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Effect of Mycobacteria on Sensitivity to the Cytotoxic Effects of Tumor Necrosis Factor

ELAINE A. FILLEY AND GRAHAM A. W. ROOK*

Department of Medical Microbiology, School of Pathology, University College and Middlesex School of Medicine, Riding House Street, London W1P 7LD, United Kingdom

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Unlike *Mycobacterium leprae*, *Mycobacterium tuberculosis* is not found inside cells other than macrophages and polymorphonuclear cells in vivo, yet previous work has revealed that in vitro it readily enters all cell lines tested. Moreover, these cells are not killed by the intracellular mycobacteria. We report here that when fibroblasts take up live (but not killed) *M. tuberculosis* H37Rv, they develop greatly increased sensitivity to the toxic effects of tumor necrosis factor (TNF) whether the cell line is inherently sensitive to TNF or not. Ultrasonically disrupted *M. tuberculosis* also has this property. The increased sensitivity is seen in the absence of metabolic inhibitors, although addition of emetine, an inhibitor of protein synthesis, causes the effect to manifest itself earlier and at a lower concentration of TNF. In contrast, infection with *Mycobacterium bovis* bacillus Calmette-Guérin induces little or no increased sensitivity to TNF, whereas *Mycobacterium avium* and *M. tuberculosis* H37Ra have intermediate sensitivities. We discuss the possibility that virulent tuberculosis strains produce a factor which distorts the normal protective function of TNF, rendering it toxic to host tissues and leading to the classical immunopathology of tuberculous lesions.

Mycobacterium leprae is found within a wide range of cell types in vivo, including muscle cells, endothelial cells, neurons, Schwann cells, and fibroblasts (2–4). In contrast, *Mycobacterium tuberculosis* is found in extracellular sites or within phagocytic cells but almost never inside nonphagocytic cells such as those which can harbor *M. leprae*. This contrast poses a paradox because, as Shepard (21) showed, in vitro *M. tuberculosis* can enter not only HeLa cells (a neoplastic line) but also human amnion and monkey kidney cells. Recent electron microscope studies confirm that the organisms are indeed intracellular (14). Logically, we must assume either that uptake of *M. tuberculosis* by nonphagocytic cells does not occur in vivo or that cells which do take up the organisms are killed. This highlights a further paradox. Macrophages usually die when they take up more than 5 to 10 *M. tuberculosis* organisms in vitro (and probably in vivo). However, the cell types studied by Shepard in vitro can take up more than 100 bacilli and yet remain superficially healthy. Therefore, the apparent absence of *M. tuberculosis* from such cell types in vivo is surprising.

The fever, weight loss, and tissue damage which are characteristic of tuberculosis may be attributable to the release of tumor necrosis factor (TNF) (20). Macrophages in tuberculous granulomata are activated by gamma interferon (IFN-γ) and by an autocrine feedback loop involving calcitriol (15, 19). Mycobacterial lipoarabinomannan is a potent trigger of TNF release from such cells (11), and alveolar lavage cells from tuberculosis patients release large quantities of TNF in vitro (18), apparently spontaneously. Moreover, the sera of tuberculosis patients contain high levels of an inhibitor of TNF related to the extracellular domain of the TNF receptor (5). We postulate here that some of the tissue damage and the paradoxical absence of *M. tuberculosis* from nonphagocytic cell types in vivo are attributable to TNF because this mediator causes destruction of cells which take up the organism or which are exposed to an unidentified component released from the bacilli.

MATERIALS AND METHODS

Reagents. Except where specifically stated, all reagents were obtained from Sigma (Poole, Dorset, United Kingdom). Lowenstein-Jensen medium was prepared in our laboratory. Recombinant murine and human TNF (rMuTNF and rHuTNF, respectively) were a gift from G. R. Adolf (Boehringer).

