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Strain Virulence and the Lysosomal Response in Macrophages Infected with *Mycobacterium tuberculosis*

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Strains H37Ra and H37Rv, attenuated and virulent variants, respectively, of the original human strain H37 of *Mycobacterium tuberculosis*, were used to infect cultures of mouse peritoneal macrophages. Bacterial viability of each strain was assessed over a 2-week period, and the cellular response to H37Ra during the first week was observed using electron microscopy. Prelabeling of secondary lysosomes with ferritin was used to facilitate the estimation of fusion of the lysosomes with phagosomes containing the bacteria. Streptomycin was excluded from the medium of cell cultures infected with H37Ra. The intracellular viability of strain H37Rv (in the presence of streptomycin) showed a lag during the first week after infection and then rose progressively to a mean figure seven times the starting level. In contrast, the viability of strain H37Ra declined, on the average, to one-fifth of the starting level during the first week; moreover, this decline occurred in the absence of antibiotics. In the second week a variable rise in the viable count took place, usually regaining the starting level. Electron microscopy of macrophages infected with H37Ra revealed a higher proportion of "damaged" bacteria 5 days after infection than at 1 day, in keeping with the decline in viability. Phagosomes containing these "damaged" (and presumed dead) organisms showed virtually universal fusion with prelabeled lysosomes. Phagosomes containing "intact" bacteria of this strain showed a prevalence of fusion varying from 38 to 56%, somewhat higher than the level previously reported for "intact" organisms of H37Rv. Nevertheless, the lysosome-phagosome fusion response to "intact" H37Ra was still far less extensive than that observed previously towards "intact" *M. lepraemurium* (around 90%). In conclusion, a difference between the macrophage lysosome-phagosome fusion response towards viable organisms of strain H37Ra and to the virulent strain H37Rv was observed, but was not pronounced, and the present findings are in keeping with the increasingly held view that H37Ra should be regarded as a low-virulence or attenuated strain rather than truly avirulent.

Mycobacteria ingested by cultured mouse peritoneal macrophages and enclosed within phagosomes can be divided, by electron microscopy of the infected cells, into two structural categories designated as "damaged" and "intact" (2). Fusion of ferritin-labeled secondary lysosomes with phagosomes containing obviously damaged (presumed nonviable) organisms is virtually universal in all mycobacterial species so far examined; but their frequency of fusion with phagosomes containing the intact (and probably viable) population varies remarkably. With the mouse pathogen *Mycobacterium lepraemurium* the great majority of the phagosomes enclosing intact bacteria show abundant evidence of lysosomal fusion, whereas most phagosomes enclosing intact bacilli of the virulent human strain H37Rv of *M. tuberculosis*, and also of another mouse pathogen *M. microti* (vole tubercle bacillus), show no sign of this fusion (2, 9).

Strains H37Rv and H37Ra of *M. tuberculosis* arose as variants of the original human strain H37 (19). Unlike H37Rv, strain H37Ra does not cause progressive disease in normal experimental animals. There is instead a slow decline in bacterial viability in the organs of mice when observed up to 4 months (3, 7, 17, 18). According to some reports, however, considerable bacterial multiplication (in mouse or guinea pig) occurs in the first few weeks, followed if observation is continued by a gradual decline in viability (1, 12, 13), though even after long periods a minority of the bacteria have been found to survive (7). For these reasons strain H37Ra, formerly termed "avirulent," is now commonly referred to as "attenuated." Strain H37Ra showed no multiplication within cul-

tured macrophages from guinea pigs (10, 20) or rabbits (14) during a week; viability was lost (14) or remained unchanged (10) during this period. In one report, however, some multiplication was recorded in rabbit macrophages (15).

The present work was undertaken to follow the viability of strain H37Ra in cultured macrophages from mice, and to see whether the macrophage lysosomes would respond by fusing more readily with phagosomes containing this nonpathogenic strain, recognizing them as different from phagosomes containing viable organisms of the pathogenic strain H37Rv. In view of evidence that streptomycin slowly penetrates into macrophages (4, 5, 8, 16) and might therefore be expected directly to inactivate intracellular organisms of the attenuated strain H37Ra (so promoting lysosome-phagosome fusion), it seemed desirable on this occasion to avoid the use of streptomycin as a means of restricting extracellular bacterial growth, using instead daily washes followed by a medium change.

