direct contact with the unit membrane that constitutes the phagosome wall. The well-known tendency for holes to develop, during exposure to the electron beam, around sectioned intracellular mycobacteria seems, from our experience, to be due to a special problem of obtaining adequate epoxy resin support for this peribacillary electron-transparent zone; it was minimized by prolonged infiltration (see Materials and Methods).

Criteria that we have adopted throughout this study for the recognition of pathological damage affecting the tubercle bacilli have been as follows: (*a*) gross cavitation notably affecting the nuclear region; (*b*) herniation of cytoplasmic contents through breaks in the plasma membrane; (*c*) lamination, often leading to myelin figure formation, involving the bacterial cell wall and its surrounding electron-transparent zone; or most commonly, (*d*) combinations of these abnormalities (Figs. 7–10). Bacilli that were not considered to be "damaged" were classified as "intact" or, in obscure cases, as "doubtful."

*Response to Infection with a Multiplying Virulent Strain of M. tuberculosis (H37Rv).—*

Bacterial multiplication: Infection of the cultured macrophages with strain H37Rv was followed by progressive intracellular multiplication, leading to destruction of the monolayer 8–10 days after a heavy infection, or 14–16 days after a light infection (see Materials and Methods). However, for the first two-thirds of the survival period after these infections the monolayers remained intact and the general appearance of the cells by light microscopy after conventional stains, e.g. methylene blue or Giemsa, was essentially normal. The progress in typical infections, revealed both by light-microscopic counts of intracellular acid-fast bacilli and by counts of viable bacterial units per coverslip monolayer, is shown in Fig. 3 *A*. Both curves rise steadily.

Ultrastructural features: Parts of an uninfected macrophage from a 2 wk old culture are shown in Fig. 4. The typical dense granules are abundant, in accordance with the high serum content of the culture medium and the duration of cultivation. They vary markedly in density and size, ranging in diameter from about 90 nm to 1 μ with the majority in the size range of 0.5–0.8 μ. Each is bounded by a well-preserved unit membrane (Fig. 5).

Gross distortions of cellular architecture inevitably complicate the terminal stages of infection, and in the present study we have concentrated our electron-microscopic observations of macrophages infected by strain H37Rv upon the period preceding terminal disintegration of the monolayers. In cells fixed and

Fig. 4. Ultrastructural features of the cytoplasm of a sectioned peritoneal macrophage from a monolayer culture maintained 15 days in high-serum medium. Dense granules, or secondary lysosomes (Gr), are abundant, varying both in size and in the density of their contents. Profiles of mitochondria (M) and of the Golgi complex (Go) are also shown. × 60,000.

Fig. 5. A dense granule at higher magnification, showing the limiting unit membrane. × 120,000.
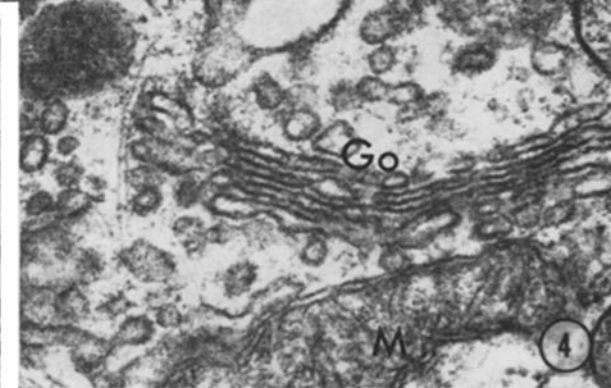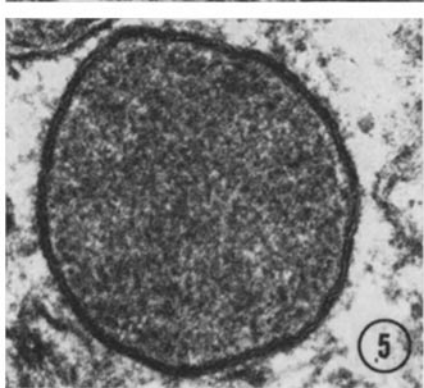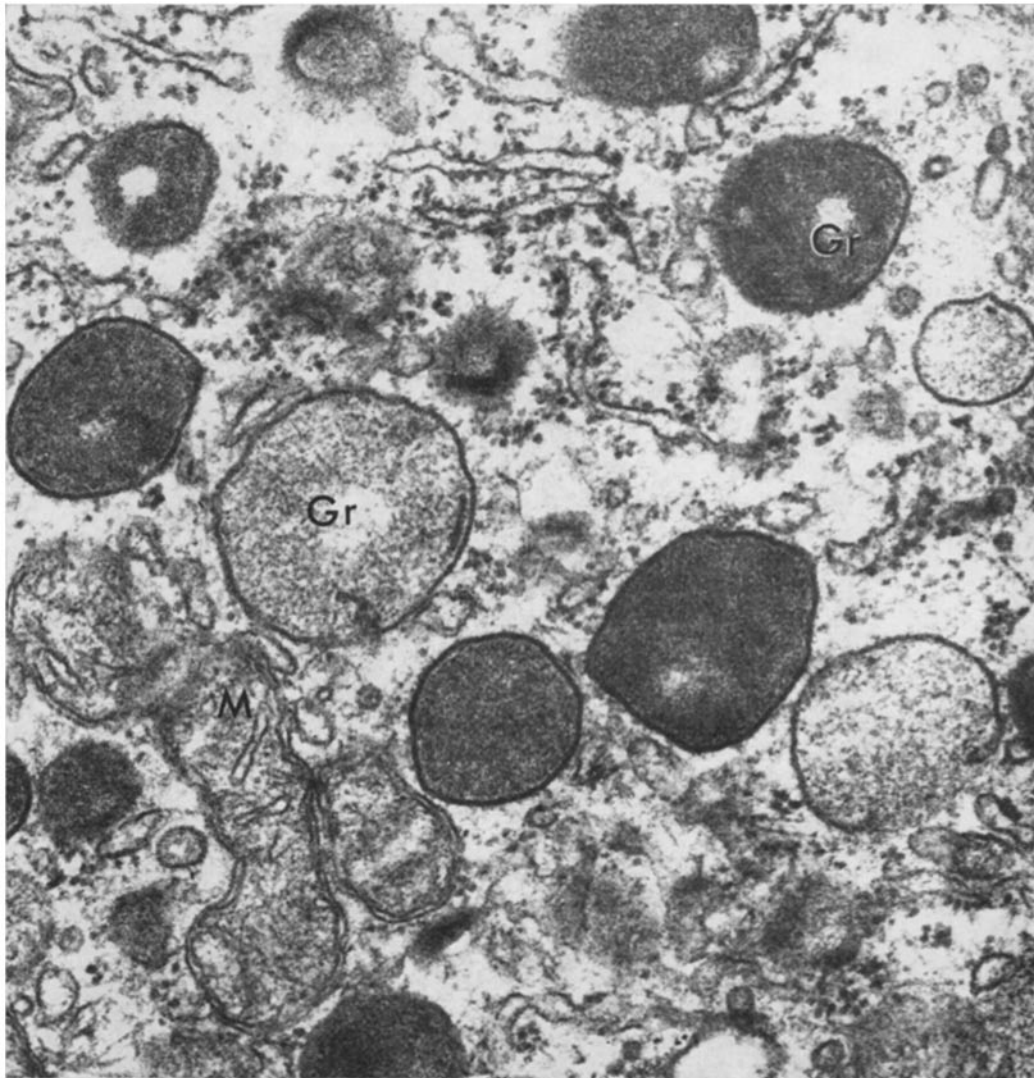
sectioned up to 4 days after a heavy infection (i.e. about half the monolayer survival period) the nucleus, mitochondria, and rough endoplasmic reticulum appeared to be generally unaffected. Phagosomes, varying in frequency from cell to cell, contained intact or damaged bacilli. These were mostly seen as one bacillus per phagosome profile (Figs. 1 and 2), but larger phagosomes containing several sectioned bacilli were also encountered. Bacteria were never seen to be free in the cytoplasm (i.e. outside phagosomes) at this stage of the infection. Unlike those of uninfected macrophages the dense granules frequently showed signs of having fused with one another giving rise to large membrane-bound confluent structures, often but not invariably located in the neighborhood of phagosomes that contained bacteria.

