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PHAGOSOME-LYSOSOME INTERACTIONS IN CULTURED MACROPHAGES INFECTED WITH VIRULENT TUBERCLE BACILLI

Reversal of the Usual Nonfusion Pattern and Observations on Bacterial Survival

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The facultative intracellular parasite *Mycobacterium tuberculosis*, after ingestion by cultured mouse peritoneal (MP)[1] macrophages, can be divided, on the basis of its appearance under the electron microscope, into two categories designated as "damaged" and "intact" (1, 2). Fusion of ferritin-prelabeled secondary lysosomes with phagosomes enclosing obviously damaged, and presumed nonviable, bacilli is virtually universal, conforming to the usual fate of large foreign bodies within phagocytes and resulting in exposure of the ingested organisms to the lysosomal digestive enzymes. In contrast, most of the macrophage phagosomes enclosing intact, and probably viable, virulent tubercle bacilli (e.g. strain H37Rv) show no sign of fusion with lysosomes during the first few days of infection. We have suggested that this apparent evasion by the bacilli of direct exposure to the action of the lysosomal contents may be associated with the ability of virulent *M. tuberculosis* to survive and multiply in cultured macrophages (1, 2). Similar nonfusion has since been reported for two species of *Chlamydiae* (3, 4) and by Jones and Hirsch (5) for the protozoal intracellular parasite, *Toxoplasma gondii*. On the other hand, not all *Mycobacteria* resemble virulent tubercle bacilli in this respect; thus, *M. lepraemurium* attracts prompt and almost invariable phagosome-lysosome fusion after ingestion by cultured macrophages (2), while *M. leprae* has a complex intracellular life-cycle, part of which is evidently spent free in the cytoplasm, outside the confines of the vacuolar system (6).

Some intriguing questions now arise, for example: (*a*) Is the nonfusion response to *M. tuberculosis* due to the surface properties of the bacterium or to an active inhibitor derived therefrom? (*b*) Does it represent a fixed character or can manipulation cause a fusion response to be substituted? (*c*) If so, what is the effect of such conversion on subsequent intracellular bacterial survival and

[1] *Abbreviations used in this paper:* B-BSS, bacteria not pretreated with rabbit serum; B-IS, bacteria pretreated with IS; B-IS-W, as B-IS but bacteria washed after exposure; B-NS, bacteria pretreated with NS; B-NS-W, as B-NS but bacteria washed after exposure; BCG, Bacille Calmette-Guérin; BSS, balanced salt solution; IS, rabbit immune (anti-BCG) serum; MP, mouse peritoneal; NS, rabbit normal serum.

multiplication? We have approached these questions by considering means of altering or coating tubercle bacilli before ingestion, so as to promote the onset of phagosome-lysosome fusion. A prerequisite is that the viability of the bacterial inoculum should not be diminished, since dead bacilli consistently induce fusion. From a suggestion by our colleague Dr. J. M. Gaugas, that presence or absence of antibody in vivo on the mycobacterial surface during infection might affect phagosome-lysosome fusion after phagocytosis, we anticipated that attachment of specific antibody to the tubercle bacillus in vitro would offer a practical means of ensuring the inclusion of nonbacterial protein within the peribacillary phagosome. We report here the effects of exposure of virulent *M. tuberculosis* to selected sera on the subsequent lysosomal response and on intracellular bacterial survival and growth in cultured normal MP macrophages.

Materials and Methods

Tissue Culture and Ferritin Labeling. Macrophages, obtained from unstimulated peritoneal cavities of normal female mice of the albino P strain (i.e. unelicited), were cultivated as cover slip monolayers in 1-ml volumes of Chang medium (containing 40% heat-inactivated horse serum) in Leighton tubes; 60 U/ml penicillin was included in the cultivation medium throughout (1, 7, 8). After 10–15 days of cultivation the medium was changed, and after a further 3–5 days the cells were exposed for 2½–3½ h to ferritin (Pentex), twice crystallized and cadmium-free (Miles Laboratories Inc., Kanakee, Ill.) in the way already detailed (1) except that the concentration of ferritin was reduced from 20 mg/ml to 10 mg/ml in the later experiments, with no apparent disadvantage.

Bacterial Strain. This was *M. tuberculosis* virulent human strain H37Rv (TMC 102), obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y. It was maintained by subcultivation on the surface of Proskauer and Beck liquid medium.

Pretreatment by Antibody and Subsequent Procedures, Including Infection of Monolayers

Rabbits weighing 2 kg were immunized by six intramuscular injections of 2 ml vaccine at weekly intervals in multiple sites, and were bled 2 wk later. The vaccine (supplied by our colleague, Dr. R. J. W. Rees) was a mixture in equal parts of Freund's incomplete adjuvant and a suspension of the BCG strain of attenuated tubercle bacillus, part of which had been sonicated in order to break the bacilli. The sera from several rabbits were pooled and stored in aliquots at −20°C, without inactivation, for periods up to 15 mo. Before use the serum was filtered through a Millipore 0.22 μm membrane. Pooled normal rabbit serum was treated similarly.