Organisms. *M. tuberculosis* H37Rv and H37Ra were obtained from J. Grosset (Faculte de Medicine Pitie-Salpetriere, Paris, France) and B. Bloom (Albert Einstein College of Medicine, New York, N.Y.), respectively, and maintained by serial passage on Lowenstein-Jensen medium. The two strains of *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (Glaxo and Pasteur) were subcultured onto the same medium from the vials of the manufacturer and passaged on it for more than 1 year before use in these experiments. The two strains of *Mycobacterium avium* were isolates from patients with AIDS and were provided by Ivor Brown (St. Mary's Hospital Medical School, London, United Kingdom). These were strain 13032 (containing no plasmids, serotype 4, isolated from the blood) and strain 14330 (serotype 4b, containing two plasmids, isolated from feces). Before use, suspensions consisting of single organisms or very small clumps were prepared from 7- to 10-day-old cultures by use of sterile techniques, washed in RPMI 1640 medium, and counted as described in detail elsewhere (17). When killed organisms were required, this suspension was divided into two aliquots, one of which was incubated overnight with rifampin at 50 μg/ml. Suspensions of killed organisms were then washed in RPMI 1640 medium and added to cell monolayers at 80 organisms per cell and 40 organisms per cell. The lack of viability of the rifampin-exposed aliquot was confirmed by culture on Lowenstein-Jensen medium.

Bacterial sonicates. Sonicates of *M. avium*, BCG, and *M.*

* Corresponding author.

tuberculosis were prepared as described previously (13) from the same organisms cultured for 3 weeks on Sauton medium. After insoluble material was removed by centrifugation at 10,000 × *g* for 30 min, the preparation was sterilized by passage through a membrane filter (2-μm pore size). The sonicate of *M. leprae* was a gift from R. J. W. Rees (World Health Organization batch CD114) prepared as described previously (24). For some experiments, aliquots were heated in sterile glass tubes at 37 (control), 56, or 80°C for 30 min before use. To test the effect of proteolytic digestion, 1-ml aliquots of sonicate (5 mg/ml) were incubated for 3 h at 37°C and then overnight at room temperature with either protease-conjugated agarose beads (10 mg/ml) (Sigma; P4531) or similar beads conjugated to *p*-aminophenyl *N*-acetylglucosamine as a control before being used in the assay.

Cell lines. Two cell lines were a gift from N. Matthews (Yamanuchi Research Institute, Littlemore Hospital, Oxford, United Kingdom). For most experiments, the cells were L929 cells (murine fibrosarcoma) selected for sensitivity to TNF. In some experiments, the UAR line was used (12). The UAR line is an adherent TNF-resistant mutant of the U937 human monocytoid line. Both lines were maintained in 80-cm^2 tissue culture flasks as a monolayer. The medium used was RPMI 1640 (GIBCO Biocult) containing 5% fetal calf serum (FCS) and L-glutamine (RPMI-FCS). No antibiotics were added at any stage. Two to three times a week, the cultures were divided into two or three portions. When confluent, the monolayer was washed with phosphate-buffered saline (PBS) and then exposed to EDTA-trypsin at room temperature for 5 min. This latter mixture was PBS containing 20 mg of disodium EDTA per 100 ml and 0.025% trypsin (GIBCO). The detached cells were then centrifuged once, resuspended in medium, and plated into two to three new flasks.

Bioassay for TNF. The results described below were obtained by using variants of a published L929 assay (22, 23), except that the metabolic inhibitor used in some experiments (as indicated in Results) was emetine (1 μg/ml) (10). Briefly, L929 cells were plated in 96 flat-bottomed microtiter wells (Nunc) at about 2.5×10^4 cells per well in 100 μl of RPMI-FCS. The precise number of cells was varied to achieve confluence at the planned time of addition of rHuTNF or rMuTNF. A full series of doubling dilutions of TNF in the same medium was then added. The range of TNF concentrations used was determined in pilot experiments so that, where possible, 50% survival of the L929 cells fell in the middle of this range. In the presence of emetine, 50% survival was seen at about 1 ng of TNF per ml, whereas the effective range was 1,000 times higher in its absence. All data in the table and bar charts were calculated from the concentration of TNF giving 50% survival. When this point could not be achieved, such as for the controls shown in Fig. 1 to 3, the whole survival curve was plotted. Survival was determined as described previously (23). Briefly, 21 or 46 h after addition of TNF (in the presence or absence of emetine, respectively), the supernatant was poured off and the monolayers were fixed with methanol for 1 min. The monolayers were then stained with 0.75% crystal violet for 5 min and rinsed with water. Finally, 50 μl of 33% acetic acid was added to each well to dissolve the dye, and the optical density at 570 nm was determined in an enzyme-linked immunosorbent assay reader. Error bars indicate standard deviations.