Evidence was frequently seen of fusion having occurred between dense granules and bacterium-containing phagosomes, as indicated by the presence of dense material resembling lysosomal contents within these phagosomes, much as reported previously for peritoneal macrophages containing ingested bacteria of various species including *Salmonella typhimurium* and *Listeria monocytogenes* (e.g. reference 19). However, a contrast soon became apparent: signs of fusion were infrequent if the bacilli were intact, but almost universal if damaged. Since a main objective was to study lysosome-phagosome fusion phenomena, this contrast indicated a need for methods of determining the occurrence or nonoccurrence of fusion more precisely than is possible by routine inspection of standard thin sections. Two approaches seemed possible, namely acid phosphatase cytochemistry and prelabeling of lysosomes with electron-opaque markers.

Acid phosphatase activity: By light microscopy the large population of lysosomes gave rise, as expected, to intense granular cytoplasmic staining in monolayers subjected to the conventional Gomori technique, after glutaraldehyde fixation; and it was therefore difficult to identify local reactions associated with groups of ingested acid-fast bacilli. If prior fixation was omitted, uninfected cells showed slight background staining and a few had brown or black granules of various sizes. Cells infected with strain H37Rv showed a more pronounced general reaction, particularly in the proportion of cells showing dark granules. Moreover, closely associated with many groups of ingested bacilli there was local activation, shown by masses of black reaction product in apparent contact with them.

This evidence of response to tuberculous infection led us to anticipate similar findings by electron microscopy, serving as a guide to the frequency of lysosome-phagosome fusion. However, although many of the characteristic lysosomal granules, both in uninfected cells and in cells infected 3 days previously, were heavily marked with reaction product after conventional glutaraldehyde fixation and Gomori staining, the poor quality of membrane preservation and erratic distribution of the reaction product (i.e. variation between cells and between lysosomes within a cell) made interpretation difficult. In particular, it frustrated entirely the quantitative approach that we wished to attempt. Nevertheless, a difference in the pattern of lysosome-phagosome fusion, according to whether phagosomes contained intact or damaged organisms, was suggested by the distribution of the reaction product in many cells and was consistent with our earlier impression.

Use of ferritin as marker: Attention has already been called to the fusion potential of

secondary lysosomes in macrophages (4). Various electron-opaque markers have been used in other experimental situations to label preexisting lysosomes in order to study their fusion phenomena (e.g. references 20, 21). Localization of ferritin in vitro within macrophage pino-cytic vesicles and channels and within "large vacuoles" was reported after adding the ferritin to the culture medium (22). We decided to examine the capacity of ferritin to prelabel macro-
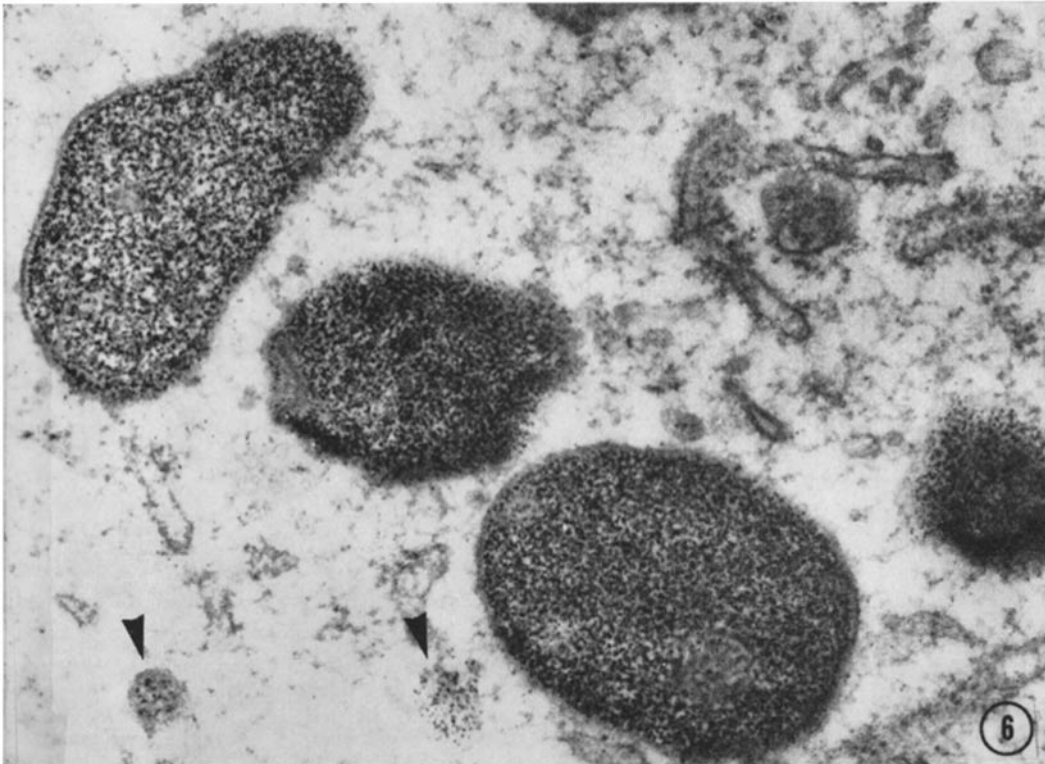

Fig. 6. Localization of electron-opaque ferritin marker in the dense granules (secondary lysosomes) of a normal cultured macrophage fixed 4 days after pulse labeling for $3\frac{1}{2}$ hr. Few if any organelles identifiable as dense granules had failed to acquire the marker as shown. Two smaller ferritin-containing profiles (arrowed) are presumed to be residual pinocytic vesicles or channels. Lead staining was omitted. X 77,000.

phage lysosomes before infection with tubercle bacilli. The result was that virtually all of the dense granules (secondary lysosomes), including the smallest, were selectively and heavily marked (Fig. 6), as well as a considerable number of small, peripheral pinocytic vesicles and canalicular elements of the cytoplasm. Traces of ferritin were usually also to be found free in the cytoplasm, though absent from the nucleus and mitochondria. Naturally occurring ferritin was not observed in cultures to which ferritin had not been added. We thus appeared to have a method for observing the fusion behavior of ferritin-prelabeled secondary lysosomes and pinocytic vesicles; but the activity of small, unlabeled categories of vesicles (e.g. Golgi-type
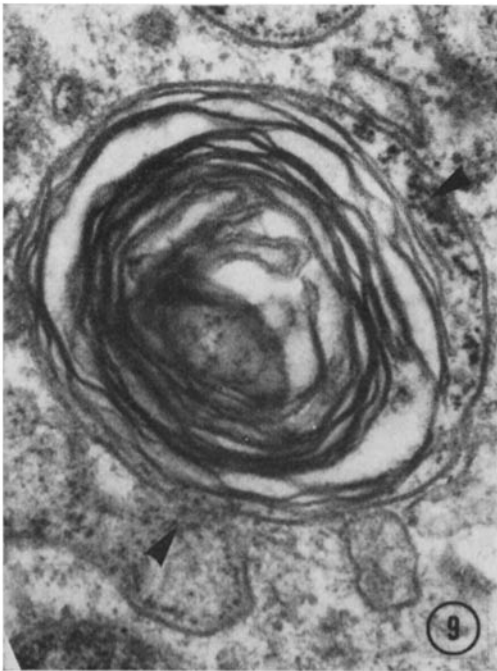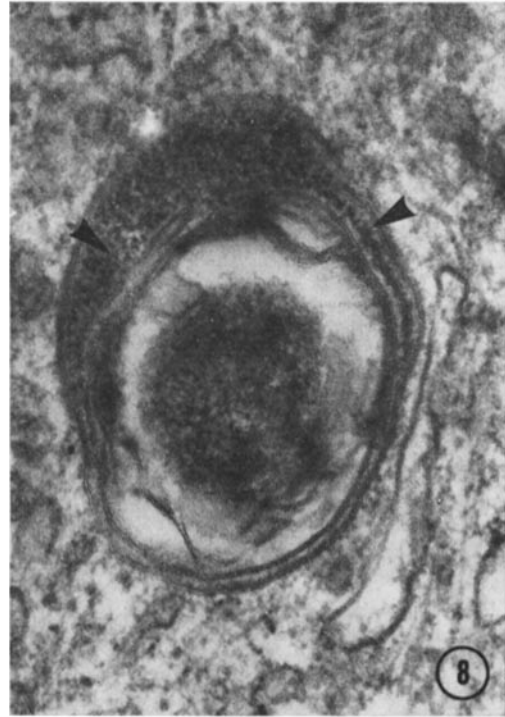
primary lysosomes [2]) could of course pass undetected. Various degrees of fusion of labeled lysosomes with phagosomes containing tubercle bacilli are shown in Figs. 7–10.

