Phagosome–Lysosome Fusion and Cyclic Adenosine 3':5'-Monophosphate in Macrophages Infected with *Mycobacterium microti*, *Mycobacterium bovis* BCG or *Mycobacterium lepraemurium*

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(Received 19 April 1978; revised 25 August 1978)

When ingested by mouse peritoneal macrophage monolayers, live *Mycobacterium microti* caused a sustained increase in monolayer cyclic AMP content and fusion of lysosomes with the bacterium-containing phagosomes was impaired. Ingested live *M. bovis* BCG caused a transient increase in cyclic AMP and the defect in phagolysosome formation was less pronounced. Dead mycobacteria and live *M. lepraemurium* neither enhanced monolayer cyclic AMP content nor inhibited phagolysosome formation. *Mycobacterium microti* and BCG exceeded *M. lepraemurium* in cyclic AMP-synthesizing activity *in vitro* but the question of whether bacterial cyclic AMP contributed substantially to the increments in infected macrophages was not resolved. Antibody-coated BCG retained the ability to synthesize cyclic AMP and to enhance monolayer cyclic AMP but lost the ability to inhibit phagolysosome formation in macrophages. The observations are discussed in terms of possible control of phagolysosome formation by cyclic nucleotides.

INTRODUCTION

Several species of micro-organism which survive and multiply inside macrophages have been found to avoid exposure to lysosomal digestive enzymes in the infected cells. Such avoidance might well contribute to the intracellular survival of these microbes. Lysosomes fail to fuse with phagosomes containing living *Toxoplasma gondii* (Jones & Hirsch, 1972), *Mycobacterium tuberculosis* (Armstrong & Hart, 1971), *Mycobacterium microti* (Hart *et al.*, 1972; Lowrie *et al.*, 1975) or *Mycobacterium bovis* BCG (Armstrong & Hart, 1971) but fuse with phagosomes when the intraphagosomal microbes are dead or are alive but coated with antibody (Armstrong & Hart, 1975). In contrast, lysosomes fuse extensively with phagosomes which contain other intracellular parasites, such as *Mycobacterium lepraemurium* (Hart *et al.*, 1972) or *Salmonella typhimurium* (Carrol *et al.*, 1979), regardless of whether these bacteria are alive or dead.

In a preliminary report (Lowrie *et al.*, 1975), we showed that living *M. microti* caused an increase in cyclic adenosine 3':5'-monophosphate (cyclic AMP) in macrophages infected with this organism and that dead *M. microti*, latex spheres or living *M. lepraemurium* in macrophages had no effect on cyclic AMP levels. We suggested, by analogy with the mode of action of some anti-inflammatory agents, that the increased amounts of cyclic AMP might be the cause of the failure of lysosome–phagosome fusion in *M. microti*-infected cells and speculated that cyclic AMP might mediate the inhibition of phagolysosome formation which is apparent in other instances of intracellular parasitism. The hypothesis was sustained by the finding that *S. typhimurium* resembled *M. lepraemurium* not only in

failing to inhibit phagolysosome formation but also in failing to increase the cyclic AMP content of infected macrophages (Carrol *et al.*, 1979).

In this paper, we present the results of quantitative comparisons of the abilities of *M. microti*, *M. bovis* BCG and *M. lepraemurium* to inhibit phagolysosome formation, to raise the cyclic AMP content of infected macrophages and to synthesize cyclic AMP in laboratory media.

METHODS

Macrophage monolayers. Resident (non-elicited) peritoneal macrophages were obtained from normal albino CFLP mice by peritoneal lavage and established as monolayers in plastic Petri dishes as described by Lowrie *et al.* (1979).