MATERIALS AND METHODS

Bacterial strains. *M. tuberculosis* strains H37Rv (TMC 102) and H37Ra (TMC 201) were obtained from the Trudeau Mycobacterial Culture Collection, Saranac Lake, N.Y. Strain H37Rv was maintained by subcultivation on the surface of Proskauer and Beck liquid medium, and H37Ra was maintained on Lowenstein and Jensen egg agar. Paper-filtered suspensions (consisting predominantly of single cells, about half of them viable) were prepared as previously described (2) and adjusted to give a ratio of bacteria to macrophages of 20:1 or 40:1; this high ratio was required so as to obtain an adequate uptake to facilitate electron microscopic quantitative assessments.

Tissue culture. Macrophages, obtained from unstimulated peritoneal cavities of normal female mice of the albino P strain, were cultivated as monolayers in Chang medium in Leighton tubes as described elsewhere (2). After about 10 to 14 days of cultivation, the cells were overlaid with the appropriate bacterial suspension within the Leighton tubes for 2 h at 37 C, the infection being preceded, where electron microscopy was to be done, by exposure to ferritin (Pentex Biochemical) as previously detailed (2). After the infection, the cells were washed twice (H37Rv) or four or five times (H37Ra) with balanced salt solution, fresh medium was introduced, and the tubes were incubated again for 6 or 12 days.

Use of antibiotics. (i) Streptomycin. For the reasons given in the Introduction streptomycin was omitted from the tissue culture medium in experiments using H37Ra (both for viability and ultrastructure). After infection of the cells, the medium was changed daily after three or four washes, the new (but used) medium being taken from "contemporary" uninfected monolayers in a parallel series of Leighton-tube cultures (so as to avoid undue stimulation of the infected cultures by daily contact with unused fresh medium). The monolayers remained intact and in a good state of preservation to the end of the 12-day period of culture.

For the experiments using H37Rv, streptomycin (6.7 μg [base]/ml) was incorporated into the medium after the infection of the cells, and no further medium change was made (see Discussion).

(ii) Penicillin. Penicillin (60 U/ml) was normally included in the tissue culture medium throughout an experiment. However, since penicillin has been reported to show some inhibition of the growth of H37Ra in vitro (21) and might be expected to penetrate into macrophages, this agent was omitted (both before and after the infection) from a few of the experiments on the viability of H37Ra, in spite of the obvious risks of using antibiotic-free cell cultures during several weeks. In these experiments the macrophages were layered on the glass interiors of the Leighton tubes instead of routinely on the cover slips, in order to improve the effectiveness of washing away free bacteria.

Assessment of intracellular bacterial viability. Counts of viable bacterial units on days 0, 6, and 12 were made on oleic-albumin agar medium after disrupting the cells by means of ultrasonic vibration. When cover slips were omitted, the procedure previously described (2) was modified by exposing the Leighton tube itself, instead of a test tube containing the crushed cover slip, to vibration.

Electron microscopy. Monolayers on cover slips were processed as previously described (2, 9). The cell profiles were surveyed, and the bacilli were scored as "intact" or "damaged," using the previously defined criteria (2). Phagosome-lysosome fusion was identified by the presence of lysosome ferritin label within bacterium-containing phagosomes.

RESULTS

Intracellular bacterial viability. Owing to the omission of one or both antibiotics from the tissue culture medium, a number of the cultures of strain H37Ra became contaminated, either by infection transmitted from the mice or adventitiously from the daily change of medium. Reduction of the number in the groups of mice and omission of cover slips assisted. Even so, it is virtually impracticable, by means of intense daily washing alone, to remove free bacteria from the external medium in Leighton tubes sufficiently to exclude all possible influence on the intracellular counts through phagocytosis or rephagocytosis; this, together with extracellular multiplication, is a risk in experiments involving prolonged cultivation (22). In view of these considerations, any decline observed in the intracellular viability of this strain can confidently be regarded as genuine, or even an underestimate, but any rise, particularly after prolonged cultivation, may be suspect.

Because of the varying starting values from experiment to experiment, the counts of viable

bacterial units per macrophage monolayer of strains H37Rv and H37Ra are shown (Fig. 1) as ratios of the count at day 6 over that at day 0 (i.e., day of infection) and at day 12 over day 0. Between day 0 and day 6 strain H37Rv (in the presence of streptomycin) showed its usual lag, followed by progressive multiplication and ultimate destruction of the monolayer; the mean increase from day 0 to day 12 was seven times. The counts of strain H37Ra (in the absence of streptomycin) showed a consistent decline between day 0 and day 6, the mean fall being one-fifth (omission of penicillin as well as the streptomycin made no discernible difference, and the results are therefore not shown separately in Fig. 1); an additional experiment showed that this decline did not start until after day 1. Between day 6 and day 12 the viable counts of H37Ra rose to a variable extent, on average regaining by day 12 about the same level as at day 0.

