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Nonadherent Cultures of Human Monocytes Kill *Mycobacterium smegmatis*, but Adherent Cultures Do Not†

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Human peripheral blood monocytes are permissive for the growth of *Mycobacterium tuberculosis*, but the fate of nonpathogenic *Mycobacterium smegmatis* in these cells is not known. Since *M. smegmatis* may be used as a host with which to express and screen for *M. tuberculosis* genes needed for survival in monocytes, we determined whether human peripheral blood monocytes could restrict the growth of *Mycobacterium smegmatis*. Adherent human peripheral blood monocytes were permissive for the growth of *M. smegmatis*, as measured by ex vivo [^{3}H]uracil uptake. However, human peripheral blood monocytes which were cultured nonadherently in Teflon wells were able to restrict the growth of *M. smegmatis* while remaining permissive for the growth of *M. tuberculosis* H37Ra. The loss of viability of *M. smegmatis* in nonadherent cells was correlated with an increase in nonspacious phagocytic vacuoles. The killing of *M. smegmatis* was not blocked by N^G-monomethyl-L-arginine, suggesting that it was not due to the production of reactive nitrogen intermediates. Incubation of the monocytes for 1 to 7 days before infection had no effect on the fate of *M. smegmatis*, suggesting that adherence versus nonadherence, and not differentiation, was the key determinant for the difference in functional ability. Nonadherent human peripheral blood monocytes may be a more appropriate model than adherent cells for the study of factors employed by bacteria to survive within monocytes and for selection screening of bacterial genes needed for intracellular survival.

The resurgence of diseases caused by facultative intracellular parasites such as *Mycobacterium tuberculosis* and *Mycobacterium avium* in immunocompromised people has caused a renewed interest in the strategies employed by mycobacteria for intracellular survival. Although the mycobacteria can infect many cell types, most in vitro studies have concentrated on the growth of mycobacteria in the monocyte/macrophage, the normal host for *M. avium* and *M. tuberculosis* in the lung and for *M. tuberculosis* in the liver. It is known that *M. tuberculosis* (9, 29), *M. avium* (6), and *Mycobacterium bovis* BCG (22) can survive in cultured human and murine monocytes in vitro: in these cells, the slow-growing mycobacteria can grow, most likely by inhibiting phagolysosome fusion (3) and/or by inhibiting acidification of the phagosome (35).

The gene products involved in the intracellular survival of pathogenic mycobacteria are not known, although several genes potentially involved in intracellular survival have been isolated (4, 24, 38). To define a gene or genes needed for survival, one strategy would be to express the genome of virulent *M. tuberculosis* within a nonpathogenic bacterium, infect human monocytes, and screen for transformants now capable of intracellular survival. This strategy has been used to select for a gene product from attenuated *M. tuberculosis* H37Ra which enables *Escherichia coli* to invade HeLa cells and to survive within cultured human monocytes (4). Other investigators have used nonpathogenic *Mycobacterium smegmatis* as a host with which to manipulate and express *M. tuberculosis* and *M. avium* genes (5, 14, 16, 30, 41), since it is fast growing, transformable (31), and still retains unique mycobacterial determinants. Genes that have been expressed in *M. smegmatis* include a functional superoxide dismutase gene from *M. tuberculosis* and *Mycobacterium leprae* (41, 42), a 19-kDa glycosylated antigen from *M. tuberculosis* (14), and *M. avium* DNA coding for the glycopeptidolipid antigens (5). *M. smegmatis* in which genes from pathogenic mycobacteria are expressed could be tested for the ability to survive intracellularly; such a strategy presumes that *M. smegmatis* would be killed by monocytes.

However, little is known about the intracellular fate of rapidly growing mycobacteria such as *M. smegmatis*. *M. smegmatis* has been reported to be killed by rabbit macrophages in vivo (18) and can be prevented from growing within Bcg^r mouse macrophages in vitro (8). Although both splenic and elicited peritoneal macrophages from Bcg^r mice can reduce *M. smegmatis* numbers by 40 or 60% within 48 h, Bcg^r macrophages were only bacteriostatic (8) and complete killing was not demonstrated.

We investigated the ability of *M. smegmatis* to survive within cultured human peripheral-blood-derived monocytes and macrophages. We found that the culture conditions of the monocytes could drastically affect the intracellular survival of *M. smegmatis*. While plastic-adherent monocytes or macrophages were permissive for growth of *M. smegmatis*, Teflon-cultured cells killed intracellular *M. smegmatis*.