Quantitative assessment of fusion: To express both bacillary morphology and the lysosomal fusion response in quantitative terms, we introduced a survey method capable of statistical evaluation. Sample monolayers were fixed at varying times after infection, and every cell profile encountered in the randomly selected thin sections was systematically surveyed. Each

TABLE I

(*A*) *Proportions of Intact and Damaged Bacteria in Macrophage Profiles 1 and 4 Days after Ingestion of Virulent M. tuberculosis (Strain H37Rv), Assessed by Electron Microscopy*

Day after infection	Cell profiles sampled	Bacilli encountered	Structural appearance of bacilli		
			Intact	Damaged	Doubtful
	No.	*No.*	%	%	%
1	186	200	46	49	5
4	156	228	55	38	7

(*B*) *Proportions of Bacterium-Containing Phagosomes (in These Cell Profiles) That Show Fusion with Ferritin-Prelabeled Lysosomes, Subdivided According to Whether the Bacteria are Damaged or Intact*

Morphology of bacilli in phagosomes	Day after infection	Cell profiles sampled	Phagosomes encountered	Fusion		
				Yes	No	Doubtful
		No.	*No.*	%	%	%
Damaged	1		85	96*	2‡	1
	4		67	99**	0‡‡	1
Intact	1		70	36*	63‡	1
	4		90	23**	71‡‡	6
Doubtful	1		(11)	(64)	(36)	(0)
	4		(13)	(54)	(46)	(0)
Total	1	186	166	69	30	1
	4	156	170	55	41	5

The figures in parentheses refer to totals of less than 15. The differences within the pairs of percentages marked *, ‡, **, and ‡‡ are significant ($P < 0.01$).

of the cell profiles thus sampled was recorded either as containing no visible bacilli, or as containing one or more visible bacilli. Where bacilli were present, each bacillus-containing phagosome was recorded, first as to whether the bacteria were damaged or intact according to the

FIGS. 7–10. Presence of ferritin in phagosomes containing tubercle bacilli (H37Rv) ingested 1 day (Figs. 7 and 10) or 4 days (Figs. 8 and 9) before fixation, all taken from the same experiment. These macrophages received a standard ferritin pulse before infection; and the presence of this marker in the phagosomes (arrowed) is indicative of lysosome-phagosome fusion having occurred. An apparent site of fusion between a dense granule (Gr) and the phagosome is shown in Fig. 7; the bacillus in this instance was scored as intact. Increasing degrees of bacillary damage, with myelin figure development, are depicted in Figs. 8, 9, and 10. × 72,000 (Figs. 7 and 9); × 90,000 (Fig. 8); × 62,000 (Fig. 10).

stated criteria and, secondly, as to whether any ferritin macromolecules were detectable in the phagosome. In this way we could ascertain (*a*) the prevalence of apparently intact and visibly damaged bacilli seen in the cell profiles (mean number per profile); (*b*) the proportions of bacilli that were intact or damaged; and (*c*) for phagosomes containing intact or damaged bacilli respectively, the proportions of each category showing evidence of fusion with lysosomes. In the few phagosomes that contained both damaged and intact bacillary profiles, the contents were recorded as damaged. (For a critique of the method, see Discussion).

The results of an experiment, incorporating macrophages sampled 1 and 4 days after a heavy infection with strain H37Rv are given in Table I. About half the intracellular bacilli on day 1 were classified as intact and half as damaged (Table I *A*). It is probable that the latter originated mostly from dead bacteria in the infecting inoculum rather than having been killed in the cells shortly after ingestion.

The reasons for this view are: (*a*) There was no significant increase in the prevalence of damaged bacilli (mean number per cell profile) between day 1 and day 4 in this and all comparable experiments. (*b*) On average about half the organisms were nonviable (ratio viable count to total acid-fast count) in sampled suspensions of *M. tuberculosis* prepared by glass-homogenization of surface cultures, as used for the inoculum in these experiments. One such suspension of strain H37Rv was fixed, embedded as a pellet, sectioned, and surveyed in precisely the same way as the tissue cultures: just under half the bacilli were scored as damaged.

Taking all bacillus-containing phagosomes together, the majority showed signs of fusion with lysosomes (69% at day 1 and 55% at day 4; Table I *B*); but the presence, concurrently, of damaged and intact bacilli permits the pattern of fusion of lysosomes with phagosomes containing the two types of organism to be compared. A striking and statistically significant difference was then observed, both at day 1 and at day 4 (Table I *B*). In almost all (96 and 99%) the phagosomes containing one or more damaged bacilli there was evidence of lysosomal fusion, whereas this was seen in only a minority (36 and 23%) of those containing intact organisms. Thus at least up to halfway through the monolayer survival period after a heavy tuberculous infection there was a marked tendency for the phagosomes containing intact bacilli not to have fused with ferritin-marked lysosomes (Figs. 11–13), at a time when those containing

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FIGS. 11–13. Absence of ferritin in phagosomes containing ingested tubercle bacilli (H37Rv) in macrophages labeled with ferritin before infection. In each case the bacilli were scored as intact.

FIG. 11. 1 day after infection: two labeled dense granules (Gr) are near to the phagosome but have not fused with it. × 62,000.

FIG. 12. On this occasion macrophages were maintained in the presence of Macrocyclon and fixed 5 days after infection. A number of surfactant-distended lysosomes are adjacent to the phagosome but have evidently not fused with it. × 135,000.

FIG. 13. Same experiment as in Fig. 12, but the cells were fixed 14 days after infection. At this stage 66% of the phagosomes containing intact bacilli were scored as unfused with ferritin-labeled lysosomes. × 130,000.