Bacteria. *Mycobacterium bovis* BCG (Glaxo) and *M. microti* OV254 (obtained from Dr P. D'A. Hart) were grown at 37 °C as nutrient-limited steady-state cultures in a continuous culture apparatus (Bioflo C2, New Brunswick Scientific Co.) in which all stainless steel in the 750 ml culture vessel had been replaced with either glass, Teflon or titanium. Culture medium (fourfold concentrated Dubos broth base, finally containing 0.08 % (w/v) Tween 80; Difco) was delivered to the culture vessel by a peristaltic pump (Watson Marlow, Falmouth, Cornwall) to give a dilution rate of 0.016 h⁻¹ which corresponds to a doubling time of 43 h. Medium delivery was continuously monitored with a meter specially designed to operate at very low flow rates (Lowrie, 1976). Air containing 5 % (v/v) CO₂ was supplied at 50 ml min⁻¹ into the culture vessel at a point above the level of the growing culture. Cultures were stirred by a magnetic impeller at a rate (350 rev. min⁻¹) just below that which caused bubbling. Preliminary tests showed that bubbles carried the bacteria out of the medium, depositing them on the vessel walls above the body of the culture where they remained; the low rates of bacterial oxygen consumption, monitored with a Clark oxygen electrode, did not necessitate vigorous aeration. Effluent gas was passed through a cooling condenser to minimize evaporative water loss. Cultures reached steady-state equilibrium within the time taken for a volume of culture medium equal to three times the culture volume to pass through the culture vessel. After this time, and until 4 weeks later (*M. microti*) or 10 weeks later (arbitrarily the longest period used with BCG), culture samples were withdrawn from the vessel for experimental use. At least 5 d were allowed for culture re-equilibration between successive samplings. Continuous cultures of *M. microti* were not run for longer than 4 weeks because growth of bacteria adherent to the vessel walls became visible after this time, indicating deviation from steady-state conditions. Microscopic counts (Thoma counting chamber) showed that steady-state *M. microti* cultures contained about 8 × 10⁸ bacteria ml⁻¹, of which more than 90 % were single or pairs of organisms; clumps containing more than 20 bacteria were rarely seen. Counts of colony-forming units (c.f.u.) on Middlebrook 7H11 agar (Difco) indicated 100 % viability. Steady-state cultures of BCG contained about 2 × 10⁹ bacteria ml⁻¹ dispersed similarly to *M. microti* but c.f.u. counts consistently revealed only about 10 % viability. This low apparent viability was probably an artefact of the assay conditions (see Discussion).

Killed suspensions of *M. microti* were prepared from culture samples by exposure either to 65 °C for 45 min or to ultraviolet (u.v.) radiation. BCG was killed by exposure to glutaraldehyde for 1 h (Lowrie *et al.*, 1979).

Mycobacterium lepraemurium, generously given by Dr R. J. W. Rees as a freshly prepared, partially purified suspension obtained from infected mouse spleen, contained 6.6 × 10¹¹ bacteria ml⁻¹ in 0.1 % (w/v) bovine serum albumin in saline. Typically the viability of bacteria in such preparations approaches 100 % (R. J. W. Rees, personal communication).

Antibody-coated BCG and non-coated control bacteria were prepared essentially as described by Armstrong & Hart (1975). A culture sample was centrifuged at 1800 g for 15 min at 4 °C, washed once in Hanks' salt solution (HSS) and divided into two parts. One part was resuspended to a concentration of 5 × 10⁸ bacteria ml⁻¹ in rabbit anti-*M. tuberculosis* serum (donated by Dr J. Stanford) and one part in HSS. Both suspensions were incubated for 1 h at 37 °C then centrifuged and resuspended in HSS. Tests with fluorescein-labelled anti-rabbit serum (Armstrong & Hart, 1975) confirmed the adherence of antibody to the bacteria.

All bacterial suspensions were finally prepared for phagocytosis by centrifugation and resuspension in phagocytosis medium which comprised 2.5 % (v/v) human cord serum or newborn calf serum in HSS.