MATERIALS AND METHODS

Isolation of monocytes for nonadherent culture. Peripheral blood monocytes (PBM) were isolated (26) from buffy coats obtained from the New York Blood Center. All isolation procedures were done at room temperature. Blood was washed three times with phosphate-buffered saline (PBS) with glucose and without calcium or magnesium, layered on 20 ml of lymphocyte separation medium (Cappel Laboratories), and spun at 400 × *g* for 25 min. Mononuclear cells harvested from the gradient were incubated in fresh human serum with 5 mM EDTA to remove platelets (27) and layered on a Nycodenz gradient (Accurate Chemical and Scientific Company) (25). After centrifugation for 15 min at 60 × *g*, the interface containing the monocytes was removed, washed twice in PBS, and resuspended in RPMI medium (BioWhittaker). Final preparations were routinely at least 95% viable. Nonadherent monocytes were cultured in 1-ml Teflon

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drop-in wells (Savillix, Minnetonka, Minn.), and adherent monocytes were cultured in 24-well plates, both in RPMI medium with 10% AB serum (C-six Diagnostics, Inc., Mequon, Wis.).

Infection of monocytes. *M. smegmatis* mc^2 155 (31) was obtained from Bill Jacobs and cultured in lipopolysaccharide (LPS)-free Proskauer Beck medium (Trudeau Institute) at 37°C with shaking at 200 rpm. *M. tuberculosis* H37Ra was obtained from Robert North (Trudeau Institute, Saranac Lake, N.Y.) and grown in Proskauer Beck medium in roller cultures. All bacterial cultures were used in the log phase of growth. To prepare single-cell suspensions, aliquots of cultures were removed to a microcentrifuge tube, vortexed briefly, and allowed to sit at room temperature to let large clumps settle. The supernatant was removed, pelleted gently, and washed and resuspended by vortexing in fresh Proskauer Beck medium. The resuspended cells were sonicated for 3 bursts of 10 s each in a water bath sonicator; the bacteria were observed microscopically and, if any clumping still occurred, sonicated again. This preparation resulted in a predominantly single-cell suspension with clumps of two or three bacteria. The bacteria were counted in a Petroff-Hauser chamber and diluted to the appropriate multiplicity of infection (MOI). Monocytes were infected 12 h after isolation and incubated for 5 h at 37°C under 5% CO_2. The cells were then pulsed with 200 μg of gentamicin for 1 h and washed three times to remove extracellular bacteria before being resuspended in fresh medium. This constituted the 0 time point of the experiment.

[^{3}H]uracil incorporation assay for microbial replication. The [^{3}H]uracil incorporation assay was adapted from one used originally by Rook and Rainbow (28) and for other mycobacterial phagocytosis studies (33). At each time point, the monocytes were washed three times with PBS, and fresh RPMI medium with 10% serum was added. The monocytes were lysed, and the bacteria were freed with 0.008% digitonin (Gallard-Schlesinger Industries, Inc., Carle Place, N.Y.) in PBS and vortexed vigorously to disrupt cellular nuclei. Five hundred microliters of LB medium (for *E. coli*) or 7H9 medium (for mycobacteria) plus 5 μCi of [^{3}H]uracil (36.4 Ci/mmol) (Amersham) was added, and the solution was incubated for 6 h at 37°C under 5% CO_2. The medium was aliquoted into 96-well plates, harvested onto filters, and counted in an LKB 1205 Beta plate liquid scintillation counter. Plate counts confirmed the validity of [^{3}H]uracil incorporation to measure growth in this system. Acid-fast smears of each sample were done routinely to determine whether any tritium counts were due to contamination of cells by other bacteria. All experiments were repeated at least four times, and representative experiments are shown. Initial (time 0) counts varied from donor to donor, but the trend was the same. For example, four consecutive experiments with nonadherent cells showed day 0 counts of 65,242, 103,682, 158,465, and 98,993 cpm, with the day 2 percentages of day 0 cpm being 6.5, 5.4, 8.5, and 8.7%, respectively.