Gr
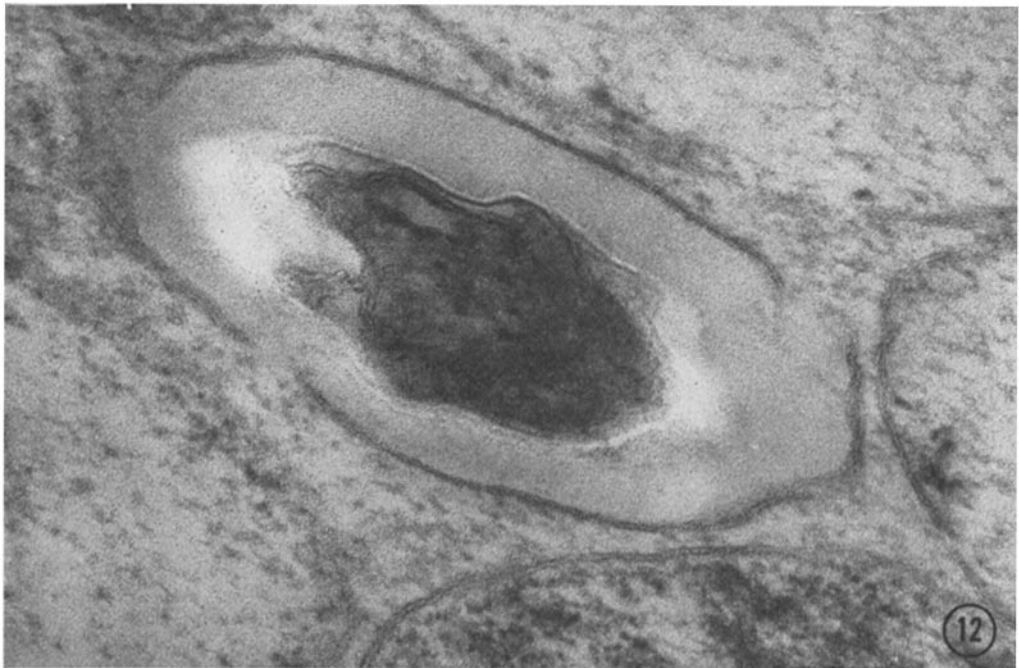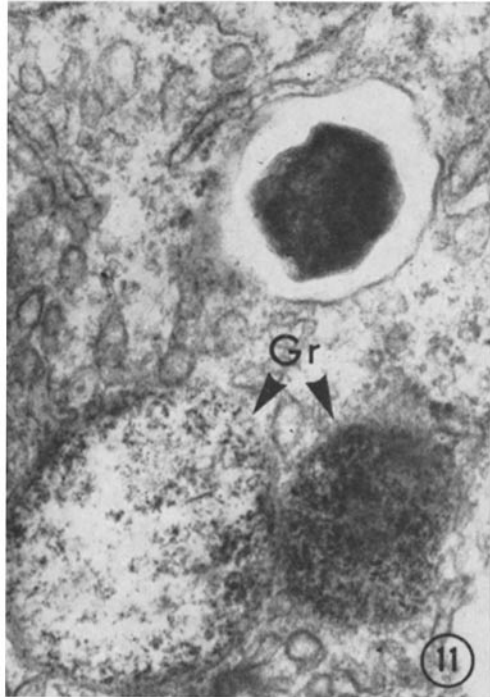
the morphologically damaged members of the same intracellular population had undoubtedly fused (Figs. 8–10).

In order to explore this phenomenon further, two experimental situations were created in which tubercle bacilli were deliberately rendered nonviable, in one case by irradiation before ingestion by the macrophages and in the other by using a live attenuated strain that would lose viability within the cells.

Response to Ingestion of Inactivated Virulent Tubercle Bacilli (Strain H37Rv).— A normal suspension of strain H37Rv was exposed to 200 *k* rad gamma radiation from a cobalt 60 source, since it had been shown in a preliminary experiment that 100 k rad was a lethal dose. A drop of the irradiated suspension was inoculated on to Loewenstein-Jensen egg medium; no colonies grew, indicating a loss of viability calculated as >99.99%. The macrophages were exposed to ferritin in the usual way, followed by a high dose of the irradiated bacilli. Sample monolayers were examined by light microscopy (Ziehl-Neelsen stain) 3 hr, 12 days, and 28 days later. By 12 days the cytoplasm had become markedly vacuolated. The number of visible acid-fast bacilli per cell declined progressively (Fig. 3 *B*); at 3 hr the bacilli appeared normal, but many of those remaining visible after 12 and 28 days appeared fragmentary or pale.

Macrophages were taken for electron microscopy 4 days after exposure to the irradiated bacilli. Numerous intraphagosomal bacteria were seen, each being surrounded by a typical electron-transparent zone indistinguishable from that observed when a live inoculum of strain H37Rv had been used. Another point of similarity to macrophages containing live bacilli was the frequent occurrence of large confluent membrane-bound structures, evidently formed by interlysosomal fusion. In Fig. 14 the morphological condition of the bacteria and the prevalence of phagosome-lysosome fusion, as judged by ferritin transfer, are shown in comparison with macrophages sampled 4 days after receiving a live inoculum of strain H37Rv. The proportion of irradiated bacilli still appearing intact at this stage was half that seen with the nonirradiated bacteria (i.e. 25% as against 53%), the difference being significant ($P < 0.01$). With the irradiated as well as the live inoculum virtually all the phagosomes containing damaged bacteria showed signs of fusion with ferritin-marked lysosomes. Moreover, in the macrophages inoculated with irradiated bacilli the majority of the phagosomes containing intact bacteria had fused (68%) and only a minority had not (19%), whereas with the live inoculum the proportions were reversed (27% fused and 65% not), these differences being statistically significant ($P < 0.01$). Thus, with the irradiated inoculum, even where the organisms were morphologically intact (though undeniably nonviable), most were associated with fusion, like the damaged organisms in the same cells; and of the total phagosome population (containing intact or damaged organisms), as many as 86% showed signs of fusion. These results with nonviable bacteria complement our previous observations by providing evidence associating viability of tubercle bacilli with nonoccurrence of lysosome-phagosome fusion.

Response to Infection with Attenuated Tubercle Bacilli (Strain BCG).—The attenuated vaccine strain BCG (substrain Moreau) did not multiply appreciably in our cultured macrophages, but was not immediately inactivated. Viable counts of bacteria released from the infected cell monolayers showed that many organisms remained viable up to 5 days, but that a rapid decline followed, with
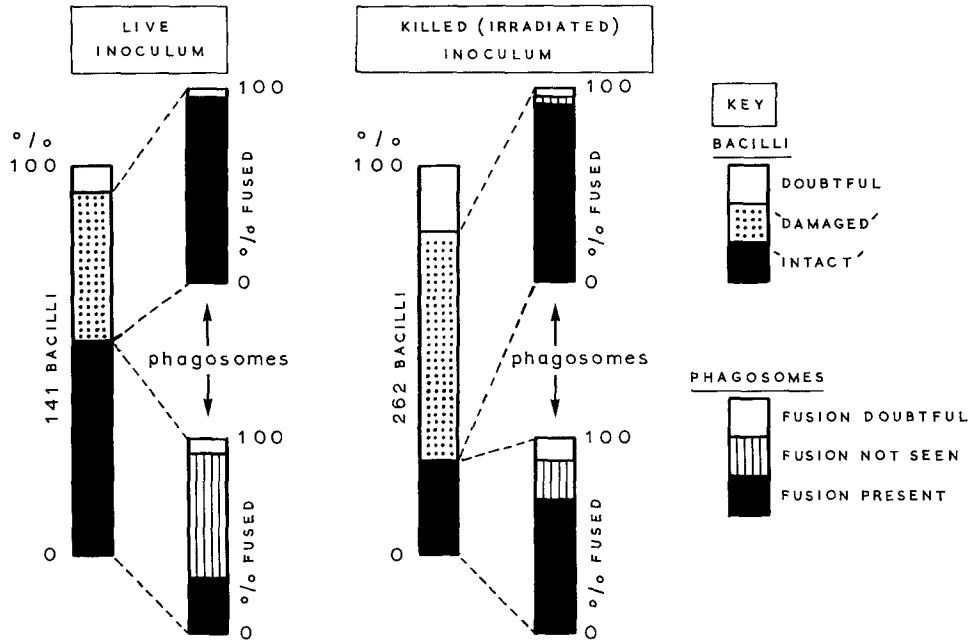

FIG. 14. The proportions of intact and damaged bacteria in macrophage profiles 4 days after ingestion of (a) live and (b) inactivated (irradiated) virulent *M. tuberculosis* (strain H37Rv), assessed by electron microscopy. Also, the proportions of bacterium-containing phagosomes in these cell profiles that show fusion with ferritin-prelabeled lysosomes, subdivided according to whether the bacteria are damaged or intact.