A well-grown culture (about 3-wk old) of strain H37Rv was homogenized in water and the suspension adjusted to a concentration of 0.2–0.8 mg (moist wt)/ml. This was subdivided equally and centrifuged, and the bacteria were resuspended in the same volumes of undiluted unheated rabbit immune serum (IS), rabbit normal serum (NS), or balanced salt solution (BSS). After dispersion these suspensions were incubated in test tubes at 37°C for 45 min and then processed as follows:

PROCEDURE A (NO WASHING OF BACILLI AFTER EXPOSURE TO SERUM). The previously incubated bacterial suspensions in serum (B-IS or B-NS) were kept on ice until the post-ferritin washes of the monolayers had been completed. Then 9 vol of BSS were added to each to give "infection media", which thus contained 10% rabbit serum. The incubated bacterial suspension in BSS (B-BSS) was diluted similarly with further BSS, and in this case 2.5% human cord serum (routinely employed in this laboratory to stimulate phagocytosis of tubercle bacilli) was finally added.

These bacterial infection media were used immediately to overlay the previously ferritin-exposed and washed macrophage monolayers, within their Leighton tubes, for 2 h at 37°C. Ratios of bacteria to macrophages ranged from 10:1 to 40:1, the high ratios being required to obtain sufficient uptake (actually a mean of 1.5–6.0/cell initially) for the electron-microscopic quantitative survey. After further washing with BSS to remove free bacteria, fresh cultivation medium, plus 6.7 μg (base)/ml streptomycin to prevent extracellular bacterial multiplication, was introduced and the tubes reincubated at 37°C without further change of medium.

PROCEDURE B (BACILLI WASHED AFTER EXPOSURE). The previously incubated bacterial suspensions

in immune or normal rabbit serum or in BSS were centrifuged, and the bacilli were washed once in excess of BSS and then resuspended in water to give 0.2 mg (moist wt)/ml. Infection media were prepared from these aqueous suspensions of washed, pretreated bacteria (B-IS-W, B-NS-W, B-BSS) by adding to each of them 9 vol of BSS, followed finally by human cord serum to give 2.5%; the bacterium:macrophage ratio was 20:1. The monolayers were then infected and further processed as for Procedure A.

Assessment of Intracellular Bacterial Multiplication. Counts of the mean number of acid-fast bacilli per macrophage on the cover slips were made after Ziehl-Neelsen staining. Counts of mean number of viable bacterial units per monolayer were made from cultures on oleic-albumin agar after disrupting the macrophages by means of ultrasonic vibration (1). The viability of serum-pretreated tubercle bacilli was determined, before ingestion, by similar cultural methods. In one experiment electron-microscopic quantitative assessments were made of the distribution of the tubercle bacilli within (*a*) fused phagolysosomes and (*b*) unfused phagosomes, both at the start and at the end of a progressive infection.

Electron Microscopy. Monolayers on cover slips were processed as previously described (1, 2), employing the combined glutaraldehyde and osmium tetroxide fixation method of Hirsch and Fedorko (9). The cell profiles were systematically surveyed in interrupted series of thin sections, and bacilli were scored as "intact" or "damaged", using the previously defined morphological criteria (1). Phagosome-lysosome fusion was identified by the presence of ferritin label, derived from the lysosomes, within the bacterium-containing phagosomes.

Checking of Antibody Attachment to the Bacilli. Samples of the tubercle bacilli, after the pretreatment with immune or normal serum, were intensively washed with phosphate-buffered saline, fixed with methanol on slides, and tested for the presence of antibody by the indirect immunofluorescence staining technique, using fluorescein-labeled sheep antibody to rabbit immunoglobulin. The fluorescence was excited by means of long-wave ultraviolet light. A similar procedure was applied to bacilli recovered after their ingestion by the macrophages, release being effected by disrupting the cells with ultrasonic vibration.

Results

Macrophage Response to Infection after Various Pretreatments

ULTRASTRUCTURAL FEATURES. Electron microscopy was used for cells fixed 1 or 2 days after infection with *M. tuberculosis* strain H37Rv, the bacilli having been pretreated with immune (anti-BCG) or normal rabbit serum, or with no rabbit serum, before ingestion. The thin sections showed phagosomes enclosing predominantly (i.e. in over 80%) single bacteria, the majority being scored as "intact" whichever the pretreatment (Table I A). The "damaged" bacteria are considered to have originated mostly from dead organisms in the infecting inoculum (1).

The secondary lysosomes were well and selectively marked by the ferritin. In every experiment, as found previously for all mycobacteria examined, ferritin was detected within almost all (98–100%) of the phagosomes containing visibly damaged bacteria, indicating that fusion had occurred with one or more of the prelabeled lysosomes (Table I B); these lysosomes would therefore appear to be efficient in their capacity to fuse, and the level of almost 100% serves as a point of comparison for the findings relating to intact bacilli.