Electron microscopy of infected monocytes. For transmission electron microscopy of adherent monocytes, monocytes were plated on Thermanox coverslips (Nunc, Naperville, Ill.) in 24-well plates. Nonadherent cells were collected from the Teflon beakers in microcentrifuge tubes for washing and fixation. At the conclusion of each experimental time point, the cells were washed three times with PBS and fixed for 1 h at 4°C in 2.5% glutaraldehyde in 0.1 M sodium cacodylate and 0.1 M sucrose (pH 7.4). The cells were then postfixed with 1% osmium tetroxide, stained en bloc with 0.25% uranyl acetate, dehydrated in graded ethanol solutions, and embedded in Epon. Thin sections were stained with lead citrate before examination with a Jeol JEM 100CX microscope. Vacuole morphology was assessed for a minimum of 100 cells for each experimental variable.

RESULTS

Growth of *M. smegmatis* in adherent and nonadherent human PBM. Studies of phagocytosis with monocytes and macrophages are usually done with cells that are adherent to plastic culture dishes or coverslips. We infected monocytes which had been allowed to adhere to tissue culture wells for 12 h with *M. smegmatis* and examined the ability of intracellular bacteria to incorporate tritiated uracil at days 0, 2, 6, and 10. *M. smegmatis* grew within adherent PBM (as shown by electron microscopy and acid-fast staining) and achieved counts threefold higher on day 10 than on day 0 (Fig. 1A). *M. tuberculosis* was not killed by the monocytes during the course of the experiment but showed approximately a threefold increase in growth, with [^{3}H]uracil counts of 1,468, 4,564, 2,882, and 5,770 cpm for days 0, 2, 6, and 10, respectively.

Human PBM cultured in Teflon containers are nonadherent or very loosely adherent to the container. The properties of human PBM cultured in Teflon containers are similar to those of adherent monocytes (34, 37, 39), and a difference in phagocytosis has not been reported. To determine whether nonadherent monocytes would restrict or permit the growth of *M. smegmatis*, we cultured monocytes in Teflon beakers (37) for the duration of the experiment. The cells were infected 12 h after isolation. *M. tuberculosis* H37Ra was able to survive within the nonadherent PBM (with counts of 2,063, 2,701, 1,390, and 2,144 cpm for days 0, 2, 6, and 10, respectively). However, *M. smegmatis* was not only unable to grow in nonadherent monocytes but was killed in those cells, with a 250-fold decrease in [^{3}H]uracil counts from day 0 to day 10 (Fig. 1B). This difference between adherent and nonadherent monocytes was not due to a difference in uptake, since zero-time [^{3}H]uracil counts were similar for both adherent and nonadherent monocytes (Fig. 1).

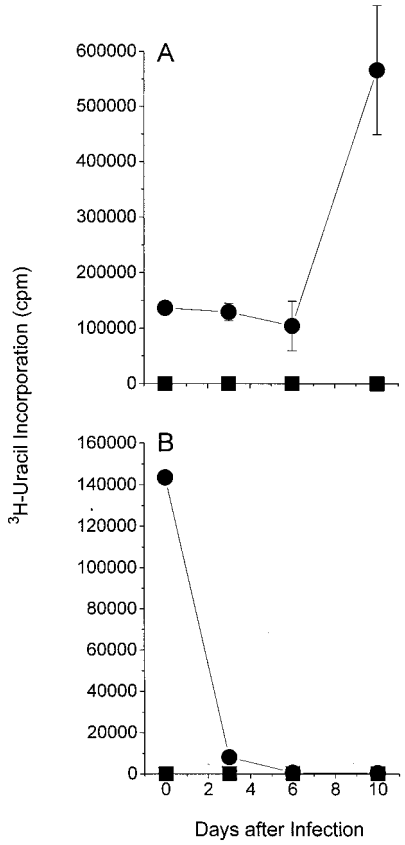

FIG. 1. Adherent and nonadherent monocytes infected with *M. smegmatis*. Human PBM were grown in either adherent cultures in 24-well plates (A) or nonadherent cultures in Teflon beakers (B). The cells were infected with *M. smegmatis* at an MOI of 5:1 (●) for 6 h, treated with gentamicin, and washed. Uninfected control cells (■) were treated in a manner identical to that of the infected cells. At the time points shown, the cells were collected and lysed, and [^{3}H]uracil incorporation into the released bacteria was measured.