Calculation of results of L929 bioassays. Optical densities and TNF concentrations were entered into a curve-fitting program (Dataplot; S. M. Fraser, University of Strathclyde), and a regression line was fitted to the linear part of the curve. The TNF dilution giving 50% survival was then derived by interpolation. When comparing TNF dilutions giving 50% kill under different conditions or in the presence of different mycobacterial preparations, data from three to eight experiments were pooled and probabilities were calculated by using the nonparametric Mann-Whitney U test.

Modifications of the TNF bioassay for use in the presence of live mycobacteria or mycobacterial components. When infected cells were required, cell monolayers were split as described above and put into fresh flasks, together with organisms, at a ratio of 40 organisms per cell. These flasks were incubated overnight to allow uptake of bacilli. The cells were then removed from the plastic with PBS-EDTA-trypsin, washed, counted, and plated as described above. Aliquots of the same infected, trypsinized, and washed cells were used for the preparation of cytospins, and, after the Ziehl-Neelsen technique was used for staining, the number of bacteria per cell and the percentage of infected cells were determined. In the experiments reported below, all cells were infected with between 5 and 50 bacilli.

Assessment of protein synthesis in infected L929 cells. Cultures of infected or control L929 cells were cultured in microtiter wells as described earlier with or without TNF, and at intervals the medium in replicate wells was removed and replaced by 50 μl of prewarmed leucine-free modified Eagle medium containing 0.5 μCi of L-[4,5-^{3}H]leucine (Amersham). After 30 min, the [^{3}H]leucine was replaced with EDTA-trypsin to detach the cells, which were immediately harvested by use of a standard semiautomated cell harvester (Skatron). (The number of organisms present in the wells was not sufficient to incorporate measurable [^{3}H]leucine during a 30-min pulse.)

RESULTS

Effect of live intracellular mycobacteria on the sensitivity of fibroblasts to TNF. L929 cells were infected with *M. tuberculosis* H37Rv, *M. avium*, or BCG (Glaxo) in tissue culture flasks. The cells were then detached from the plastic with trypsin-EDTA and washed, and aliquots were plated into microtiter trays or used to assess the number of bacilli per cell as described above. All cells contained 5 to 50 organisms. When the results were assessed at 24 h (Fig. 1a), a marked increase in sensitivity to rHuTNF in the presence of emetine, caused by infection with *M. tuberculosis* H37Rv, was found. The shift in the concentration of TNF required to give 50% kill represents approximately five doubling dilutions or a 32-fold decrease in TNF concentration. In this experiment, infection with an *M. avium* strain had a weaker effect, while infection with BCG (Glaxo) appeared to reduce the toxicity of TNF (Fig. 1a). Results were similar with rMuTNF (data not shown). To find out whether the phenomenon occurred only in inherently TNF-sensitive cell lines, the UAR line was also tested. This line is totally resistant to TNF with or without metabolic inhibitors (12), so there was 100% survival of control cells at all rHuTNF concentrations (Fig. 1b). However, when infected with H37Rv, it was killed by rHuTNF, although the TNF concentration required was about four times that required for infected L929 cells. The killing of both infected cell lines by TNF was strictly dose related, and the cells survived when the TNF was sufficiently diluted. Thus, H37Rv alone was not toxic to the cells under these conditions.