The effects of the different pretreatments, with or without subsequent washing (Materials and Methods), on the fusion response to intact bacteria are shown for representative experiments in Table I B. For phagosomes enclosing bacilli that had received no pretreatment with rabbit serum (B-BSS), the prevalence of phagosome-lysosome fusion was 28%, a low figure similar to those previously reported by us (1, 2) for strain H37Rv (around 25–30%), and confirming the

TABLE I A

Proportions of Intact and Damaged Bacteria (Assayed by Electron Microscopy) in Macrophage Profiles after Ingestion of M. tuberculosis (Strain H37Rv); Bacteria Previously Exposed to Immune, Normal, or No Rabbit Serum

Experiment no.	Prior treatment of bacteria	Bacilli encountered	Structural appearance of bacilli	
			Intact	Damaged
			%	%
74/1(a)	B-BSS	110	58	42
	B-NS	187	65	35
73/18	B-NS	103	77	23
	B-IS	70	67	33
74/1(b)	B-NS-W	223	76	24
	B-IS-W	254	69	31

TABLE I B

Proportions of Bacterium-Containing Phagosomes (in These Cell Profiles) that Showed Fusion with Ferritin-Prelabeled Lysosomes, Subdivided According to Whether the Bacteria were Damaged or Intact

Experiment no.	Prior treatment of bacteria	Appearance of bacilli in phagosomes	Phagosomes encountered	Phagosome-lysosome fusion	
				Yes	No
				%	%
74/1(a)	B-BSS	Damaged	40	100	0
		Intact	60	28[1, 2]	72
	B-NS	Damaged	57	98	2
		Intact	105	73[1, 3]	27
73/18	B-NS	Damaged	15	100	0
		Intact	50	82	18
	B-IS	Damaged	13	100	0
		Intact	21	90	10
74/1(b)	B-NS-W	Damaged	51	98	2
		Intact	153	33[4, 3]	67
	B-IS-W	Damaged	64	100	0
		Intact	157	73[4, 2]	27

In experiment 74/1 the macrophages were fixed at 1 day, and in experiment 73/18 at 2 days, after the infection. The differences within the pairs of percentages marked 1, 2, 3, and 4 are significant ($P < 0.01$).

predominantly nonfusion pattern associated with this organism. When, however, the bacilli had been pretreated with normal rabbit serum, without washing them after exposure (B-NS), a high prevalence of fusion (73% and 82%) was evident, the difference from B-BSS being significant ($P < 0.01$), and the figure was

similar (90%) when rabbit immune serum had been used (B-IS); furthermore, the quantity of the ferritin found around these bacilli in the phagolysosomes was often very large. Thus, under these conditions exposure to either serum had promoted phagosome-lysosome fusion where it would not otherwise have occurred.

The situation was strikingly transformed when the bacilli had been washed after exposure to the two rabbit sera and before permitting ingestion by the macrophages. Comparisons (Table I B) now show a low prevalence of fusion (33%) for normal serum (B-NS-W), similar to that observed in the same experiment without serum pretreatment (B-BSS, 28%), whereas for immune serum (B-IS-W) the prevalence was maintained at a high level (73%); the differences are again significant ($P < 0.01$). Thus, under these conditions enhancement of phagosome-lysosome fusion was associated only with the immune serum. Representative ultrastructural features are illustrated in Figs. 1–3.

CHECK OF ANTIBODY ATTACHMENT. Immunofluorescence tests (see Materials and Methods) for the presence of rabbit antibody to *M. tuberculosis* showed strong fluorescence around bacteria pretreated with immune rabbit serum, but weak or none after pretreatment with normal serum. Positive tests, though reduced in intensity, were also given by immune serum-pretreated bacilli that had been ingested by macrophages 1–2 h previously, indicating that the attachment was still holding and the antibody not yet digested away.

Bacterial Survival after Serum Pretreatments

EXTRACELLULAR AND INTRACELLULAR BACTERIAL VIABILITY. Samples of the infection media containing suspensions of tubercle bacilli that had been pretreated with immune or normal rabbit serum, or no rabbit serum, and both unwashed and washed subsequently (B-IS, B-NS, B-IS-W, B-NS-W, B-BSS), were taken before infection of the monolayers. Counts of viable bacterial units were made and were repeated again 6 or 7 days later, after incubation of these preparations at 37°C. No significant change was shown for any category, indicating that the pretreatments had no direct effect on bacterial viability in the cell-free medium. Incidentally, it may be noted that little or no bacterial aggregation was produced by exposure to immune serum.

There was no consistent difference in the phagocytosis of strain H37Rv in any of the pretreatment categories. The infection of the monolayers was followed, after a lag period, by progressive intracellular multiplication, leading to disintegration of the cell sheet commencing between 7 and 10 days. This capacity to multiply (after the initial lag period) was shown about equally by bacteria exposed to the fusion-promoting pretreatments (B-IS, B-NS, B-IS-W) and by the others (B-NS-W, B-BSS), and was revealed both by light-microscopic counts of intracellular acid-fast bacilli and by counts of viable bacterial units per cover slip monolayer (Table II). The acid-fast counts (exemplified by a representative experiment with B-IS-W, B-NS-W and B-BSS, Table II B) were in monolayers containing ferritin, the electron microscopy being done in parallel; it was possible that the presence of this substance in the lysosomes might have prevented or impeded an antibacterial effect of their enzymes. However, in the experiment shown in Table II A (Experiment 73/7) the monolayers had received