It was possible that the functional ability of the monocytes was influenced by the method of isolation and not by the method of culture. We isolated PBM by the more traditional method of E-rosetting and adherence (22) and repeated the experiment. The results were the same, suggesting that it was the method of culture and not the method of isolation that led to the difference in function of the adherent and nonadherent cells.

Human PBM cultured adherently and nonadherently differentiate into macrophages during culture (39). To determine whether the killing ability of the nonadherent monocytes was dependent upon the length of culture and therefore upon differentiation, we incubated nonadherent monocytes for 0 or

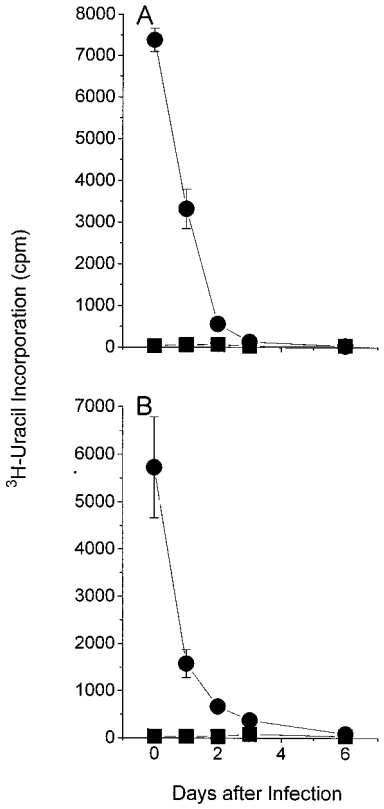

FIG. 2. Killing by monocytes and macrophages. Monocytes were cultured in Teflon wells at a concentration of 2×10^6/ml. (A) Half of the cells were infected after 12 h with *M. smegmatis* at an MOI of 5:1 (●), and intracellular bacterial growth was determined at the specified time points by [^{3}H]uracil incorporation. (B) The remainder of the cells were incubated at 37°C under 5% CO_2 for 7 days, at which time they were infected and intracellular bacterial growth was determined as for the day 0 cells. Uninfected control cells (■) were treated in a manner identical to that of the *M. smegmatis*-infected cells.

7 days before infection and examined the effect upon killing of *M. smegmatis*. As shown in Fig. 2, the length of incubation of the monocytes did not affect the rate or extent of killing of *M. smegmatis*, since infection of monocytes 12 h after isolation (Fig. 2A) or 7 days after isolation (Fig. 2B) resulted in very similar kinetics of killing. This suggests that it is the culture conditions rather than the length of incubation and the extent of differentiation that affect the ability of the Teflon-cultured cells to kill *M. smegmatis*. There was no difference in the ability of adherent monocytes/macrophages, cultured for 0 to 7 days after infection, to support the growth of mycobacteria (data not shown). Furthermore, transfer of infected Teflon-cultured cells to plastic culture dishes resulted in the loss of the killing ability of the cells (data not shown).

Electron microscopy of adherent and nonadherent cultured monocytes. The morphologies of the vacuoles surrounding virulent and avirulent *M. tuberculosis* organisms are different (2), suggesting that the morphology of the vacuole may parallel a difference in the capacity of the phagocyte to restrict the growth of the bacteria. The tight juxtaposition of the membrane to the single organisms, with an electron-transparent zone, has been seen with BCG-infected rabbit macrophages (19) and with *M. tuberculosis* (2, 11). We examined the morphology of the vacuole surrounding *M. smegmatis* in both adherent and nonadherent cultures of human PBM to determine whether the difference in function between the cells was reflected in a gross difference in the vacuole surrounding the ingested bacteria. To quantitate differences in vacuole morphology, we counted the number of single versus multiple bacteria per vacuole and the number of vacuoles with host membranes closely juxtaposed (CJ) or not CJ (NCJ) to the bacteria; we did this with adherent and nonadherent infected monocytes at a time when the fate of the intracellular bacteria was known to see whether we could correlate vacuole morphology and the presence of single versus multiple bacteria per vacuole with the killing or survival of mycobacteria (Table 1).