The reduction in viability of strain H37Ra during the first week of culture in the absence of streptomycin (and in some cases of penicillin also) would seem unequivocal. The bacterial survival and apparent multiplication during the second week may also be genuine, the consequence of intracellular growth; alternatively, it may be attributable to the continued presence of free bacteria in the medium (in spite of the precautions taken). With these considerations in mind, electron microscopic studies with this strain were confined to the first week of culture.

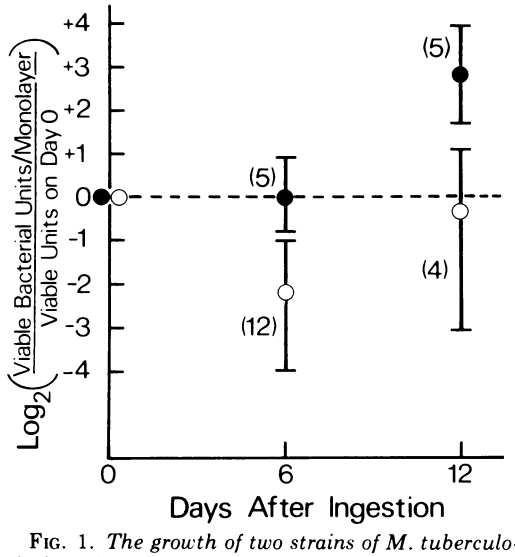

FIG. 1. *The growth of two strains of M. tuberculosis in cultured mouse peritoneal macrophages, assessed by cultural counts of viable bacterial units in the monolayers at 0, 6, and 12 days after ingestion. Symbols: —○—, attenuated tubercle bacilli (strain H37Ra); —●—, virulent tubercle bacilli (strain H37Rv). The figures in parentheses indicate the numbers of experiments performed. Penicillin (60 U/ml) was normally included in the tissue culture medium throughout. For strain H37Rv, after the ingestion the medium also contained streptomycin (6.7 μg/ml); for strain H37Ra streptomycin (and in some experiments also the penicillin; see text) was omitted. In a further experiment (not shown) with H37Ra, however, streptomycin was included as with H37Rv; the corresponding logarithmic figures after 6 and 12 days were then −3.4 and −5.3, respectively.*

The influence of antibiotics on the outcome of experiments with H37Ra was demonstrated by a further experiment in which streptomycin (6.7 μg/ml) was included in the postinfection medium, together with penicillin, and in all other respects the conditions and procedures were the same as for cultures infected with H37Rv. The viability then fell to one-tenth by day 6 and continued to fall to one-fortieth of the original level by day 12 (Fig. 1, footnote). This greater decline may have been due both to control of extracellular bacterial growth and to the penetration of streptomycin into the macrophages themselves.

Ultrastructural features of macrophage response. Electron microscopy was used for cells fixed 1 and 5 days after infection with strain H37Ra. Ultrastructural appearances of the mycobacteria, phagosomes, lysosomes, and lysosome-phagosome fusion were similar to those illustrated in a previous publication (9). The thin sections showed phagosomes enclosing predominantly single bacteria, but clusters of up to five bacilli were encountered. About two-thirds of the organisms were scored as "intact" on day 1 but only one-third on day 5 (Table 1); this difference is consistent with the initial decline in bacterial viability already noted. The electron-transparent zone that surrounds bacteria of strain H37Rv, as well as those of *M. microti* and *M. lepraemurium*, was less prominent in strain H37Ra and sometimes could not be definitely seen. The secondary lysosomes were well and selectively marked by the ferritin. As found previously for other mycobacteria (2, 9), ferritin was detected within almost all (97 to 100%) of the phagosomes containing "damaged" bacteria, indicating fusion with one or more of the prelabeled lysosomes (Table 2). For phagosomes enclosing intact bacteria of H37Ra the prevalence of phagosome-lysosome fusion was 56% (day 1) and 38% (day 5), significantly lower than for the damaged bacteria ($P < 0.01$). These figures for intact organisms lie between those previously reported (2, 9) for intact bacteria of H37Rv (25 to 30%) and those for intact *M. lepraemurium* (around 90%); the figure of 56%

TABLE 1. *Proportions of intact and damaged bacteria in macrophage profiles after ingestion of M. tuberculosis strain H37Ra*[a]

Day after infection	No. of bacilli encountered	Structural appearance of bacilli		
		Intact (%)	Damaged (%)	Doubtful (%)
1	193	63	35	2
5	135	29	69	2

[a] Assayed by electron microscopy.