The time course of the enhanced susceptibility to rMuTNF of H37Rv-infected cells in the presence of emetine

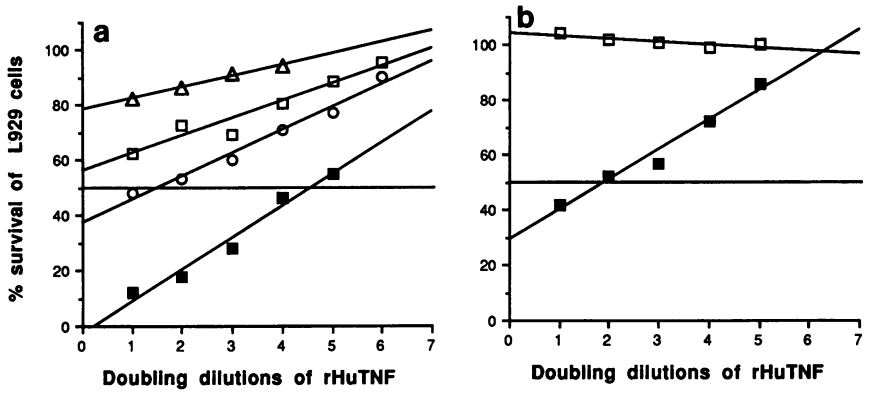

FIG. 1. Effect of intracellular mycobacteria on the sensitivity to rHuTNF of L929 (a) and UAR cells (b) in the presence of emetine (1 μg/ml). Of the cells, 100% were infected with 5 to 50 organisms per cell. The organisms used were *M. avium* 14330 (○), BCG (Glaxo) (△), and *M. tuberculosis* H37Rv (■). Control cells (□) were not infected.

is shown in Fig. 2. The concentrations of TNF used had no significant effect on the noninfected cells, but infected cells died progressively from 12 h on. Infection in the absence of TNF had little effect, and, as TNF was diluted out, the viability of the infected cells returned to control levels.

The enhanced sensitivity to rMuTNF induced by H37Rv was not dependent on the presence of emetine. In the absence of this metabolic inhibitor, the percentage of infected cells dying began to be significant at 36 h and was striking at 42 h (Fig. 3), whereas the TNF dilution causing 50% killing of the infected cells had no significant effect on noninfected cells at this time (Fig. 3). As in the experiments performed in the presence of emetine, when TNF was diluted further, the infected cells survived as well as the noninfected ones, so *M. tuberculosis* H37Rv did not cause cell death without TNF. The metabolic integrity of the infected cells was confirmed by pulsing with [^{3}H]leucine for 30 min. Cells infected for 24 h previously incorporated 19,327 ± 2,401 cpm (mean ± standard deviation), which was similar to the 21,446 ± 2,365 cpm incorporated by noninfected controls. In the absence of TNF, the incorporation by infected cells increased to 26,429 ± 2,218 cpm 30 h later ($P < 0.025$; Student's t test), while in the presence of TNF it fell to 17,509 ± 3,073 cpm at this time, when cell death was just beginning to occur (Fig. 3).

In an additional eight experiments summarized in Fig. 4, it emerged that, although the presence of emetine resulted in a more rapid effect and a lower rMuTNF requirement, the change in TNF sensitivity induced by the four organisms studied was the same whether assessed at 21 h with emetine or at 42 h without emetine. *M. tuberculosis* H37Rv had the largest effect ($P < 0.001$, with or without emetine), while H37Ra had an intermediate effect and the two BCG strains were without significant effect. Results with rHuTNF were again similar, and the murine preparation was used for all of the remaining experiments on the murine L929 cell line.

Effect of killed intracellular *M. tuberculosis* organisms on sensitivity of L929 cells to TNF. When the suspension of *M. tuberculosis* in RPMI-FCS was incubated overnight at 37°C with rifampin at 50 μg/ml before addition to the cell monolayer, the organisms were killed but were taken up by the cells as readily as live bacilli. Thus, when infected at 40 organisms per cell, 98% of the cells contained 5 to 50 organisms per cell. However, the ability of the killed organisms to increase the sensitivity of the cells to TNF was greatly diminished (Fig. 5), even when used at twice the normal dose (40 bacilli per cell) of live bacilli (Fig. 5).