TABLE 1. Morphology of *M. smegmatis*-containing vacuoles in adherent and nonadherent monocytes[a]

Time postinfection (h)	MOI	Adherent		Nonadherent	
		SV/MV	CJ/NCJ	SV/MV	CJ/NCJ
3	5:1	0.3	1.5	1.0	3.1
	20:1	0.3	1.3	1.1	1.1
24	5:1	1.1	3.6	2.7	12.5
	20:1	1.2	3.0	1.0	12.7

[a] Adherent and nonadherent cultures of monocytes were infected at an MOI of 20:1 or 5:1 and prepared for transmission electron microscopy at 3 and 24 h after infection. Electron micrographs of cells were used for enumeration of the ratio of vacuoles containing a single organism (SV) to those containing more than one organism (MV). The ratio of vacuoles closely juxtaposed (CJ) to the enclosed bacteria to those in which a bacterium is greater than a bacterial radius away from other bacteria and the vacuole membrane (NCJ) was also determined. For both the SV/MV and CJ/NCJ ratios, a number higher than one indicates a majority of SV or CJ vacuoles.

Figure 3 shows adherent and nonadherent monocytes infected with *M. smegmatis*. With Teflon-cultured monocytes, the general morphologies of the monocytes and the vacuole surrounding the bacteria were the same as that of adherent monocytes. *M. smegmatis*, which is killed by nonadherent monocytes only, is found in single-organism and multiorganism vacuoles (SV and MV, respectively) in both adherent and nonadherent monocytes. However, as shown in Table 1, there were approximately three times more SV than MV in nonadherent monocytes at both 3 and 24 h. Both adherent and nonadherent monocytes demonstrated a tendency towards increasing the number of SV with *M. smegmatis* infection over time, with at least a 2.5-fold increase from 3 to 24 h in the ratio of SV to MV. The SV/MV ratio did not change when an MOI of 20:1 instead of 5:1 was used, suggesting that the tendency towards MV early in phagocytosis was not due to clumping or phagocytosis of groups of organisms at a time but reflected a cellular change in compartmentalization.

We also examined the morphology of the vacuole surrounding the mycobacteria. In particular, we documented the degree of juxtaposition of the bacteria to the vacuole membrane, with bacteria either CJ or NCJ to the monocyte vacuole membrane. *M. smegmatis* was found, for both adherent and nonadherent monocytes, more frequently in CJ vacuoles than in NCJ vacuoles (Table 1; Fig. 3). As for the SM/MV ratio, there was a tendency for the CJ/NCJ ratio to increase over time for both adherent and nonadherent monocytes. Most strikingly, in nonadherent cells, the CJ/NCJ ratio increased from 3.1 to 12.5 at 24 h, indicating that the great majority of bacteria in the nonadherent cells were in CJ vacuoles (Fig. 3D) at a time when the bacteria were losing the capability of incorporating tritiated uracil (Fig. 1).

DISCUSSION

We have shown that nonadherent cultures of human PBM can kill *M. smegmatis*, while adherent cultures of monocytes