Note. The number of cell profiles sampled (and the number of bacilli encountered in the samples) were as follows (at 4 days): live inoculum, 108 cells (141 bacilli); killed inoculum, 324 cells (262 bacilli).

90% loss of viability by day 15 and 99% by day 28. By light microscopy the cell structure appeared normal, and counts of acid-fast bacilli per cell on the cover slips remained steady during 4 wk of observation (Fig. 3 C).

Macrophages fixed for electron microscopy 2, 5, and 15 days after a heavy BCG infection showed intraphagosomal bacteria as before. Interlysosomal fusion seemed less frequent than in response to H37Rv infection. The progress of the BCG infection is shown in Fig. 15. The findings at 2 and 5 days were very similar, with 40–50% of the bacteria intact and a similar proportion

damaged. Of the total phagosome population, 50–55% showed signs of fusion with ferritin-marked lysosomes; once again this phenomenon was evident in the great majority (80–90%) of the phagosomes containing damaged bacteria, but in only a minority (23%) of those containing intact bacteria.
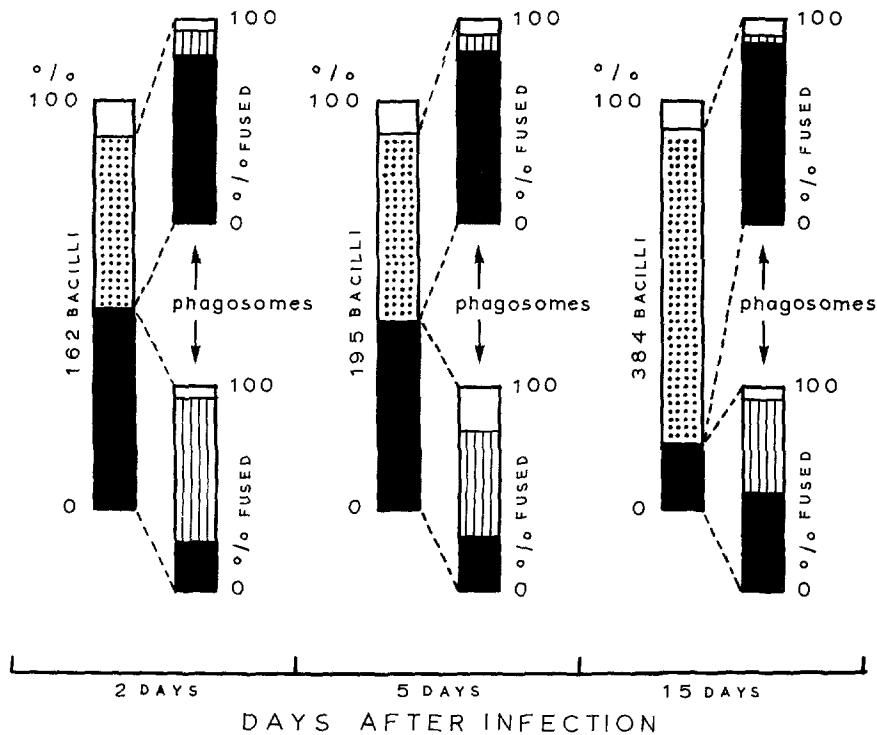

Fig. 15. The proportions of intact and damaged bacteria in macrophage profiles 2, 5, and 15 days after ingestion of attenuated *M. tuberculosis* (strain BCG), assessed by electron microscopy. Also, the proportions of bacterium-containing phagosomes in these cell profiles that show fusion with ferritin-prelabeled lysosomes, subdivided according to whether the bacteria are damaged or intact.

Notes. The key is as for Fig. 14. The number of cell profiles sampled (and the number of bacilli encountered in the samples) were as follows: 2 days, 118 cells (162 bacilli); 5 days, 171 cells (195 bacilli); 15 days, 243 cells (384 bacilli).

At day 15 the position had changed, in conformity with the decline in the viability counts. Most of the bacteria scored were now damaged (77%) and correspondingly fewer (16%) were intact (Fig. 15), the changes being significant ($P < 0.01$). As before, fusion of ferritin-marked lysosomes was detected with nearly all (90%) of the phagosomes containing morphologically damaged bacteria, and now with an increased proportion (48%) of the few phagosomes

that still contained morphologically intact bacteria, the increase being significant $(0.01 < P < 0.05)$. Thus in the early stage of this BCG infection most phagosomes containing intact bacteria were apparently unfused, but in a later stage the great majority (84%) of the total phagosome population (containing intact or damaged organisms) had fused with lysosomes.

It is also interesting to consider in this experiment the actual prevalences of intracellular bacilli; between days 2 and 5 the mean number of damaged bacilli per cell profile did not increase (the values being 0.6 and 0.5 respectively). As with the multiplying strain H37Rv, it is probable that most of the damaged BCG bacteria at this early stage were dead from the outset. On the other hand, between days 5 and 15 the mean number of damaged bacilli per cell profile doubled (to a value of 1.2); many of the newly damaged bacilli were therefore presumably derived from organisms that had been viable in the early stage of this infection.

Our observations on BCG-infected macrophages, following upon those in H37Rv infection, provide suggestive evidence that lysosomes may not fuse with phagosomes containing tubercle bacilli so long as these are viable; detectable fusion seems to be associated in time sequence with a loss of bacterial viability.

A different experimental situation is presented where virulent bacilli survive within macrophages for a prolonged period and without apparent harm to the host cells; this can be achieved by adding to the culture medium the surfactant compound Macrocyclon, which has cell-mediated bacteriostatic properties.

Response to Infection of Macrocyclon-Treated Macrophages with M. tuberculosis (Strain H37Rv).—Macrophages were heavily infected in the usual manner; but for 10 days before, and also subsequent to the ingestion of ferritin and tubercle bacilli, the medium contained Macrocyclon 6 mg/ml. Control cultures were treated with Macrocyclon in the absence of infection. Light microscopy showed moderate swelling of the cells, which is a characteristic effect of exposure to the surfactant (9); in other respects the monolayers remained apparently healthy throughout the postinfection period. The mean number of acid-fast bacilli per cell on the cover slips, based on counts made immediately after infection and again 15 days later, had no more than doubled (Fig. 3 D). Viable counts of bacilli released from the infected cell monolayers showed that during 3 wk of observation the level did not alter appreciably, except possibly for an initial slight rise (Fig. 3 D). These observations are consistent with a virtually steady state. Bacilli recovered from a Macrocyclon cell culture and used to infect fresh untreated monolayers showed undiminished virulence.