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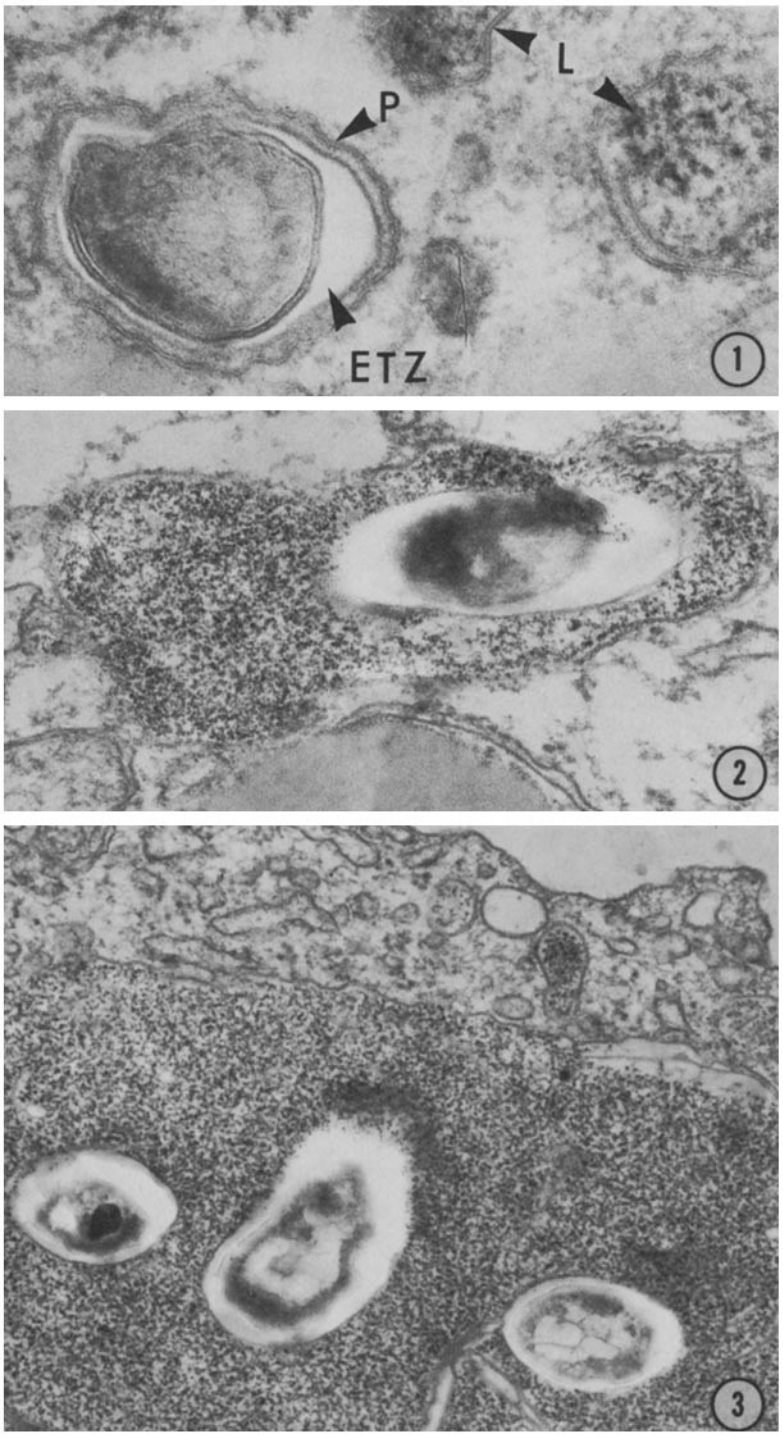

TABLE II A

Growth of M. tuberculosis (Strain H37Rv) in Cultured MP Macrophages, Assessed By Counts of Viable Bacterial Units in the Monolayers at Different Periods after Ingestion; Bacteria Previously Exposed to Immune, Normal, or No Rabbit Serum

Experiment no.	Day after ingestion	Mean viable bacterial units per cover slip monolayer $\times 10^{-3}$* after prior treatment as follows:		
		B-BSS	B-NS	B-IS
73/7	0	13	29	24
	6	22	52	43
	11	180	360	350
Calculated generation time	Days 0–11	2.9	3.0	2.9
	Days 6–11	1.7	1.8	1.7

* Assessed by culture.

no ferritin, and the similarity of trends to those in all other experiments appears to exclude this possibility.

This indication that the intracellular survival and multiplication of *M. tuberculosis* is unaffected by serum pretreatments that induce phagosome-lysosome fusion is inevitably an overall assessment, based as it is upon light-microscopic and bacterial cultural counts. The intracellular bacterial population after ingestion is distributed between phagolysosomes and phagosomes unfused with the secondary lysosomes; and it was conceivable that the overall picture of active multiplication after the fusion-promoting treatments (B-IS, B-NS, B-IS-W) was accounted for by the minority (some 5% to 25%) of the intact bacilli initially in the unfused phagosomes, while the majority (in the phagolysosomes) were inhibited in their growth or even killed by the lysosomal contents now delivered into their immediate environment.

It seems unlikely that such a situation should not have been revealed by a delay in the progress of overall multiplication compared with the control cultures (B-NS-W, B-BSS), in which the majority of the intact bacilli were initially in unfused phagosomes; but no such delay is apparent. The evidence for this view is from Table II, and is as follows.