Effect of sonicates and culture filtrate on sensitivity of L929 cells to TNF. The fact that killed organisms enhanced the toxicity of TNF less than live ones could be due either to the

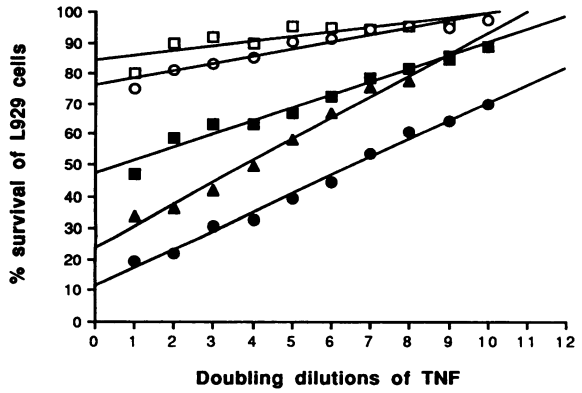

FIG. 2. Time course of rMuTNF-induced killing of control L929 cells (open symbols) or L929 cells infected with *M. tuberculosis* H37Rv (closed symbols) in the presence of emetine (1 μg/ml). Results are shown for 12 (□, ■), 15 (▲), and 21 (○, ●) h.

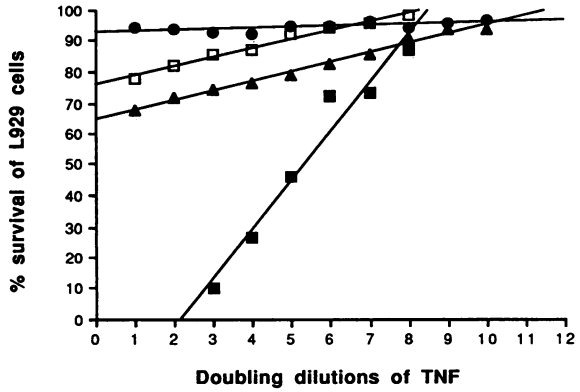

FIG. 3. Time course of rMuTNF-induced killing of control L929 cells (open symbols) or L929 cells infected with *M. tuberculosis* H37Rv (closed symbols) in the absence of emetine. Results are shown for 18 (●), 36 (▲), and 42 (□, ■) h.

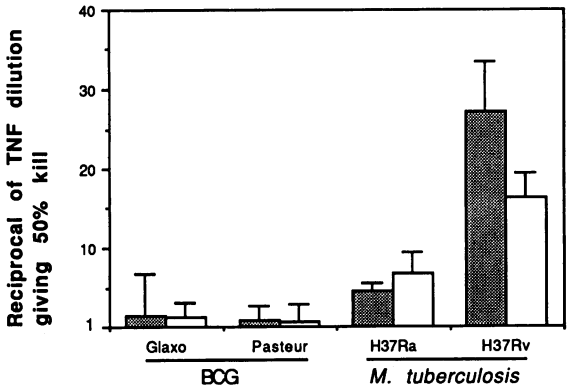

FIG. 4. Effect of infection with BCG or *M. tuberculosis* strains on the concentration of rMuTNF required to give 50% kill of L929 cells in the presence (shaded bars, assessed at 21 h) or absence (open bars, assessed at 42 h) of emetine (1 μg/ml) (standard deviations indicated by error bars). Data from eight experiments are pooled. To normalize the data, the dilution of TNF required for 50% kill of noninfected cells is regarded as 1 U/ml. This dilution corresponded to concentrations between 0.3 and 2.1 ng/ml in the presence of emetine and between 0.24 and 3.2 μg/ml in its absence. Relative to this control value, in the presence of emetine, cells infected with H37Rv require 27-fold-less TNF.

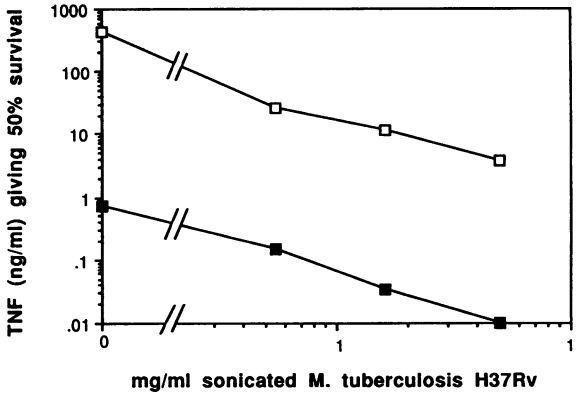

FIG. 6. Effect of sonicated *M. tuberculosis* H37Rv on the rMuTNF concentration (nanograms per milliliter) required to give 50% kill of L929 cells. The experiment was performed in the presence of emetine (1 μg/ml), assessed at 21 h (■), and without emetine, assessed at 42 h (□).