Interrelationships between bacterial numbers, cyclic AMP changes and phagolysosome formation in macrophages containing bacteria. The experimental procedures were essentially as described by Carrol *et al.* (1979) and Lowrie *et al.* (1979). Briefly, 7 to 14 d after establishment of the macrophage monolayers, macrophage lysosomes were labelled with ferritin, to allow subsequent identification of lysosome contents by

electron microscopy, and then the macrophages were allowed to ingest the bacterial suspensions for 2 h. Immediately at the end of this period, or after subsequent maintenance of the monolayers for 90 h (*M. microti* experiments) or 42 h (BCG experiments), the amount of cyclic AMP (Carrol *et al.*, 1979) and the numbers of acid-fast bacteria (a.f.b.) per monolayer macrophage (Lowrie *et al.*, 1975) were assessed. Monolayers were maintained in the absence of streptomycin. Accordingly, at approximately 24 h intervals after phagocytosis the maintenance medium was removed, the monolayers were rinsed with warm HSS (2×2 ml) and then replenished with fresh medium. This procedure minimized continued phagocytosis of extracellularly multiplying bacteria. Cyclic AMP increments in monolayers which had ingested bacteria were calculated by subtracting the average cyclic AMP content of control (phagocytosis medium alone) monolayers from the cyclic AMP content of experimental monolayers. Regressions of cyclic AMP increment against number of a.f.b. per monolayer macrophage were calculated by the least-squares method. Monolayers were prepared for quantitative electron microscopical surveys of phagolysosome formation 90, 42 or 18 h after phagocytosis of, respectively, *M. microti*, BCG or *M. lepraemurium*. In several experiments with *M. microti* and BCG the numbers of monolayer-associated and monolayer-free c.f.u. per dish were also assessed as follows. Maintenance medium was removed from the dishes and stored on ice for subsequent determination of monolayer-free c.f.u. Monolayers were rinsed with ice-cold HSS (2×2 ml) and the cells were then scraped free into ice-cold HSS (2×1.5 ml) using a silicone rubber 'policeman'. Cell suspensions were dispersed by brief ultrasonication (Biosonik microprobe, Bronwill Scientific Co., Rochester, N.Y., U.S.A.; 60 W for 6 s). Cell dispersions and maintenance medium were diluted 10-fold into 0.02% (w/v) sodium dodecyl sulphate at room temperature to liberate cell-associated bacteria and further serial 10-fold dilutions were immediately made in saline. Preliminary tests showed that this brief exposure of the bacteria to the detergent did not appreciably decrease viability. Counts of c.f.u. were done using 7H11 agar.

Bacterial cyclic AMP content and synthetic capacity. Samples of steady-state cultures of *M. microti* and BCG were centrifuged at 1800 g for 15 min at 4 °C and the pellets were washed once by resuspension in ice-cold HSS and recentrifugation. Membrane filter-sterilized culture supernatants and perchloric acid extracts of the washed bacteria were partially purified by ion-exchange chromatography before assay for extracellular and intracellular cyclic AMP, respectively (Carrol *et al.*, 1979). The *M. lepraemurium* suspension was similarly assayed for intracellular cyclic AMP. The capacity of the three mycobacterial species to synthesize cyclic AMP during incubation in phosphate-buffered saline (Dulbecco A; Oxoid) was assayed. The bacteria were washed once in the ice-cold buffer then resuspended to a concentration of 1×10^8 bacteria ml⁻¹ or, with *M. lepraemurium*, 6.6×10^8 bacteria ml⁻¹ and incubated at 37 °C. Intracellular and extracellular cyclic AMP were assayed at intervals as described above. Synthetic responses in phagocytosis medium were tested in a similar manner.

RESULTS

Cyclic AMP increase in macrophages containing mycobacteria

Phagocytosis of glutaraldehyde-killed BCG did not cause an increase in cyclic AMP in macrophages but phagocytosis of untreated BCG produced a significant rise which was proportional to the number of a.f.b. per macrophage (Fig. 1). Although the basal cyclic AMP content of the control monolayers varied between experiments from 5 to 17 pmol (10^7 macrophages)⁻¹, this did not influence the magnitude of the infection-induced increase. The cause of the variation in basal cyclic AMP levels between experiments is not known. The cyclic AMP increment seen immediately after 2 h phagocytosis had markedly diminished after subsequent maintenance of the monolayers for 42 h, even though the numbers of total and viable monolayer-associated organisms increased slightly (< 10%) during this period. In contrast, phagocytosis of up to an average of 16 living *M. microti* per macrophage caused an increase in cyclic AMP which persisted for at least 90 h, during which time the total and viable monolayer-associated bacteria increased about twofold.