FIG. 1–3. Electron micrographs showing lysosome-phagosome relationships in macrophages containing intact tubercle bacilli that had been exposed to rabbit immune serum (B-IS-W) before ingestion. Secondary lysosomes were prelabelled with ferritin.

FIG. 1. An example of nonfusion. The ferritin marker, though present in nearby lysosomes (L), has not entered the phagosome (P). A characteristic electron-transparent zone (ETZ) surrounds the bacillus.

FIGS. 2 and 3. Examples showing evidence of lysosome-phagosome fusion (the predominant response after antiserum pretreatment). Abundant ferritin surrounds a single bacillus in Fig. 2, separating the phagosome wall from the peribacillary electron-transparent zone. Fig. 3 shows an extreme case, with three bacilli embedded within a massive phagolysosome. × 120,000 (Fig. 1); × 80,000 (Fig. 2); × 61,000 (Fig. 3).

TABLE II B

Growth of M. tuberculosis (Strain H37Rv) in Cultured MP Macrophages, Assessed by Counts of Intracellular Acid-Fast Bacilli, in the Monolayers, at Different Periods after Ingestion; Bacteria Previously Exposed to Immune, Normal, or No Rabbit Serum

Experiment no.	Day after ingestion	Prior treatment of bacteria							
		B-BSS		B-NS-W		B-IS-W		B-IS-W light infection*	
		(a)‡	(b)§	(a)‡	(b)§	(a)‡	(b)§	(a)‡	(b)§
			%		%		%		%
74/4	0	2.2	—	1.9	—	3.0	—	0.4	—
	8	3.0	20	4.5	25	4.9	19		
	10	13.6‖	64	10.9‖	56	9.6‖	55	1.0	12
	11	11.6	80	13.4	80	10.2	80		
	12	15.6	>80	12.9	>80	15.4	>80	7.1‖	59
	13							11.9	83
	14							13.6	80

* Bacterium/macrophage ratio one-fifth that for B-IS-W in adjacent columns.

‡ Mean bacilli per cell in monolayer; stained by Ziehl-Neelsen method.

§ Proportion of cells with > 20 bacilli shed into medium, assessed at same time as bacilli per cell in monolayer.

‖ Commencing destruction of monolayer.

If (Table II A) the starting viable count of those bacteria in the B-IS culture that were in unfused phagosomes, say one-fifth of the total of 24,000, were 5,000, and if it were assumed that they were responsible for all the multiplying population, then (applying the calculated generation time of the control B-BSS culture) the expected count 11 days after infection for B-IS would have been about 75,000 instead of the actual 350,000. Similarly, comparison of the last two columns of Table II B show that a light infection, giving a starting acid-fast count about one-eighth of that after the comparable usual heavy infection, was followed by a delay of 2–3 days before "catching up"; but there was no evidence of this delay in the principal B-IS-W culture, compared with the control cultures (B-NS-W and B-BSS).

COMPARISON OF BACILLARY MULTIPLICATION IN PHAGOLYSOSOMES AND IN UNFUSED PHAGOSOMES. In spite of this further evidence that intracellular multiplication was unaffected by a raised level of phagosome-lysosome fusion, it seemed important to investigate the matter more precisely. An experiment (suggested by Dr. Thomas C. Jones, Cornell University Medical College, New York) was therefore performed, using ultrastructural assessments and relating bacterial counts to phagolysosomes and unfused phagosomes separately, both at the start and the end of a progressive infection by immune serum-pretreated *M. tuberculosis* strain H37Rv (B-IS).

The technical procedures were those already described. The macrophages were fixed for electron microscopy on the day after infection (day 1) and on days 7, 8, and 9. At the end of this period parallel observations by light microscopy showed

a four- or fivefold increase of mean acid-fast bacilli from the two per cell seen immediately after infection; in addition, destruction of the cell sheets was commencing. Moreover, the thin sections showed that many of the cells now exhibited cytolysis, and a small proportion of the bacilli were free in the cytoplasm. However, from days 1 to 8 the cell profiles retained a reasonable architecture and intact membranes, so that ultrastructural assessments could be made. The secondary lysosomes were still heavily labeled with ferritin [incidentally confirming a previous observation made 14 days after introduction of the label (1)]. The usual scoring of bacilli, phagosomes, and cell profiles was in this experiment arranged so as to show survival (intactness) and change of prevalences (of intact and of damaged bacilli) both in phagosomes fused with ferritin-labeled lysosomes (phagolysosomes) and in unfused phagosomes.