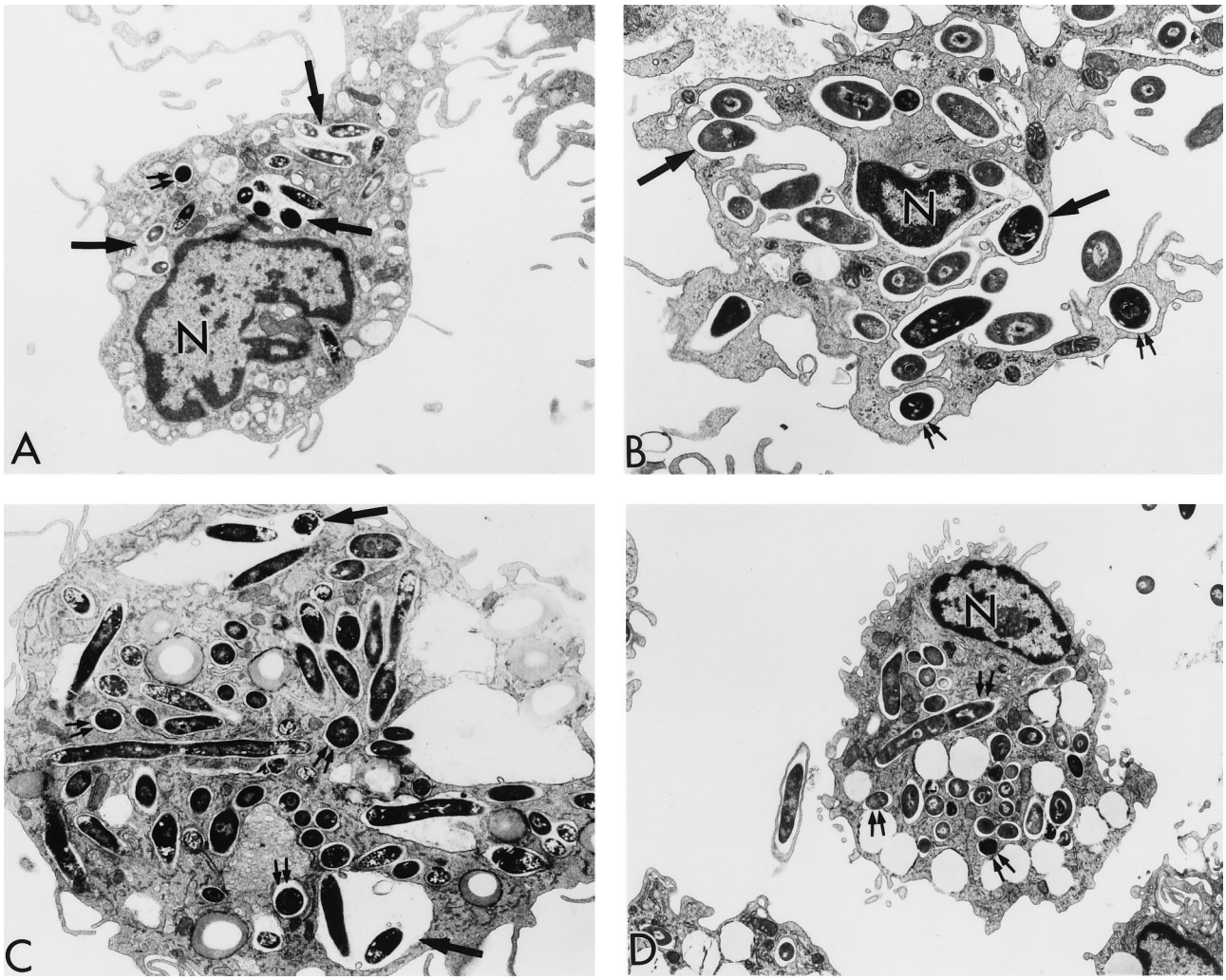

FIG. 3. Electron microscopy of adherent and nonadherent monocytes after infection with *M. smegmatis*. Monocytes were cultured either on plastic coverslips (adherent cells) (A and C) or in Teflon beakers (nonadherent cells) (B and D) and infected with *M. smegmatis*. (A) Adherent monocyte infected with *M. smegmatis* at an MOI of 20:1 for 3 h; (B) nonadherent monocyte infected with *M. smegmatis* at an MOI of 20:1 for 3 h; (C) adherent monocyte infected with *M. smegmatis* at an MOI of 5:1 for 24 h; (D) nonadherent monocyte infected with *M. smegmatis* at an MOI of 20:1 for 24 h. Large arrows indicate multibacterial vacuoles, while small arrows indicate single-bacterium vacuoles. N, cell nucleus. The multibacterial vacuoles shown are all NCJ to the bacteria. Magnifications of panels: A, ×5,000; B, ×8,300; C, ×6,600; D, ×5,000.

are permissive for the intracellular growth of *M. smegmatis*. Comparison of Teflon-cultured monocytes with adherent cultures of monocytes has to date suggested that the cells develop and function similarly. Nonadherent cultures of mouse peritoneal macrophages and bone marrow macrophages have been found to behave in the same way as their adherent-culture counterparts with respect to Fc and complement receptor expression and pinocytosis capacity (37). Cell surface studies with human Teflon-cultured monocytes have demonstrated that the function and differentiation of Teflon-cultured cells is comparable to that of cells maintained in adherent cultures on glass or plastic. Between days 3 and 5 of culture, Teflon-cultured monocytes, like adherent-culture monocytes, show a 10-fold increase in β-glucuronidase and lose peroxidase granules (39), indications of differentiation into macrophages. However, in other cases, the adherence of monocytes can alter the cellular phenotype. For example, culture of human monocytes on glass versus collagen changes their ability to kill tumor cells (17). The adherence of human monocytes to plastic or extracellular matrix components induces the tyrosine phosphorylation of pp76 and the upregulation of interleukin-1β mRNA (20), but the consequences of these differences have not been demonstrated. Ours is the first report suggesting a dramatic difference in function between adherent-culture and Teflon-cultured human monocytes and macrophages. It is interesting that for human neutrophils, adherence also strongly modifies function but appears to upregulate phagocyte function (23).