For electron microscopy, macrophages were taken 5 and 14 days after infection. In both control and infected cell cultures the surfactant had a profound effect on the secondary lysosomes (Fig. 16); they were increased in num-
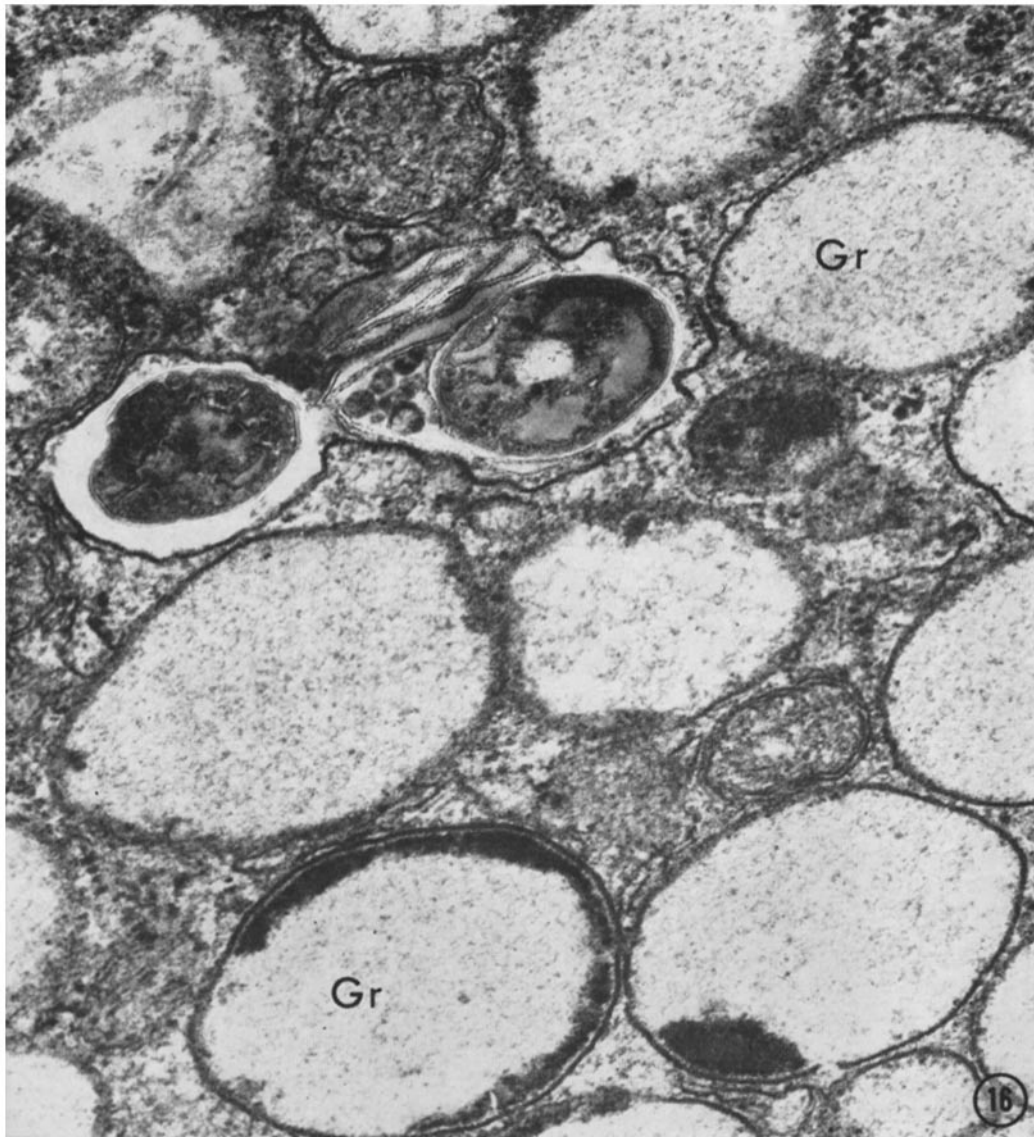
Fig. 16. The effect of Macrocyclon on the cytoplasm of macrophages maintained in the presence of the surfactant, fixed 4 days after ingestion of tubercle bacilli. The dense granules (Gr) are grossly distended, but their limiting membranes are intact. Profiles of two intra-phagosomal bacilli are seen, one of which was scored as intact and the other as damaged. It may be noted that in contrast to the dense granules, the phagosomes were not visibly affected by the surfactant. × 60,000.

ber and were markedly distended, but their limiting membranes were intact. Their normal dense contents, as well as the ferritin, were concentrated peripherally, usually at one side, giving an electron-lucent appearance to the lysosomes very similar to that resulting from exposure of macrophages to sucrose (23). The morphology of the macrophage nuclei, mitochondria, endoplasmic

TABLE II

(A) *Proportions of Intact and Damaged Bacteria in Profiles of Macrocyclon-Treated Macrophages 5 and 14 Days after Ingestion of Virulent M. tuberculosis (Strain H37Rv), Assessed by Electron Microscopy*

Day after infection	Cell profiles sampled	Bacilli encountered	Structural appearance of bacilli		
			Intact	Damaged	Doubtful
	No.	No.	%	%	%
5	84	98	67	22	10
14	166	135	66	28	6

(B) *Proportions of Bacterium-Containing Phagosomes (in These Cell Profiles) That Show Fusion with Ferritin-Prelabeled Lysosomes, Subdivided According to Whether the Bacteria are Damaged or Intact*

Morphology of bacilli in phagosomes	Day after infection	Cell profiles sampled	Phagosomes encountered	Fusion		
				Yes	No	Doubtful
			No.	%	%	%
Damaged	5		15	93*	7‡	0
	14		35	97**	3‡‡	0
Intact	5		54	31*	61‡	7
	14		86	21**	66‡‡	13
Doubtful	5		(8)	(50)	(50)	(0)
	14		(7)	(71)	(29)	(1)
Total	5	84	77	45	49	5
	14	166	128	45	47	9

The figures in parentheses refer to totals of less than 10. The differences within the pairs of percentages marked *, ‡, **, and ‡‡ are significant (P < 0.01).

reticulum, and other cytoplasmic organelles remained unaffected by the surfactant, apart possibly from some hypertrophy of the Golgi complex. These selective changes are consistent with the view that Macrocyclon (like the structurally related "Triton WR-1339" [Rohm and Haas Co., Philadelphia, Pa.] [24]) accumulates within cellular secondary lysosomes (9).

In infected cells interlysosomal fusion was not a conspicuous feature, in contrast with its frequency in the absence of the surfactant. The bacteria were located as usual in phagosomes, the morphology of which seemed not to differ from phagosomes in cells cultured without Macrocyclon; in particular, they

were not subject to the gross distension shown by the secondary lysosomes (Fig. 16). The proportion of bacteria scored as intact at day 5 approached 70%, due perhaps to a higher than usual proportion of viable organisms in the inoculum. The proportions of damaged and intact bacilli were essentially the same at days 5 and 14 (Table II *A*). Furthermore, the mean numbers of intact and of damaged bacilli per cell profile showed no significant increases between days 5 and 14, in agreement with earlier observations in vivo which indicated a steady state of tubercle bacilli in the lungs of Macrocyclon-treated mice (14, 25).

Table II *B* shows that under Macrocyclon treatment the usual, nearly universal fusion of ferritin-marked lysosomes with those phagosomes containing damaged bacilli was evident both at day 5 and at day 14. But the greater interest lies in the phagosomes containing intact bacilli. Not only had the large proportion of bacteria scored as intact not changed over the intervening period of 9 days, but the phagosome-lysosome fusion rate for intact bacteria remained low, over 60% of their phagosomes escaping fusion (Figs. 12 and 13). The fusion pattern already described for progressively multiplying bacteria of this virulent strain is thus maintained when the infection has been "stabilized" by Macrocyclon.

The possibility that the number of ferritin-marked lysosomes fusing with phagosomes increases at a slow rate, and that this phenomenon was therefore underestimated in our short-term study of multiplying bacilli of strain H37Rv, cannot be adequately tested with a heavy progressive infection, since sampling later than 4 days inevitably coincides with commencing disintegration of the monolayers. However, by this use of Macrocyclon to maintain bacterial viability without injury to the host cells, an intracellular population of intact H37Rv bacilli has been observed for 2 wk, with no evidence of late developing fusion. Assuming, therefore, that bacillary "intactness" and cultural viability run closely parallel, the association of viability of *M. tuberculosis* with nonoccurrence of phagosome-lysosome fusion seems inescapable.