Daily rinsing of the monolayers essentially prevented continued phagocytosis of extracellularly multiplying bacteria. The numbers of viable bacteria which were monolayer-free did not exceed 1% of the numbers which were monolayer-associated at any time after phagocytosis of either BCG or *M. microti* and the numbers of monolayer cells seen to contain no a.f.b. did not decrease.

Mycobacterium lepraemurium resembled glutaraldehyde-killed BCG and heat- or u.v.-killed *M. microti* in not enhancing monolayer cyclic AMP content, despite phagocytosis

Fig. 1. Correlation of monolayer cyclic AMP increment with number of monolayer-associated BCG. Macrophages were allowed to ingest glutaraldehyde-killed BCG (a) or live BCG (b) for 2 h and immediately after phagocytosis, or after subsequent incubation for 42 h in maintenance medium, individual monolayers were assayed for both monolayer-associated a.f.b. and cyclic AMP. The average cyclic AMP content of corresponding control monolayers, which did not receive bacteria, was subtracted from the values for individual test monolayers and the difference was expressed as change in cyclic AMP $(10^7 \text{ macrophages})^{-1}$ using estimates of monolayer DNA to calculate the number of monolayer macrophages present. Data were pooled from six separate experiments in which bacterium:macrophage ratios during phagocytosis were varied between 2:1 and 126:1. Each point represents results from an individual test monolayer assayed either immediately after phagocytosis (O), or 42 h later (●). Linear regressions were fitted by the least-squares method.

of up to an average of 20 a.f.b. per macrophage. The effects of the three mycobacterial species on monolayer cyclic AMP are summarized in Table 1.

Mycobacterial cyclic AMP synthesis

Because the additional cyclic AMP found in infected macrophages might have been either of bacterial origin or synthesized by the macrophages in response to the bacteria (or both), the capacities of the three species of mycobacteria to synthesize cyclic AMP were compared. Cultures of *M. microti* growing in the continuous-culture apparatus contained 10 times as much cyclic AMP as similarly growing BCG cultures and net cyclic AMP synthesis was 10 times faster (Table 2). Both organisms released over 80% of the synthesized cyclic AMP into the culture medium per unit time. The bacteria harvested from cultures and suspended in phagocytosis medium did not contain sufficient cyclic AMP [about 1.0 and 0.1 pmol $(10^8 \text{ a.f.b.})^{-1}$ in *M. microti* and BCG, respectively] to account for the extra cyclic AMP found in macrophages which had ingested the bacteria [5.7 and 8.8 pmol $(10^8 \text{ a.f.b.})^{-1}$; Table 1]. Incubation of the bacteria for 2 h at 37 °C in phagocytosis medium did not cause an increase in the amount of cyclic AMP present. However, stimulation of cyclic AMP synthesis occurred when the bacteria were incubated in phosphate-buffered saline and, as during growth in culture medium, a substantial proportion of the nucleotide was simultaneously released into the medium (Fig. 2). *Mycobacterium microti* had over 10 times the activity of BCG and synthesized more than 20 pmol $(10^8 \text{ a.f.b.})^{-1}$ in 2 h. *Mycobacterium microti* thus had the potential to synthesize sufficient cyclic AMP to account for the increment in infected macrophages; this potential was not apparent in BCG. The cyclic AMP content of *M. lepraemurium* suspensions increased during incubation for 2 h in phosphate-buffered saline from 0.01 to only 0.04 pmol $(10^8 \text{ a.f.b.})^{-1}$.