TABLE 2. *Proportions of bacterium-containing phagosomes*[a] *that showed fusion with ferritin-prelabeled lysosomes*

Appearance of bacilli in phagosomes	Day after infection	No. of phagosomes encountered	Phagosome-lysosome fusion		
			Yes (%)	No (%)	Doubtful (%)
Damaged	1	64	97	3	0
	5	81	100	0	0
Intact	1	84	56	44	0
	5	29	38	55	7
Doubtful	1	3	(100)[b]	(0)	(0)
	5	2	(100)	(0)	(0)

[a] In the cell profiles of Table 1.

[b] Numbers in parentheses refer to totals less than 10.

for H37Ra is significantly higher than that for H37Rv and both figures for this strain are lower than for *M. lepraemurium* ($P < 0.01$).

DISCUSSION

Throughout this work, as in our previous studies, fusion of macrophage lysosomes with phagosomes has been detected by observing transfer of ferritin label. The fusion response observed therefore involves, by definition, vesicles in the category of secondary lysosomes, which are abundant in macrophages cultured in high-serum medium (6) and very variable in size. As discussed at length previously (2), it is not possible to be sure that some phagosomes containing viable tubercle bacilli, with which secondary lysosomes do not fuse, may nevertheless have fused with small unlabeled enzyme-containing vesicles, i.e., smooth-surfaced Golgi vesicles or primary lysosomes. However, experiments using the Gomori-type acid phosphatase technique after H37Rv infection (2) suggested that the associated fusion pattern was similar, in relation to intactness or damage of intraphagosomal bacteria, to the pattern revealed by the ferritin-labeling technique. More recently, convincing evidence leading to the same conclusion was reported for macrophages containing *Toxoplasma gondii* (11).

As explained earlier, certain differences in the culture conditions were adopted for the two strains: H37Ra had no streptomycin but daily washes; H37Rv had streptomycin but no daily washes. Thus the systems were not identical. However, one of the ultrastructural examinations was made on monolayers fixed 1 day after the H37Ra infection and therefore before the daily washes had been started. Moreover, the inclusion of streptomycin in the H37Rv experiments seemed justified by our regular experience that, at the concentration used, this antibiotic controls extracellular bacillary multiplication of H37Rv but does not prevent its progressive intracellular multiplication after an initial lag period. There is, furthermore, no evidence that streptomycin has any direct influence upon lysosome-phagosome fusion phenomena. Hence, our comparisons of these two host-parasite systems are considered valid within the limits selected.

The differing trends of intracellular bacterial viability observed in the cultured macrophages during 2 weeks after their infection by strains H37Rv and H37Ra of *M. tuberculosis*, as expressed by viable counts, are in reasonable accord with the progressive disease caused by the former strain and with the regression of lesions and survival of a minority of the bacteria in vivo after infection by the latter strain (7). The moderate decline in viability of strain H37Ra during the first week of culture was accompanied by an increase in the proportion of bacteria appearing damaged in the thin sections; moreover, in this strain the proportion of phagosomes containing "intact" organisms that had fused with ferritin-prelabeled lysosomes was higher than is characteristic of strain H37Rv, but at the same time fusion remained far below the level reported previously for *M. lepraemurium* (9).

Whereas a difference in "recognition" by the macrophage lysosomes as a whole between ingested organisms of strain H37Ra and those of the virulent strain H37Rv was observed, it was not pronounced. The limited differences found, like the viability observations, are in accord with the view that H37Ra is of lesser virulence than H37Rv but is not avirulent (1, 7). The greater incidence of phagosome-lysosome fusion for H37Ra could have been brought about by its bacterial surface properties, and the effect, by

bringing lysosomal contents into contact with more intraphagosomal bacteria, could be to cause their inactivation. It cannot yet be decided whether this is the correct sequence or whether, on the other hand, the bacterial inactivation was mediated in some other way and the increased fusion was no more than the characteristic lysosomal response towards phagosomes containing already dead organisms. If the latter were true, then inactivation might perhaps be caused by some unidentified diffusible intracellular metabolite, or it might be a passive defect in adapting to the new environment. Such a lack of adaptation, rather than susceptibility to a hypothetical bactericidal factor, might well be the basis of diminished virulence of strain H37Ra (17).

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