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The effect of *Mycobacterium tuberculosis* on the susceptibility of human cells to the stimulatory and toxic effects of tumour necrosis factor

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SUMMARY

It has previously been shown that the inherently tumour necrosis factor-α (TNF-α)-sensitive L929 murine fibroblast cell line becomes much more sensitive to the cytotoxic effect of this cytokine after exposure to *Mycobacterium tuberculosis* in culture. In this study it is now shown that normal human cells of types likely to be involved in tuberculous lesions are affected in a similar way. Growth of normal human fibroblasts is usually stimulated by TNF-α *in vitro*, but after exposure to *M. tuberculosis* or to extracts of this organism, these cells are killed rather than stimulated by subsequent exposure to TNF-α. Similarly, human endothelial cells become susceptible to doses to TNF-α which do not normally affect viability. Moreover this enhancement of sensitivity to TNF-α is not confined to its toxicity. Endothelial cells and HeLa cells exposed to *M. tuberculosis* express increased levels of ICAM-1 after subsequent exposure to TNF-α, implying synergy between the two stimuli. It is suggested that these effects contribute to the ability of *M. tuberculosis* to distort the normal protective role of TNF-α so that the cytokine becomes detrimental to the host.

INTRODUCTION

Evidence that tumour necrosis factor-α (TNF-α) plays a central role in the immunopathology of tuberculosis is strong, but mostly indirect. Thus these patients have macrophages activated by interferon-γ (IFN-γ), other lymphokines and calcitriol,[1-4] and such macrophages can be shown *in vitro* to be strongly primed for TNF-α release[1,4] and to be expressing TNF-α *in vivo*.[3] Moreover *Mycobacterium tuberculosis* contains at least one and probably several powerful triggers of cytokine release.[5,6] Alveolar lavage cells from these patients release massive quantities of TNF-α *in vitro*,[7] and develop high serum levels of TNF-α inhibitors,[8] which probably arise as a response to chronic TNF-α release *in vivo*. These points, together with the fact that patients can be febrile and cachectic argue that TNF-α may be released locally into the lesions, and spill over into the systemic circulation.

TNF-α does not always cause tissue damage, but two factors contribute to the likelihood that TNF-α does so in tuberculosis. First, cetain types of inflammation, such as that induced by endotoxin[9] or some tumours[10] cause the tissue to become sensitive to TNF-α. Sites containing T-cell-mediated responses to mycobacterial antigens share this property, and are TNF-α sensitive.[11-13] Secondly, individual cells can become hypersensitive to TNF-α when exposed to *M. tuberculosis*.[14] Thus it was demonstrated recently that when the fibroblast line L929 is infected with *M. tuberculosis* or exposed to culture supernatants (treatments which have little direct effect on cell survival), the cells are killed by much lower doses of TNF-α.[14] This ability to sensitize L929 cells to TNF-α is shared by *M. fortuitum* and *M. vaccae*, but not by *M. avium*, *M. kansasii*, or bacillus Calmette–Guérin (BCG) (ref. 14; E. A. Filley, H. A. Bull, P. M. Dowd and G. A. W. Rook, unpublished observations). However the L929 line is of murine origin, and it is inherently TNF-α sensitive. Therefore these observations did not show whether the factor(s) produced by *M. tuberculosis* can also increase the TNF-α sensitivity of human cells, and more importantly, of normal cell types likely to be directly involved in the inflammatory process *in vivo*. The present study set out to answer these questions using normal human fibroblasts and normal human umbilical vein endothelial cells (HUVEC). It was also the aim of this study to find out whether the sensitization to TNF-α led only to increased susceptibility to its toxicity, or also, during the 40–50 hr which elapse before cell death in the *in vitro* system, to enhancement of other parameters known to change following exposure of these cells to TNF-α. For this purpose changes in expression of the intracellular adhesion molecule ICAM-1 were studied. TNF-α has been shown to increase expression of ICAM-1 on endothelial cells in a concentration- and time-dependent manner.[15]

Abbreviations: HUVEC, human umbilical vein endothelial cells; HFF, human foreskin fibroblasts; TNF, tumour necrosis factor.