absence in the killed organisms of the relevant component or to their failure to release it. We therefore tested sonicated *M. tuberculosis* H37Rv. The sonicate was able to enhance the toxicity of rMuTNF with or without emetine, although to achieve an effect comparable to that induced by infection with live bacilli, it was necessary to add a large concentration (500 μg/ml) of the sonicate. The dose-response relationships of this effect are shown for sonicated H37Rv in Fig. 6, where the results are expressed in nanograms of TNF per milliliter rather than as dilutions. The presence of emetine reduced the TNF requirement approximately 1,000-fold at all concentrations of sonicate. Results were similar with one culture filtrate (3 weeks) of H37Rv (data not shown), although such filtrates contain many intracellular components which leak from dead organisms (1) so that the significance of this observation is uncertain. However, these experiments show that uptake of bacilli is not essential for induction of increased sensitivity to TNF.

Further experiments revealed that heating the sonicate of H37Rv to 80°C or pretreating it with the insolubilized protease preparation reduced its ability to enhance the toxicity of TNF by 50 to 80% relative to control preparations which had been heated to 37°C or incubated with agarose beads conjugated to an irrelevant material (*p*-aminophenyl *N*-acetylglucosamine). The size of this reduction was similar whether the experiments were performed in the presence of emetine over a low range of TNF concentrations and assessed at 21 h or they were performed in the absence of emetine over a higher range of TNF concentrations and assessed at 42 h.

Sonicates derived from BCG (Glaxo), BCG (Pasteur), and the two strains of *M. avium* all had some ability to enhance the toxicity of TNF when added at the high final concentration of 500 μg/ml. The effect was more variable than that seen with whole live bacilli, and the results from three to eight experiments, reported as the median and range of the percentage of the effect observed when sonicated *M. tuberculosis* H37Rv was used on the same occasion, are summarized in Table 1. All of these sonicates had ~20% of the activity of the H37Rv sonicate and were significantly less

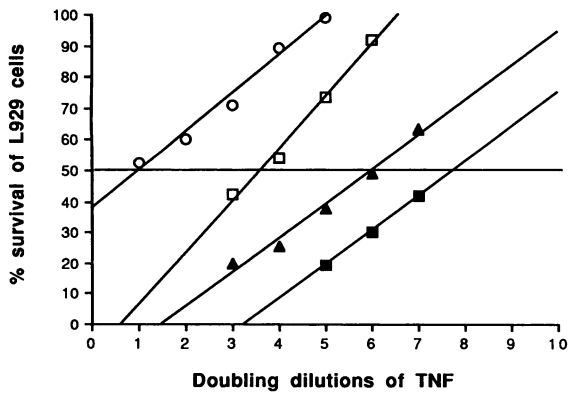

FIG. 5. Effect of killing *M. tuberculosis* H37Rv on its ability to enhance the toxicity of TNF for L929 cells. Symbols: ○, medium alone; □, 80 rifampin-killed *M. tuberculosis* organisms per cell; ▲, 40 live *M. tuberculosis* organisms per cell; ■, 80 live *M. tuberculosis* organisms per cell.

TABLE 1. Ability of mycobacterial sonicates to enhance sensitivity of L929 cells to TNF

Sonicate organism (*n*)[a]	Median % of effect (range)[b]	
	With emetine	Without emetine
BCG Pasteur (8)	18.8 (10.2–76.9)	17.5 (7.7–83.0)
BCG Glaxo (6)	13.0 (5.5–58.8)	19.6 (10.3–54.9)
M. avium (5)	7.5 (2.5–62.5)	14.6 (2.7–76.9)
M. avium (6)	27.7 (16.3–95.0)	19.4 (7.4–98.0)
M. leprae (2)	—[c] (17.5)	11.7 (10.9–12.5)

[a] Organism from which sonicate was derived. All sonicates were tested at 500 μg/ml. *n*, number of experiments.