DISCUSSION

Critique of Methodology.—The identification and study of lysosomes by means of electron-opaque particulate materials, including ferritin, is well documented (26). In the present work this approach has been extended to the study of phagosome-lysosome fusion behavior in infected cell cultures. In the high concentrations used, ferritin labels virtually all the dense granules (secondary lysosomes) encountered in mouse peritoneal macrophages. Even the smaller granules (<100 nm) are labeled and so we classify them as secondary lysosomes, although similar small dense granules in peritoneal macrophages were regarded by Carr (22) as possibly primary lysosomes. In the system that we have employed, even small transfers of lysosomal contents into a phagosome are capable of detection, because even a few ferritin macromolecules can be identified by electron microscopy on account of the characteristic size and morphology of the iron core (diameter about 55 A). On the other hand, where fusion is minimal, the marker will not necessarily be observed in all planes of section through a phago-

some. In such cases the fusion status of the latter might be misjudged, and this occurrence cannot be excluded in individual instances. But the statistical evidence of nearly universal fusion when the bacteria are damaged, in contrast to the low prevalence of fusion when they are intact, remains valid; hence such error is probably minor.

Selective marking of the lysosomal dense granules persisted for at least 2 wk after pulse-labeling with ferritin, although the concentration of the marker appeared to diminish in some of the lysosomes during that period. An important consideration is that secondary lysosomes formed subsequent to the ferritin ingestion might remain unlabeled, giving rise to an observational error increasing with time. However, observations made 2 wk after the initial massive labeling suggest that new lysosomes do by some means also become labeled. Thus sections made 15 days after the BCG infection showed no appreciable number of un-labeled typical dense granules. Similarly, 14 days after the tuberculous infection of Macro-cyclon-treated macrophages, virtually all the dense granules contained ferritin as well as surfactant. Finally, an experiment was made specially to test this point, in which the macro-phages were infected with strain H37Rv 14 days after ferritin labeling, instead of the same day, and were fixed 2 days later. Nearly all the dense granules were labeled to a greater or lesser degree, though some had clearer centers with ferritin only at the periphery, and a few were unlabeled. However, it was remarkable that even after this postponed infection the contrast in fusion pattern was as marked as ever, with over 90% of phagosomes containing damaged bacteria showing signs of having fused with ferritin-labeled lysosomes as against 25% of phagosomes containing intact bacteria; this finding could be explained by the fusion of several lysosomes with each phagosome and indicates persisting competence of the labeled granules.

Ferritin also marks other organelles, notably smooth-surfaced channels and small low-density vesicles which we have interpreted as pinocytic elements probably in transit to fusion with existing secondary lysosomes. The possibility that ferritin-labeled nonhydrolase-contain-ing pinocytic vesicles can fuse directly with bacterium-containing phagosomes cannot be excluded on the evidence available to us. Nevertheless, it can be said that profiles undoubtedly demonstrating the fusion of typical large secondary lysosomes with phagosomes were fre-quently found, and we have no reason to doubt that these were the main source of the ferritin found in the bacterium-containing phagosomes. Small coated vesicles are numerous in the cytoplasm of the cultured peritoneal macrophages, especially in the Golgi area, but ferritin marker was rarely seen in this category of vesicle. The scattered ferritin molecules observed free in the cell cytoplasm did not increase with time; their precise origin and significance remain obscure. A possible limitation of some importance, as discussed below, is that fusion with phagosomes by unlabeled although enzyme-containing vesicles, i.e. smooth-surfaced Golgi vesicles or primary lysosomes (2), would be unaccompanied by ferritin transfer and so be undetected.

The structural scoring of the bacilli seen within phagosomes is crucial to our analyses. Damaged bacilli are so designated with reasonable assurance, but as the bacillary thin sections are mostly transverse or oblique it must occur in some instances that a bacterium scored as intact has evidence of damage in other planes. This could account in part for lysosomal fusion where it occurred with phagosomes containing intact bacilli, many of these organisms being in reality damaged.

The basis of our quantitative assessment was the indiscriminate sampling of the macro-phages by random sectioning of pelleted monolayers. The extent to which the phagosomes and bacilli scored were representative depends of course on how far the cell profiles surveyed themselves were typical, as well as on the survey procedure. Widely spaced series of sections from 2 or 3 epoxy resin blocks represented each point of assessment, and care was taken to survey systematically all the cell profiles encountered on each grid. In one experiment involv-ing infection with strain H37Rv, the data were submitted to more detailed statistical analysis:

the frequencies of bacteria in the cell profiles followed a negative binomial distribution in 11 of the 14 sets of data. In all experiments the changes in prevalence of the bacilli (mean number per cell profile) have approximately paralleled those of the cultural viable counts and of the counts of intracellular acid-fast bacilli. Further reassuring evidence of the reliability of the methods was that in each experiment the data derived from the different blocks and at different scoring sessions showed little variation provided enough phagosomes and bacteria were counted.

Interpretation of Findings.—The viability status of an individual tubercle bacillus cannot be judged from its ultrastructural morphology alone; but the results of manipulating overall population viability, together with monitoring by cultural counts of viable bacilli, suggest the following general relationships: (*a*) that in any intracellular population tubercle bacilli classified as damaged by our criteria are nonviable bacilli; (*b*) that where the population is multiplying or surviving, a substantial proportion of the intact bacilli are living organisms; but (*c*) that where the population is demonstrably nonviable (e.g. after lethal irradiation or late in the BCG infection), the remaining bacilli still scored as intact are likely to be dead or inactivated.

The quantitative approach adopted, based on differentiating ingested tubercle bacilli into visibly damaged and apparently intact categories, and on the use of ferritin as a lysosome marker, has revealed a low incidence of fusion between macrophage dense granules (secondary lysosomes) and phagosomes containing intact bacteria, in contrast to almost universal fusion when the bacteria are damaged. In our experiments with live strain H37Rv (with or without Macro-cyclon), and also with BCG, half or even more of the bacilli seen in the first few days after infection were in the intact category; and the associated low incidence of phagolysosome formation persisted so long as the proportion of bacilli scored as intact, and the bacterial viability counts, did not fall. It is difficult, therefore, to attribute survival of tubercle bacilli in the macrophages to a resistance to hydrolases within the phagolysosomes; rather it is associated with a lack of contact with these enzymes, owing to the nonoccurrence of lysosomal fusion.

Two hypotheses to explain this pattern may be considered, namely, that many bacilli are intact and alive because of a failure of fusion or, conversely, that fusion does not occur because the bacilli are intact and alive. The first hypothesis presupposes a scarcity or relative inefficiency of the lysosomes. According to this, fusion fails to keep pace with a progressive H37Rv infection, whereas in the BCG infection the increasing incidence of fusion at a late stage succeeds in bringing about declining bacterial viability. The persistence for several wk of viable intact bacilli at a steady level in the presence of Macrocyclon could be accounted for if the surfactant's action was to inhibit fusion. Against this hypothesis is that the macrophages are well provided with secondary lysosomes; and there is no evidence of inefficiency, for they fused readily with those phagosomes containing the part of the bacillary population believed dead in the

inocula, and also with the great majority of those containing bacilli deliberately inactivated by irradiation. In the case of Macrocyclon, strong evidence against its action being dependent on inhibition of phagosome-lysosome fusion is provided by a further experiment, in which the incidence of fusion was compared in Macrocyclon-treated and in normal macrophages 1 and 4 days after an infection with strain H37Rv; no significant difference was found. In the light of these considerations the second hypothesis seems the more likely, fusion by secondary lysosomes being somehow dependent on recognition of the status of the intraphagosomal organisms. If this were so, the nonoccurrence of fusion with phagosomes containing intact bacilli in the progressive H37Rv infection would be due to the bacterial viability, and the persistence of this pattern in the presence of Macrocyclon a response to the parallel maintenance of the bacterial viability; while fusion in the later stages of the BCG infection would be because the bacilli were then recognized as an increasingly nonviable population.

In these experiments, no particular evidence of a regular topographic association of primary lysosomes with bacterium-containing phagosomes has been obtained, even after staining for acid phosphatase. Nevertheless, it is conceivable that small primary lysosomes, unlabeled by ferritin, perform a crucial role in macrophage response by fusing with phagosomes indiscriminately, whatever the state of the enclosed mycobacterium; and that the subsequent response of the secondary lysosomes depends upon the outcome of this initial event. Further experiments are desirable in order to test this possibility.