The results are given in Tables III and IV. Table III shows that the tendency for

TABLE III*

Proportions of Bacterium-Containing Phagosomes that Showed Fusion with Ferritin-Prelabeled Lysosomes in Profiles of Cultured Macrophages at the Start and End of an Infection with Virulent M. tuberculosis (Strain H37Rv); Bacteria Previously Exposed to IS

Appearance of bacilli in phagosomes	Day after ingestion	Phagosomes encountered	Phagosome-lysosome fusion	
			Yes	No
			%	%
Damaged	1	33	100	0
	7	17	100	0
	8	37	100	0
Intact	1	94	79	21
	7	120	68	33
	8	171	65	35

* Experiment 74/9.

lysosomes to fuse with the phagosomes containing intact bacilli, found in the previous experiments 1–2 days after infection with antiserum-treated *M. tuberculosis* (B-IS and B-IS-W), was evident also in this experiment (B-IS) on day 1, fusion being seen in 79% of these phagosomes. This tendency was still evident on days 7 and 8, 68% and 65% being seen to be fused, though the decrease ($P = 0.05$) suggests the possibility of a small drift towards more unfused phagosomes. The drift could have been due to the production of daughter-phagosomes enclosing bacterial progeny that retained insufficient antibody to induce fusion. Because of this possibility that as multiplication proceeded the new bacilli might not be always retained in their parental phagosomes, as well as the possible contrary tendency of some phagosomes to amalgamate, in Table IV the bacillary counts are expressed in relation to the cell profiles (IV A) as well as to the phagosomes (IV B). Table IV A shows no increase in the proportion of bacteria damaged, nor of the mean number of damaged bacteria per infected cell

TABLE IV*

Proportions of Intact and Damaged Bacteria, and their Prevalences and Distributions within Phagolysosomes and Unfused Phagosomes,‡ in the Cell Profiles of Table III at the Start and End of the Infection; Bacteria Previously Exposed to IS

(A) Bacterial Numbers Expressed in Relation to Cell Profiles

Day after ingestion	Cell profiles encountered enclosing bacilli	Bacilli		Mean bacilli per 10 cell profiles				
				Intact bacilli				Damaged bacilli
		All	Intact§	Total	Free in cytoplasm	In phagolysosomes	In unfused phagosomes	Total‖
			%					
1	102	138	75	10**	0	8‡‡	2§§	3
7	107	250	92	21	0	17	4	2
8¶	129	359	86	24**	2	16‡‡	6§§	4

(B) *Bacterial Numbers Expressed in Relation to Phagosomes*

Day after ingestion	Phagosomes encountered containing bacilli	Fused phagosomes (phagolysosomes)		Unfused phagosomes		Intact bacilli encountered	
		Total	With ⩾2 intact bacilli	Total	With ⩾2 intact bacilli	Total	In phagolysosomes
			%		%		%
1	127	107	6‖ ‖	20	5	103	80
7	137	98	38	39	18	230	80
8¶	208	148	30‖ ‖	60	20	307	69

* Experiment 74/9.

‡ That is, bacterium-containing phagosomes that, respectively, showed fusion with ferritin-prelabeled lysosomes, and those with no such evidence.

§ The remainder were scored as damaged.

‖ All in phagolysosomes.

¶ On day 9, destruction of the monolayer was commencing.

Note. The differences within the pairs marked**, ‡‡, and §§, are significant ($P < 0.001$); and that within the pair marked ‖ ‖ is also significant ($P < 0.0001$).

profile, during the progress of the infection, whereas the intact bacteria per profile increased significantly ($P < 0.001$) from 1.0 (day 1) to 2.4 (day 8); this is evidence against a progressive killing of bacteria. It will be seen that the increase in total prevalence of intact bacteria was contributed to by those observed to be enclosed within phagolysosomes as well as by those within the apparently unfused phagosomes, each doubling or more and the increases also being significant ($P < 0.001$). Table IV B provides further evidence that the intact bacteria observed within phagolysosomes, as well as those within unfused phagosomes, had survived and multiplied during the infection. Most (70–80%) of the intact bacteria encountered were observed to be in phagolysosomes and relatively few in unfused phagosomes, not only at the start of the infection (as had been found consistently previously with B-IS and B-IS-W) but also at its end (days 7 and 8). Moreover, the proportions both of phagolysosomes and of unfused phagosomes

scored as enclosing two or more intact bacilli increased from 6% on day 1 to 30% on day 8 for the former ($P < 0.0001$) and from 5% to 20% for the latter category.

Finally (again using the data in Table IV), if the extreme hypothesis were true that the overall multiplication of bacteria in the monolayers was confined to those initially in phagosomes unfused with lysosomes, then with, say, 20% intact bacilli initially in these (unfused) phagosomes and 80% in phagolysosomes, and an overall multiplication of 2.4 × in 8 days, one might expect 100 bacteria to have produced 240 of which 80 (33%) were in phagolysosomes and the remainder, i.e. 160 (67%), in unfused phagosomes. In fact, the distribution was the reverse.

From these considerations, it seems highly probable that in these experiments the tubercle bacilli were able to survive and multiply even when they were in phagolysosomes, and that the overall picture of bacterial multiplication observed after antiserum pretreatment did not conceal a differential behavior as between bacilli in the phagolysosomes and those in the phagosomes remaining unfused with lysosomes. Thus, there is good support for the view that in normal MP macrophage monolayers conversion of the usual nonfusion response to extensive phagolysosome formation did not affect the subsequent fate of *M. tuberculosis*.

Discussion

Modification of the Lysosome Fusion Response. Employing the same quantitative approach to assessment of phagosome-lysosome fusion as in our previous investigations of macrophages infected with *Mycobacteria* (1, 2, 10), it is now shown that pretreatment of virulent *M. tuberculosis* bacilli with rabbit sera, under suitable conditions, can substitute a fusion pattern for the predominantly nonfusion response towards "intact" bacteria that is characteristic in cultured normal MP macrophages after ingestion of this microorganism.