[b] Reduction in TNF concentration required for 50% kill of the L929 cells in the presence of each sonicate, expressed as a percentage of the reduction induced by *M. tuberculosis* H37Rv sonicate in the same experiment. All were significantly less effective than the H37Rv sonicate ($P < 0.001$; Mann-Whitney U test).

[c] Only two experiments were done because of lack of material. In one experiment, *M. leprae* caused an enhanced TNF requirement.

active ($P < 0.001$; Mann-Whitney U test). However, pretreatment of the cell monolayers with BCG sonicate for 4 h did not reduce the subsequent ability of the H37Rv sonicate to enhance sensitivity to TNF (data not shown). Only two experiments were performed with the sonicate of *M. leprae*, which had insignificant activity in the presence of emetine and only ~10% of the activity of H37Rv sonicate in the absence of emetine. However, it is not certain that the *M. leprae* preparation can be regarded as equivalent to the other preparations since relevant components may have been lost during the extraction procedure (24).

DISCUSSION

We have shown that fibroblasts which take up *M. tuberculosis* become very sensitive to the toxic effect of TNF. Work published while the present study was in progress, using fibroblasts infected with *Listeria monocytogenes*, *Shigella flexneri*, and *Salmonella typhimurium* (7), has demonstrated a similar phenomenon. Moreover, various virus-infected or transformed cell lines show increased sensitivity to this cytokine (8, 9, 25). It is, therefore, possible that one of the protective roles of TNF is the elimination of cells which have become defective for any reason. It will be of particular importance to discover how the increased sensitivity to TNF is mediated.

Since the enhanced toxicity of TNF is also induced by sonicates and culture filtrates, it seems likely that tissue cells in the vicinity of a tuberculous lesion become sensitive to TNF. It is also conceivable, although less probable, that *M. tuberculosis* enters some nonphagocytic host cells in vivo and that these are eliminated in the TNF-rich environment of a tuberculous lesion. Together, these points help to explain the paradox described in the introduction. We also suggest that the toxicity of *M. tuberculosis* for macrophages both in vitro and in vivo is due to a similar phenomenon. The macrophage may become sensitive to its own TNF, released in response to mycobacterial lipoarabinomannan (11), as the organism is taken up. This hypothesis could be tested if TNF expression in a suitable *M. tuberculosis*-sensitive macrophage line could be blocked.

The results with whole live bacilli correlated well with the known hierarchy of virulence of *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and BCG. Used as live bacilli, the avirulent BCG strains did not significantly sensitize the cells to TNF. Therefore, it is interesting that sonicates of the BCG strains did have some activity, although this was always less than that seen with an equivalent concentration of sonicated *M. tuberculosis* H37Rv. It is, therefore, possible that BCG contains less of the active molecule(s) or that BCG fails to release these components unless it is disrupted. Preliminary experiments have shown that both *M. tuberculosis* and BCG survive within L929 cells, but the rate of growth of these two organisms within nonprofessional phagocytes has not, to our knowledge, been compared.

The ability of *M. tuberculosis* to increase the toxicity of TNF may be an important virulence mechanism which helps to explain the dual role of TNF in tuberculosis. We have argued at length that TNF is responsible for much of the immunopathology in this disease (20) and it causes striking hemorrhagic necrosis in mycobacterial lesions (16). On the other hand, experiments with TNF-neutralizing antibodies have revealed that TNF plays an essential protective role in mice infected with BCG (6). These apparently contrasting findings can now be reconciled. We suggest that *M. tuberculosis* (in contrast to BCG) increases the toxic effects of TNF so that the net effect of this cytokine is detrimental to the host. This hypothesis can be tested by treating tuberculous mice with the same neutralizing antibody to TNF that was used previously in the experiments with BCG (6). Experiments of this type are in progress in collaboration with Georges Grau (Geneva, Switzerland), and preliminary results suggest that the effects of anti-TNF in mice infected with the H37Rv strain studied here may be different from the effects in mice infected with BCG.

In conclusion, we suggest that the ability of *M. tuberculosis* to enhance the sensitivity of nonprofessional phagocytic cells to the toxicity of TNF may be a critical virulence factor which distinguishes it from BCG. We now intend to identify the factors responsible and to extend the studies to a range of normal tissue cell types.

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