Table 1. *Change in macrophage cyclic AMP content with increase in number of monolayer-associated bacteria*

Macrophage monolayers were allowed to ingest bacteria for 2 h and immediately after phagocytosis, or after subsequent incubation of the monolayers for 42 h (BCG experiments) or 90 h (*M. microti* experiments), individual monolayers were assayed for both a.f.b. and cyclic AMP. Control monolayers did not receive bacteria. Data were pooled from many experiments and the regression coefficient for the slope of the difference in macrophage cyclic AMP content (test minus control) against the number of a.f.b. per macrophage in the test monolayer was calculated by the least-squares method from the equation $y = bx$.

Test bacterium	Regression coefficient*	
	Immediately after phagocytosis	After subsequent incubation
<i>M. microti</i> , untreated	(22) $5.71 \pm 0.42 \dagger \ddagger$	(6) $5.54 \pm 0.58 \dagger$
<i>M. microti</i> , heat-killed	(14) 0.40 ± 0.30	(5) -1.34 ± 0.39
<i>M. microti</i> , u.v.-killed	(4) 1.03 ± 0.33	Not tested
BCG, untreated	(15) $8.82 \pm 0.71 \dagger \ddagger$	(13) $0.86 \pm 0.15 \dagger$
BCG, glutaraldehyde-killed	(10) 0.40 ± 0.46	(11) 0.47 ± 0.52
<i>M. lepraemurium</i> , untreated	(4) 1.42 ± 0.42	Not tested

* The units are pmol cyclic AMP increment $(10^8 \text{ macrophages})^{-1}$ (a.f.b. per macrophage) $^{-1} \pm \text{s.e.}$ The total number of test monolayers is shown in parentheses in each instance.

† Significant regression ($P < 0.001$) by Student's t test.

‡ The difference between these coefficients is significant ($P < 0.01$).

Table 2. *Cyclic AMP content and synthetic activity of mycobacterial cultures*

Samples from steady-state continuous cultures of *M. microti* or BCG growing at a dilution rate of 0.016 h^{-1} in fourfold concentrated Dubos broth base were centrifuged and assayed for intracellular (pellet) and extracellular (supernatant) cyclic AMP. Bacterial numbers were estimated by phase-contrast light microscopy. The total cyclic AMP detected (intracellular plus extracellular) was multiplied by the dilution rate and termed the net rate of synthesis. Values are the mean of triplicate determinations $\pm \text{s.d.}$ The intracellular cyclic AMP content of a suspension of *M. lepraemurium* prepared from infected mouse spleen is shown for comparison.

Test bacterium	Cyclic AMP content [pmol $(10^8 \text{ bacteria})^{-1}$]			Net rate of cyclic AMP synthesis [pmol $(10^8 \text{ bacteria})^{-1} \text{ h}^{-1}$]
	In bacterium	In culture medium	Total	
<i>M. microti</i>	0.83 ± 0.54	8.2 ± 2.4	9.0 ± 2.9	0.144
BCG	0.13 ± 0.06	1.1 ± 0.3	1.3 ± 0.3	0.021
<i>M. lepraemurium</i>	0.01	NA	NA	NA

NA, Not applicable.

Phagolysosome formation in mycobacterium-containing macrophages

The electron microscopical observations were broadly similar to those reported by Hart and co-workers (Armstrong & Hart, 1971; Hart *et al.*, 1972). Essentially all of the large (secondary) lysosomes present in cell profiles were seen to be labelled with ferritin. Untreated (living) *M. microti* had a markedly greater ability to avoid exposure to lysosome contents than did killed *M. microti* (Table 3). In contrast, although in each experiment with BCG the untreated bacteria tended to be less frequently in phagolysosomes than did the killed bacteria, the difference was less and more variable than with *M. microti*, attaining statistical significance in only one of three experiments (Table 3). In this experiment the basal cyclic AMP content of the monolayers was higher than usual [17 compared with 5 to $8 \text{ pmol } (10^7 \text{ macrophages})^{-1}$]. *Mycobacterium lepraemurium* resembled killed *M. microti* and killed BCG in eliciting extensive fusion of lysosomes with phagosomes. Examples of fusion and non-fusion with ferritin-labelled lysosomes are illustrated in Fig. 3.