It has been established already that the ingestion of previously killed *M. tuberculosis* leads to the onset of prompt and extensive phagosome-lysosome fusion (1, 2). However, in the present experiments the possibility of a lethal effect attributable to pretreating the bacteria with serum was excluded by the results of the extracellular viability tests (see Results). These results confirm the earlier literature (e.g. 11) which indicated that exposure of tubercle bacilli to specific immune serum did not in itself reduce their viability. Thus, we have to look for reasons other than prior killing to account for the fusion-promoting effects of the serum pretreatments.

An obvious alternative explanation lies in some influence of the serum constituents at the interface between the ingested bacterium and the phagosomal membrane of the host cell. It is proposed that exposure of the tubercle bacilli to the immune serum (B-IS) results in specific binding of antibody at the bacterial surface; this survives ingestion and promotes a positive fusion response towards the phagosome by nearby secondary lysosomes. A similar effect results from pretreating with normal serum (B-NS), due to a loose association of serum protein, perhaps similar to the slight affinity described between mycobacterial glycopeptide and normal guinea pig γ_2-globulin (12). A clear difference is revealed, however, when the bacilli are washed after the treatment and before ingestion: normal serum protein is readily washed away (B-NS-W) and the familiar nonfusion pattern then prevails, whereas specific antibody remains firmly attached (B-IS-W) and its fusion-promoting effect is retained.

It remains to be determined whether the conversion to a fusion response is due to neutralization, by the attached serum constituents, of a fusion inhibitor produced by the living bacterium; or quite simply to the presence of these constituents alongside the bacterium within its phagosome. The concept of an inhibitor being responsible for the usual nonfusion pattern gains credence from the report of Edelson and Cohn (13, 14) that pinosomes associated with the ingestion of Concanavalin A by MP macrophages fail to fuse with primary and secondary lysosomes, due to direct interaction of this lectin with the enclosing pinocytic membrane. The possibility that *M. tuberculosis* itself produces an inhibitor of membrane fusion should therefore be considered.

Survival of the Tubercle Bacilli. The present findings raise anew the question of how this facultative intracellular parasite is able to survive and multiply within macrophages, whether in vitro or in the intact animal. Our earlier hypothesis (1), that in cultured normal MP macrophages the continuing intracellular viability of tubercle bacilli is linked to the regularly observed evasion of direct exposure of these organisms to the digestive enzymes and other lysosomal contents, inevitably left open the question as to whether a viable bacillus would in fact be susceptible to the effects of direct lysosomal attack, or whether, like *M. lepraemurium* (2), it would in any case be resistant. It is now seen that such exposure of the bacilli to the lysosomal contents, resulting from serum pretreatment, does not appreciably impede (nor for that matter accelerate) intracellular multiplication of *M. tuberculosis* in the normal macrophage; the organisms seem to thrive even in the phagolysosomes, which now predominate. The earlier hypothesis must be reconsidered. It would now appear that at least two factors may operate within the cultured normal MP macrophage, enabling ingested viable tubercle bacilli to survive: first, evasion of exposure to the lysosomal contents and, second, resistance to the attack should exposure occur. Thus, the capacity to evade lysosomal fusion would, of course, only become crucial in a situation in which this resistance of the tubercle bacillus could be overcome; the possibility of this occurring in the immune situation is discussed later.

Previous Reports on Serum Pretreatments. Some previous studies of the fate of microorganisms within cultured macrophages also showed that prior treatment with specific immune serum, before ingestion, had no apparent effect on the outcome, or even offered some protection to the organism.

Intracellular multiplication of antibody-coated *Salmonella typhimurium* was not inhibited in normal rabbit peritoneal macrophages, though cytotoxic effects on the host cells were actually diminished (15). A strain of *Escherichia coli* that was normally killed and digested after entering rabbit peritoneal macrophages was temporarily protected against this digestion (though not against killing) if previously exposed to rabbit immune serum, itself not lethal, but not if exposed to normal serum (16). A similar delay in degradation occurred with a strain of *Staphylococcus albus*. Antibody-coated *Leishmania* parasites were not destroyed by normal guinea pig macrophages (17). Particularly relevant to the present observations is the report that pretreatment of *M. tuberculosis*, strain H37Rv, with the serum from BCG-immunized rabbits or with normal serum failed to alter the subsequent bacterial multiplication within infected peritoneal macrophages derived from immunized rabbits (18).

On the other hand, other reports show that, with a variety of organisms, prior exposure to immune, or even to normal, sera can promote intracellular death and destruction.

A strain of *E. coli* that proliferated within MP macrophages was killed if pretreated with heated normal horse serum, itself nonbactericidal (19). Pretreatment of virulent *Salmonella* with rabbit specific antiserum had a similar effect (20). *Corynebacterium ovis* retained viability and destroyed MP macrophages if it had been previously exposed to fetal calf serum, but was itself lysed by the cells if rabbit immune serum was used (21). Preincubation of *Rickettsia mooseri* with fresh or heated human antiserum (not itself lethal) rendered the organisms susceptible to destruction within nonimmune human macrophages, whereas exposure to normal serum failed to prevent intracellular multiplication (22). *Toxoplasma gondii* pretreated with heat-inactivated human antiserum (whose effect on extracellular viability was no different from that of normal human serum as judged by incorporation of tritiated uridine into parasite nucleic acids) before ingestion by normal human monocytes was inhibited in growth or killed intracellularly; after similar treatment with normal serum the intracellular organisms proliferated (23). Similar findings were reported after exposure of *T. gondii* to heat-inactivated mouse antitoxoplasma antibody before ingestion by normal MP macrophages (24–26).