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MATERIALS AND METHODS

Cell lines

HUVEC were prepared in medium 199 with modified Earle's salts (Gibco, Paisley, U.K.) containing added glutamine and 20% pooled human serum. The method was as described previously.[16] They were used as primary cultures. The HeLa cells were a CD4-transfected line prepared as part of the Medical Research Council's AIDS-directed programme, which were obtained from E. Puszczynska (National Institute for Biological Standards and Controls, South Mimms, U.K.). These were cultured in RPMI-1640 (Gibco Biocult, Uxbridge, U.K.) with 5% foetal calf serum (FCS), but no antibiotics. The normal human foreskin fibroblasts (HFF) were obtained from Dr T. Meager (NIBSC), and cultured in RPMI-1640 with 10% FCS, without antibiotics. These had been passaged $\times 8$ when used.

Reagents

Except where specifically stated, all reagents were obtained from Sigma (Poole, U.K.). Lowenstein–Jensen medium was prepared in this laboratory. Recombinant murine (rm) TNF-α was a gift from Dr G. R. Adolf (Boehringer, Vienna, Austria). Monoclonal antibody to ICAM-1 was RR1/1.1.1 obtained from Boehringer as ascites-derived IgG1 at 2·5 mg/ml.[17] An irrelevant monoclonal with no known binding specificity, of the same subclass (monoclonal antibody P3), was used as control.

Organisms

Mycobacterium tuberculosis strain H37Rv was obtained from Professor J. Grosset (Faculte de Medicine Pitie-Salpetriere, Paris, France) and maintained by serial passage on Lowenstein–Jensen medium. The Glaxo strain of BCG was subcultured onto the same medium from the manufacturer's vials, and passaged on it for more than 1 year before use in these experiments. Suspensions of organisms consisting essentially of single bacilli were prepared from 7-day cultures as described previously,[18] and sonicates of BCG, and *M. tuberculosis*[19] were prepared from the same organisms cultured for 3 weeks on Sauton's medium.

Assays for TNF-α sensitivity of the cell lines

These were based on a published TNF-α bioassay.[14] Briefly, cells were plated in 96-well flat-bottomed microtitre wells, (Nunc, Roskilde, Denmark) at $1\text{–}2 \times 10^4$ cells/well in the appropriate medium (described above). Two hours later, when the cells were adherent, a further 10 μl of RPMI was added, containing nothing, or a suspension of *M. tuberculosis* or BCG Glaxo adjusted to give 40 organisms/cell, or sonicated *M. tuberculosis* to give a final concentration of 0·5 mg/ml. This organism/cell ratio resulted in uptake of >3 bacteria by at least 96% of the cells. The sonicate concentration was selected from dose–response studies published previously.[14] After incubation for a further 16 hr, some of the wells to which whole bacteria had been added were rinsed three times with medium and treated with EDTA/trypsin to remove the cells. Cytospins of these cells were prepared, and the per cent infected cells and the number of bacilli/cell were checked after staining with the Ziehl–Neelsen technique, as described previously.[14] To the remaining replicate wells, a full series of rTNF-α dilutions was added in 100 μl of RPMI. The appropriate range of TNF-α concentrations for each of the three cell lines was predetermined in pilot experiments. Metabolic inhibitors such as emetine and actinomycin, usually used in TNF-α bioassays, were not used in the experiments reported here. Then 46 hr after addition of TNF-α (i.e. 62 hr after addition of *M. tuberculosis* or control medium), the supernatant was poured off, the monolayers were fixed with methanol for 1 min, and cell survival was determined as described previously.[14] Briefly the monolayers were fixed with methanol for 1 min, 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 ELISA reader. Error bars indicate ±SD.