In our system the macrophage lysosomal response to *M. tuberculosis* differs from the usually observed participation of dense granules in the formation of phagolysosomes enclosing ingested digestible or indigestible particulate matter (3) or microorganisms (7). However, some examples of nonfusion of lysosomes with phagosomes containing viable or multiplying microorganisms have been reported.

Mouse peritoneal macrophages showed by phase contrast large phagocytic vacuoles and degranulation when infected in vitro by various avirulent nonmultiplying bacteria, but not when the bacteria were virulent and capable of multiplication, e.g. *M. tuberculosis* (27). Ultrastructural observations of hamster peritoneal macrophages after phagocytosis of *Histoplasma capsulatum* in vivo showed that although lysosomal fusion did occur, other vacuoles containing normal-looking organisms were also encountered in which there was no evidence of such fusion (28). In an electron-microscopic study of *Staphylococcus aureus* after phagocytosis by liver Kupffer cells both in vivo and in the perfused isolated organ, phagosome-lysosome fusion was shown in the former but not in the latter; the organisms were apparently intact in both cases (29). If mice were exposed to an aerosol of viable spores of *Aspergillus flavus* after cortisone treatment, which renders them highly susceptible to this infection, the lysosomes of their alveolar macrophages showed little or no fusion with phagocytic vacuoles; lysosomes of macrophages from normal mice, which are resistant to this infection, showed extensive fusion (30).

Investigations directly comparable to the present observations, embracing both progressive and modified forms of tuberculous infection in cultured mam-

malian cells and correlating ultrastructural features with bacterial viability data, seem not to have been undertaken hitherto.

However, the ultrastructure of granulomas developing in the lungs and other tissues of mice after intravenous injection of *M. tuberculosis* strain H37Rv has been described (31). Mycobacteria were identifiable within histiocytic cells over a period of many wk after infection. Numerous abnormal cytoplasmic bodies (variously referred to as "lamellated osmiophilic bodies," "opaque droplets," "Y bodies," etc.) were broadly interpreted as lysosomal elements concerned in the cellular response to the foreign organisms. Tubercle bacilli within or closely attached to such organelles were almost entirely degenerate and fragmentary. In some of the illustrations, however, representing the early stages of infection, some well-preserved bacillary forms, with typical electron-transparent zones around them, can be seen in phagosomes showing no obvious signs of lysosomal fusion having occurred, and resembling those containing intact bacilli in the present experiments. In a rather similar study (32) on the cellular response in peritoneal granulomas of the hamster but to heat-killed bacilli of strain H37Rv and to attenuated strain BCG, there was again evidence that dense lysosomes approach and fuse with bacillus-containing phagosomes, producing digestion vacuoles in which stages of bacterial degradation were seen.

It is only to be expected that in vivo systems such as these, subject also to acquired immunity, would reveal a complex and varying pattern of host-parasite relationships. The findings, while broadly consistent with the concept of lysosomal participation in the digestion and disposal of inactivated tubercle bacilli, give little idea of the sequence of events after ingestion of living *M. tuberculosis* or of the relationship between the organisms and the host cells during the period of active bacterial multiplication. In particular, correlations between lysosomal fusion pattern and bacterial morphology and viability were not attempted. Even in our in vitro system there was plentiful evidence of phagosome-lysosome fusion; only by quantifying the proportions of intact and damaged organisms and the lysosomal response to each, as well as correlating the data with cultural counts, did the association of lysosomal fusion to viability of tubercle bacilli become apparent.

A different state of affairs is suggested by ultrastructural and cytochemical studies of infection due to the obligate intracellular parasites, *Mycobacterium lepraemurium* and *Mycobacterium leprae*. Direct examination of biopsied human leprosy lesions (e.g. references 33, 34) and of tissues of mice infected experimentally with *M. lepraemurium* (35) has produced convincing evidence of lysosomal fusion with phagosomes containing the ingested mycobacteria, many of which nevertheless remained intact and seemed even to be multiplying successfully in spite of direct exposure to a microenvironment containing acid phosphatase and probably other lysosomal hydrolases. The resistance of *M. lepraemurium* to lysosomal enzymes has also been clearly demonstrated by multiplication of this organism in cultured rat fibroblasts infected in vitro. Ultrastructural examination revealed frequent fusion of lysosomes with phagosomes, although the contained bacteria (derived from infected mouse liver)

appeared "solid" and were presumably viable (36). This is in striking contrast to the finding now reported for *M. tuberculosis*.

Thus, among the mycobacteria, leprosy bacilli can survive and multiply within phagolysosomes of cultured cells, whereas tubercle bacilli evidently do so mostly in phagosomes without fusion with secondary lysosomes. Further studies on similar lines can be expected to provide comparative information on these relationships after ingestion by macrophages of other mycobacteria including saprophytic species, and of unrelated pathogenic organisms.

It was pointed out recently by Hirsch and Fedorko (37) that mechanisms proposed to account for inactivation of microorganisms in polymorphonuclear leukocytes, involving peroxidases and cationic proteins present in the granules of these cells, may not apply to macrophages, and that "the mechanism of bactericidal action within macrophages remains completely unknown." The present findings, while in full accord with the concept that in cultured macrophages the characteristic dense granules are directly involved in digestion and disposal of dead or inactivated tubercle bacilli, indicate that probably other factors than direct exposure of the bacilli to the contents of these granules (that is, after phagosome-lysosome fusion) actually determine the fate of living tubercle bacilli ingested by the macrophages.

SUMMARY

The cytological response to the ingestion of tubercle bacilli by cultured mouse peritoneal macrophages has been studied by electron microscopy. Methods included a quantitative assessment based on systematic surveying of cell profiles, and of phagosomes and their contained bacteria, encountered in thin sections; classification of the sectioned bacteria into visibly damaged and apparently intact categories; prelabeling of dense granules (secondary lysosomes) with ferritin as an aid to identifying the occurrence and frequency of phagosome-lysosome fusion; and monitoring of bacterial growth and viability by light microscopy and cultural counts. The situations studied were as follows: progressive infection with the multiplying virulent strain H37Rv; ingestion of the same strain previously inactivated by gamma radiation; infection with an attenuated strain (BCG); and a stabilized virulent infection induced by the surfactant Macrocyclon.

In the bacterial suspensions used routinely for inoculation, about half the bacilli were viable, matching closely the proportions of intact and damaged organisms identified with the electron microscope. In the inoculated macrophages, some phagosomes containing intact bacilli and others containing damaged bacilli were always to be found; but the proportion of organisms scored as damaged increased, and that of intact organisms decreased, in situations where the population as a whole had been rendered nonviable before inocula-

tion, or where they became so intracellularly as in the late stages of a BCG infection.

Evidence of fusion of ferritin-marked lysosomes with some bacterium-containing phagosomes was obtained in all experiments, but a significant difference was regularly observed according to whether the bacilli were damaged or intact. Virtually all phagosomes containing damaged bacilli showed signs of fusion; but when many phagosomes were present containing apparently intact organisms (as with actively multiplying strain H37Rv or with this strain held at a steady level of viability by Macrocyclon, and also with strain BCG at an early stage of that infection), signs of fusion of lysosomes with these phagosomes were infrequent.

From these findings it is inferred that intracellular survival of *M. tuberculosis* in cultured macrophages is associated with a tendency to nonfusion of dense granules with the phagosome, thus avoiding direct exposure of the bacilli to the contents of these organelles. It is suggested, further, that fusion of dense granules with the phagosome, leading to digestion, is determined by recognition of the bacillus as nonviable. The possibility is discussed that the cytological response to different mycobacterial infections may reflect differences of a basic nature between facultative and obligate intracellular parasitism.

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