Heat-killed *M. microti* was often seen to be structurally degenerate in macrophages 90 h

Fig. 2. Cyclic AMP synthesis and release by *M. microti* (a) and BCG (b) during incubation in phosphate-buffered saline. Both organisms were taken from steady-state cultures growing under identical conditions in a continuous culture apparatus. The bacteria were washed once at 0 °C by centrifugation, resuspended in the buffer to a concentration of 1×10^9 bacteria ml $^{-1}$ on the basis of microscopic counts and incubated at 37 °C. At intervals, samples were centrifuged and the pellets and supernatants, respectively, were assayed for intracellular (○) and extracellular (●) cyclic AMP. These two values were summed to indicate the total cyclic AMP present (□). Each point represents the average of duplicate samples.

Table 3. Proportions of bacterium-containing phagosomes that were fused with ferritin-labelled lysosomes after phagocytosis of mycobacteria

Macrophage monolayers that had ingested untreated (live) or killed bacteria were incubated for 90 h (*M. microti*), 42 h (BCG) or 18 h (*M. lepraemurium*) and then phagolysosome formation was assessed electron microscopically.

Test bacterium	No. of macrophages examined	No. of bacterium-containing phagosomes found	Phagosome-lysosome fusion	
			Fused (%)	Not fused (%)
<i>M. microti</i> , untreated	39	154	40*	60
<i>M. microti</i> , heat-killed	45	244	85*	15
<i>M. microti</i> , untreated	52	68	35†	65
<i>M. microti</i> , heat-killed	61	67	88†	12
BCG, untreated	23	147	37‡	63
BCG, glutaraldehyde-killed	34	152	60‡	40
BCG, untreated	61	207	70	30
BCG, glutaraldehyde-killed	52	112	79	21
BCG, untreated	66	291	73	27
BCG, glutaraldehyde-killed	42	141	75	25
<i>M. lepraemurium</i> , untreated	16	72	86	14

*† The differences within these pairs of percentages are highly significant ($P < 0.001$).

‡ The difference between these percentages is significant ($P < 0.01$).

after phagocytosis but untreated *M. microti* was not. Ultrastructural damage was seldom recognized in either glutaraldehyde-killed or untreated BCG 42 h after phagocytosis. Frequency of bacterial damage was not quantified.

The abilities of the different mycobacteria to avoid exposure to lysosomal enzymes correlated with their different abilities to cause and sustain increased amounts of cyclic

Fig. 3. Transmission electron micrographs showing phagosome-lysosome relationships in thin sections of macrophages that had ingested living *M. microti* 4 d before fixation. (a) An example of non-fusion: the ferritin marker, although present in nearby lysosomes (L), has not entered the phagosome (P) containing the bacterium. This non-fusion is typical with living *M. microti*. (b) An example of fusion: both ferritin and the bacterium are present within a single membrane-bound vesicle – a phagolysosome (PL). Bar markers represent 0.2 μm .

Table 4. *Phagolysosome formation in macrophages containing antibody-precoated or non-coated BCG*

Fusion of ferritin-labelled lysosomes with bacterium-containing phagosomes was assessed electron microscopically 42 h after phagocytosis.

Pre-treatment of test bacteria	No. of macrophages examined	No. of bacterium-containing phagosomes found	Phagosome-lysosome fusion	
			Fused (%)	Not fused (%)
Immune serum	44	275	95*	5
Hanks' salts	28	256	53*	47
Immune serum	41	109	85†	15
Hanks' salts	34	92	54†	46

*† The differences within these pairs of percentages are highly significant ($P < 0.001$).

Fig. 4. Effect of antibody coating on cyclic AMP synthesis by BCG in phosphate-buffered saline. Suspensions of antibody-coated or non-coated (control) BCG in buffer were incubated at 37 °C and assayed for intracellular (○, ●) and total (□, ■) cyclic AMP as described in Fig. 2. Each point represents the average of duplicate samples from either control (open symbols) or antibody-coated (closed symbols) preparations.