Semiquantitative assay for expression of ICAM-1

To assess expression of ICAM-1 an extra stage was added to the protocol outlined in the previous section. After the 46 hr of incubation with TNF-α, the monolayers were fixed with 0·1% glutaraldehyde in phosphate-buffered saline (PBS) for 10 min. ICAM-1 expression was then detected by 'cell ELISA' as described previously.[20] Briefly, the wells were blocked for 30 min with PBS containing 0·1% bovine serum albumin (BSA), and 5 mM EDTA. Then the anti-ICAM-1 monoclonal antibody at 0·5 μg/ml or the same concentration of an isotype-matched control was added to the wells, which were incubated for a further 1·5 hr at room temperature. The wells were then washed three times with PBS/EDTA/BSA, and peroxidase-conjugated antibody to murine Ig (Dako P260; Dako, High Wycombe, U.K.) was added at a final dilution of 1/500 in PBS/EDTA/BSA for 1·5 hr at room temperature. After further washes the binding was assessed using standard protocols, with ABTS (Sigma A1888) as chromogen. After reading the ELISA result, the same wells were washed, and fixed again in methanol, before survival was assessed as described in the previous section, to allow correction of the values for TNF-α-induced changes in cell numbers.

RESULTS

The effect of culture with *M. tuberculosis* on the growth and survival of the cell lines when subsequently exposed to TNF-α

TNF-α was not inherently toxic to the three cell lines used (Fig. 1). In some experiments HeLa were stimulated by 10 ng/ml TNF-α. Growth of the normal fibroblasts was routinely stimulated by TNF-α as reported by others,[21] and peaked between 10 and 30 ng/ml. Growth of HUVEC was not significantly affected by TNF-α except when they were plated at very low cell densities when, like the other lines, their growth was stimulated (data not shown).[22]

All three lines took up *M. tuberculosis* as described previously[14,23] using other 'non-phagocytic' cell lines *in vitro*. In the experiments shown in the figures, at least 96% of the cells contained >3 bacilli, and most cells contained too many for precise counting.

When infected with *M. tuberculosis* all three types showed modestly decreased cell numbers at the end of the experiment (i.e. 62 hr after infection) even in the absence of TNF-α. Thus mean absorbance values calculated from seven replicate experiments ranged from 7·5% (HUVEC) to 20% (fibroblasts) below those of uninfected cells (Fig. 1).

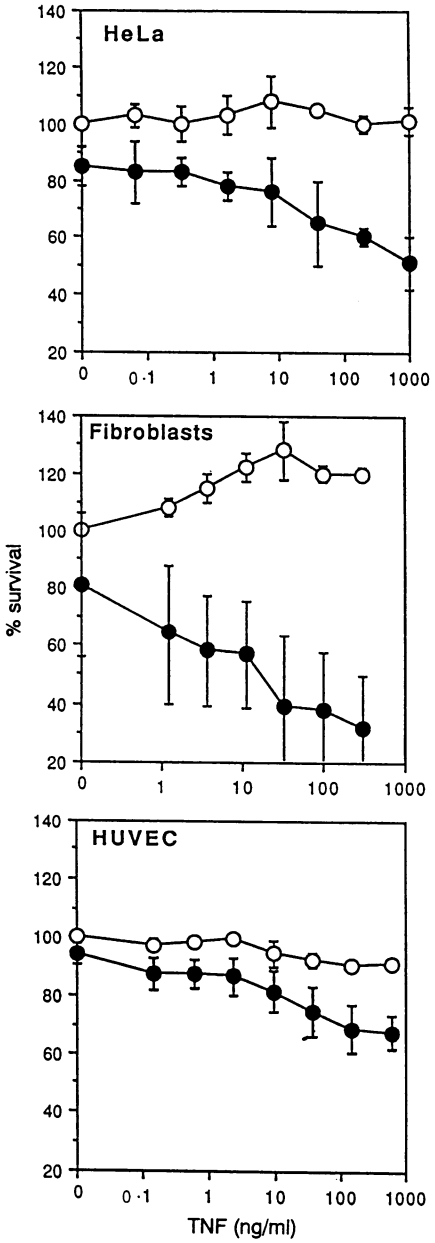

Figure 1. Human HeLa, fibroblasts, or HUVEC were infected with *M. tuberculosis* (●) or left as uninfected controls (○), and cultured for 16 hr. Then the indicated concentration of TNF-α was added, and the cells were cultured for a further 46 h. Cell density was then assessed, and calculated as a percentage of that seen in the non-infected control wells, cultured without TNF-α. The results are plotted as the mean ± SD of seven experiments.