The reason for the difference in function between adherent- and nonadherent-culture monocytes is not yet known. It is unlikely that the release of reactive oxygen intermediates is responsible for the difference in killing of *M. smegmatis* between nonadherent and adherent monocytes. Nitroblue tetrazolium reduction, a measure of the respiratory burst, is similar for nonadherent and adherent monocytes (4a, 36). Furthermore, the production of reactive oxygen intermediates has been shown not to be correlated with the fate of *M. bovis* (13), *M. tuberculosis* (12), *M. avium* (7), or *M. smegmatis* (8) in murine macrophages.

It is also unlikely that reactive nitrogen intermediates (RNI) are responsible for the killing of *M. smegmatis* by Teflon-

cultured monocytes. The release of inducible RNI from human monocytes or macrophages is controversial, but there have been several recent reports that human monocytes can release RNI (7, 10, 21, 40). Infection of adherent human monocytes with *M. smegmatis* has been shown not to induce the formation of nitric oxide (10). We found that, while human monocytes are capable of releasing RNI in response to *M. tuberculosis*, RNI are neither induced in response to *M. smegmatis* (data not shown) nor involved in the killing of *M. smegmatis*. Also, the addition of N^G-monomethyl-L-arginine, an antagonist of L-arginine oxidation and inhibitor of NO production, had no effect on the fate of *M. smegmatis* in human monocytes, suggesting that low but nondetectable levels of RNI were not responsible for the killing of *M. smegmatis* by Teflon-cultured monocytes.

A CJ membrane of the vacuole around mycobacteria has been thought to be associated with inhibition of phagosome-lysosome fusion (15) and, by inference, with virulence. In rabbit alveolar macrophages infected in vivo with *M. smegmatis* before preparation for electron microscopy, both large vacuoles and CJ vacuoles have been observed (18), and the presence or absence of a distinct peribacillary space at early time points was not associated with apparent later digestion of *M. smegmatis*. However, 5 days after intratracheal administration of *M. smegmatis*, intact organisms were usually found in vacuoles that were CJ to them, and no organisms at all could be cultured after 10 days, suggesting that the CJ vacuole was correlated with bacterial death. It is clear that gross vacuole morphology is not always indicative of mycobacterial fate. For example, in mouse macrophages infected with *Mycobacterium lepraemurium*, vacuoles were CJ to the bacteria 2 to 4 weeks after infection of mice but were much less so 5 months after infection, when organisms continued to proliferate (1). *M. smegmatis* was found more frequently in CJ vacuoles under conditions in which it is killed, suggesting that there is a correlation between killing and vacuole morphology for this organism. It is also clear that it is not necessary for killing for *M. smegmatis* to reside in a vacuole CJ to it. It may be that the tendency of a monocyte to singly compartmentalize bacteria reflects a general change in monocyte physiology and that vacuole morphology does not parallel vacuole biochemistry.

Transfer of infected Teflon-cultured cells to plastic culture dishes results in the loss of killing ability, which indicates that differential phagocytic receptor expression and usage do not influence the fate of the phagocytosed *M. smegmatis*. The cytoskeleton in nonadherent cells is profoundly different from that in adherent cells, and this will influence many cellular events, such as signaling cascades and vesicle movement and fusion. The difference in function between plastic- and Teflon-cultured cells might also be due to energy-dependent mechanisms, cell activation state and cytokine release, or to vacuole makeup (32).

The fact that human nonadherent monocytes and macrophages can kill *M. smegmatis* permits the use of human monocytes for a screen with *M. smegmatis* for the first time. The use of nonadherent monocytes may actually be more appropriate than the use of adherent cultures of monocytes for any assay of bacterial killing. Although BCG and *M. tuberculosis* can survive within both adherent and nonadherent cell cultures, the various morphological and biochemical changes of the monocyte may be different for each culture condition, and survival within a monocyte which is no longer capable of killing effectively may not be relevant to the in vivo situation.

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