AMP in the macrophages: *M. microti* both increased cyclic AMP and inhibited phagolysosome formation for at least 90 h after phagocytosis; BCG caused only a transient increase in cyclic AMP and was less able to inhibit fusion; *M. lepraemurium* and dead mycobacteria neither increased cyclic AMP nor inhibited fusion. The different abilities of the mycobacteria to elevate monolayer cyclic AMP could in turn be correlated with their ability to synthesize cyclic AMP which was greatest in *M. microti*, less in BCG and least in *M. lepraemurium*.

Phagolysosome formation and cyclic AMP in macrophages infected with serum-precoated BCG

When BCG was precoated with immune serum, fusion of lysosomes with phagosomes was greatly promoted (Table 4). Enhanced fusion did not impair intracellular survival. Unexpectedly, however, the initial cyclic AMP increment in the monolayers was only slightly decreased (by about 23%) when the bacteria had been precoated, and declined, as normal with this organism, by 42 h after phagocytosis. Synthesis of cyclic AMP during incubation of the bacteria in phosphate-buffered saline was retarded but not prevented by serum coating (Fig. 4).

DISCUSSION

The poor ability of BCG, in relation to *M. microti*, to synthesize cyclic AMP in buffer and to enhance cyclic AMP and inhibit phagolysosome formation in macrophages could have been due to low bacterial viability. The low apparent BCG viability (about 10%) might, however, have been an artefact of an unidentified defect in the viability assay that acted against BCG and not *M. microti*. High BCG viability could be inferred from the low incidence of ultrastructural damage seen in intracellular and inoculum bacteria (Armstrong & Hart, 1971). Furthermore, calculations (Tempest, 1970) based on the detected 10% viability in the continuous culture apparatus imply improbably short doubling times for the viable bacteria (4.3 h and 2.1 h at dilution rates of 0.016 and 0.033 h⁻¹, respectively); about 12 h is generally accepted to be the minimum doubling time for tubercle bacilli (Long, 1958). Irrespective of viability, the finding that 'live' BCG was intermediate between *M. microti* and *M. lepraemurium* both in ability to enhance cyclic AMP and to inhibit fusion in macrophages is consistent with the possibility that cyclic AMP mediated fusion inhibition.

The elevation of cyclic AMP during phagocytosis of the live fusion-inhibiting mycobacteria was an unusual consequence of phagocytosis. The absence of increase with fusion-eliciting phagocytic particles (including latex; Lowrie *et al.*, 1975) was consistent with reports that ingestion of latex spheres by rabbit alveolar macrophages or antibody-coated erythrocytes by rat peritoneal macrophages did not enhance cyclic AMP content (Manganiello *et al.*, 1971; Gemsa *et al.*, 1975*a*). Stimulation of cyclic AMP with latex phagocytosis in rabbit alveolar macrophages has also been reported (Seyberth *et al.*, 1973; Schmidt-Gayk *et al.*, 1975) but has perhaps been explained by the presence of contaminating lymphocytes in the macrophage preparations (Atkinson *et al.*, 1975).