Unfortunately, in nearly all the foregoing situations, no ultrastructural evidence of a change in lysosomal behavior towards the microorganisms was sought. However, in the case of *T. gondii* some insight into its intracellular death and digestion after pretreatment with the antibody has been provided by the observation of Jones, Len, and Hirsch (24–26) that the characteristic nonfusion response of the lysosomes of cultured macrophages towards phagosomes containing healthy toxoplasmas was reversed after this pretreatment, and that this was associated with a demonstrable increase in the incidence of phagolysosomes enclosing clearly degenerating organisms. Yet with the similar pretreatment of *M. tuberculosis* and resulting reversal of phagosome-lysosome nonfusion after its ingestion, many of the phagolysosomes, both at the start and end of a progressive infection, contained intact bacilli, apparently capable of active proliferation. Thus, on the evidence available, it must be concluded that the biological consequences of experimentally reversing the normal lysosome response towards *T gondii* and *M. tuberculosis* are strikingly different, presumably because of a marked difference in inherent susceptibility to the lysosomal contents between the protozoal and the bacterial parasite.

The Immune Situation. Although the "turning off or on" of phagosome-lysosome fusion now seems not to play a decisive role in the fate of the living tubercle bacillus in cultured normal MP macrophages, the same cannot, on present knowledge, be presumed for the intact host, especially after the acquisition of specific immunity. The indications are that in the complex immune situation obtaining in vivo, macrophages can kill tubercle bacilli, and that these macrophages contain an increased complement of lysosomal enzymes (27); it has also been suggested that the properties of the lysosomal contents are in some respects altered (28). If it be assumed that antibody becomes attached to the bacilli before their ingestion by macrophages, we should have the conditions in vivo for "turning on" of phagosome-lysosome fusion and the consequent

exposure of the bacteria to lysosomal contents, possibly capable of overcoming the bacterial resistance to normal lysosomal enzymes. An interdependent role for the cellular lysosomes and circulating antibody would then emerge.

Following on from this reasoning, and turning again to the situation in vitro, there are reports of limited and variable bacteriostasis by immunologically activated MP macrophages in culture after ingestion of virulent *M. tuberculosis* (29–31), but none of truly bactericidal action. Nevertheless, the ideal of reproducing in vitro a bactericidal cell system, or at least one that halts the bacillary multiplication, in cultures of "immune" macrophages is still worth pursuing. If, in achieving this end, it proved necessary to "turn on" or enhance phagosome-lysosome fusion by pretreatment of the challenging organisms with specific antibody, phagolysosome formation would be seen as a crucial factor in the host's defenses. If, however, in an immune macrophage test system the fate of ingested tubercle bacilli were found to be uninfluenced by "turning on or off" of the phagosome-lysosome fusion, as seems to be the case with our nonimmune system, it would have to be concluded that some factor other than direct exposure to the lysosomal contents determines initially the intracellular survival or death of the organisms, the lysosomes themselves being directly concerned only with the digestion and disposal of the killed bacilli.

Summary

Tubercle bacilli of the pathogenic human strain H37Rv had previously been shown to multiply, after ingestion by cultured mouse peritoneal macrophages, within phagosomes that tended to remain unfused with secondary lysosomes. Means were sought therefore for promoting experimentally a modification of the host response so as to attain a high level of phagolysosome formation, enabling tests to be made of any effects on the course and outcome of the intracellular infection. This was achieved by exposing viable bacilli to specific rabbit antiserum before their ingestion. Quantitative assessments, using electron microscopy, now showed that a majority of the phagosomes containing intact bacilli had fused with ferritin-labeled lysosomes, and frequently the fusion was massive.

Bacterial viability studies established that the serum pretreatment was not itself bactericidal. In the course of progressive infections with strain H37Rv, monitored by counts both of viable bacterial units and of intracellular acid-fast organisms, no appreciable difference was found between the intracellular growth rates of control and antiserum-treated bacilli. Concurrent electron microscopy showed that bacilli could remain intact and multiply both in phagolysosomes and in unfused phagosomes, ruling out the possibility of selective growth of antiserum-pretreated bacilli within the minority of phagosomes that remained unfused.

It was concluded that "turning on" phagosome-lysosome fusion in normal macrophages did not influence the outcome of infection with virulent *M. tuberculosis*; lysosome contents manifestly failed to exercise an antibacterial effect on this organism. Nevertheless, the possibility remains that the lysosomes of specific immune macrophages have antituberculous potentiality. In that case the experimental "turning on or off" of fusion could be a decisive factor in the

outcome of a virulent challenge. Should it not be, the antibacterial capabilities of immune cells would need to be ascribed to factors other than lysosomal attack, the latter being essentially for disposal of the dead organisms.

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