The question of whether the additional cyclic AMP found in infected macrophages was synthesized by the bacteria or by the macrophages (or both) was not answered. Although BCG appeared to differ from *M. microti* in not being able to synthesize enough cyclic AMP to account for the initial increment which accompanied phagocytosis, preliminary experiments have indicated that BCG grown at high dilution rates (again with 10% apparent viability) had a cyclic AMP synthetic capacity quantitatively similar to that of *M. microti*. Therefore both BCG and *M. microti* were probably potentially able to synthesize the extra cyclic AMP. The minimal cyclic AMP synthetic activity detected in *M. lepraemurium* suspensions might be attributable to loss of precursor ATP during isolation of these in-vivo grown host-dependent bacteria (Hanks, 1968). The possibility that the extra cyclic AMP was produced by the macrophages requires that the rate of synthesis, destruction or loss of the cyclic nucleotide by the host cell was altered by some surface or secretory product of the bacteria, products which might reasonably have been lost from or denatured in the variously killed bacteria. The most obvious location of targets for such effectors would be the phagosomal membrane. Adenylate cyclase, the enzyme which catalyses cyclic AMP formation from ATP, has been found on plasma membranes in a wide variety of cell types (Perkins, 1973) and might therefore be expected to be present in the plasma membrane-derived phagosomal membrane of macrophages (Davies & Stossel, 1977). Macrophage adenylate cyclase activity can be stimulated by hormones via specific membrane receptors (Gemsa *et al.*, 1975*a, b*; Schmidt-Gayk *et al.*, 1975; Welscher & Cruchaud, 1976) and mycobacterial products might be envisaged as acting directly or indirectly on such receptors to stimulate the enzyme. Whether macrophages have a plasma membrane-located phosphodiesterase capable of converting cyclic AMP to AMP is not known, but the enzyme has been found on plasma membranes in other cell types (Appleman *et al.*, 1973). Although certain acidic wall lipids extracted from *Mycobacterium tuberculosis* have been shown to inhibit phagolysosome formation when introduced into macrophages (Goren *et al.*, 1976), the possibility that they affect cyclic AMP metabolism has not been examined.

The argument that an increase in cyclic AMP might have inhibited fusion in infected

macrophages is by analogy with the role of cyclic nucleotides in phagocytic degranulation in other systems. The fusion of lysosomes with developing phagosomes or external plasma membrane in human polymorphonuclear leukocytes was shown to be modulated by endogenous or exogenous cyclic nucleotides (Weissmann *et al.*, 1975; Ignarro, 1975). The extent of fusion was dependent on the balance between opposing effects of cyclic AMP (fusion-inhibitory) and cyclic GMP (fusion-promoting). This crucial role of the cyclic nucleotides in the normal control of phagosome-lysosome fusion was recently corroborated by studies of the degranulation defects in polymorphonuclear leukocytes, monocytes and fibroblasts in the Chediak-Higashi syndrome (Boxer *et al.*, 1976, 1977; Oliver & Zurier, 1976; Oliver *et al.*, 1976) and in monocytes of a patient with malakoplakia (Abdou *et al.*, 1977).

Although cyclic AMP has been found to inhibit degranulation in mouse peritoneal macrophages which were ingesting zymosan (Weissmann *et al.*, 1971), less encouraging observations have also been reported. Hormonally elicited increases in cyclic AMP in rat peritoneal macrophages did not impair intracellular digestion of antibody-coated erythrocytes (Gemsma *et al.*, 1975a). Neither cyclic AMP nor cyclic GMP affected degranulation during phagocytosis of opsonized zymosan by guinea-pig alveolar macrophages (Ackerman & Beebe, 1975). Only modest inhibition of degranulation by cyclic AMP was observed in peptone-elicited mouse peritoneal macrophages ingesting heat-aggregated gamma-globulin complexes (Welscher & Cruchaud, 1976) and this inhibition was paralleled by depressed phagocytosis. Nevertheless, cyclic AMP and cyclic GMP might in some circumstances exert mutually antagonistic controlling influences on phagolysosome formation in mouse peritoneal macrophages similar to those in other cells (Henson, 1974). If this were so, then antibody precoated on to tubercle bacilli might have promoted fusion by stimulating an unusually large synthesis of cyclic GMP, nullifying the postulated inhibitory effect of cyclic AMP. Precedent for such an effect may be available in the promotion of cyclic GMP synthesis and degranulation in polymorphonuclear leukocytes via stimulation of antibody and complement receptors on the plasma membrane (Ignarro & George, 1974; Weissmann *et al.*, 1975).

We thank Dr P. D'A. Hart for helpful discussions and advice, Miss M. E. W. Carrol for technical assistance and Professor A. P. Waterson for the generous provision of facilities for electron microscopy.

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