When the infected cells were exposed to increasing concentrations of TNF-α for 46 hr (added 16 hr after addition of organisms as described above), there was a dose-related increase in dead and detached cells, evident as a decrease in cell numbers at 62 hr. The cytotoxic effect was similar for HeLa and fibroblasts, but somewhat less marked for HUVEC. When tested, exposure to BCG had a detectable but very weak effect, while the effect of sonicated *M. tuberculosis* was intermediate (data not shown), as already reported for L929 cells.[14]

The effect of *M. tuberculosis* on the expression of ICAM-1 and on the induction of ICAM-1 by TNF-α

Expression of ICAM-1 in each well was corrected for changes in cell survival in the same well. Infection of HUVEC with *M. tuberculosis* led to an increase in background ICAM-1 expression, even in the absence of TNF-α (Fig. 2). The mean increase with *M. tuberculosis* but no TNF-α was +45·8 ± 29% relative to uninfected controls in seven replicate experiments. This was not due to contaminating endotoxin, since although addition of polymyxin B sometimes reduced the background ICAM-1 expression in wells not exposed to *M. tuberculosis*, addition of this antibiotic to wells containing infected cells tended to augment the increase in ICAM-1 expression to +53 ± 39%, although this difference was not significant.

Background expression of ICAM-1 by HeLa cells was similar to that of HUVEC. Again this was slightly increased after exposure to *M. tuberculosis* (+25·5 ± 15·1%), and further (but not significantly) increased if polymyxin was also present (+32 ± 13%).

Addition of TNF-α caused a dose-related increase in ICAM-1 expression, which was significantly greater in HeLa or HUVEC exposed to *M. tuberculosis*. The effect of exposure to sonicated *M. tuberculosis* was intermediate. These experiments were not performed with the fibroblasts.

DISCUSSION

The results reported here indicate that *M. tuberculosis*, or factors related from it, may be able to alter the pattern of response of normal tissue cells to TNF-α. First, cells for which the cytokine is normally a growth stimulator, such as fibroblasts,[21] may, after exposure to *M. tuberculosis*, be inhibited or killed by it. Secondly, endothelial cells may become not only more susceptible to TNF-α-induced damage, but also more likely to promote the extravasation of inflammatory cells via increased expression of adhesion molecules. The mechanism of these effects of *M. tuberculosis* is not clear. One possibility is that it is partly due to release of other cytokines such as interleukin-1 (IL-1), which then act synergistically with the TNF-α.

The effects demonstrated are not attributable to contaminating endotoxin. Addition of polymyxin reproducibly increased rather than decreased the effects of *M. tuberculosis*. Further examination of this unexpected finding using the more TNF-α-sensitive L929 murine fibroblast line has shown that the effect is real, but is seen only when live *M. tuberculosis* is used. Thus polymyxin does not alter the TNF-α-enhancing effect of sonicated *M. tuberculosis* (data not shown). The most likely explanation is the known ability of the polymyxins to damage the cell membrane and cause leakiness of mycobacteria.[24] The possibility that the TNF-α-enhancing activity is released in greater quantities from such damaged organisms is now being investigated.

These studies also add to the list of cell types known to take up *M. tuberculosis in vitro*. Shepard first showed that *M. tuberculosis* can enter not only HeLa cells (a neoplastic line) but also human amnion and monkey kidney.[23] Recent electron microscope studies confirm that mycobacteria apparently taken up by 'non-phagocytic' cells are indeed intracellular.[25] It is now shown that they are also taken up by normal human fibroblasts and endothelial cells. It remains unexplained why *M. tuberculosis* is not seen inside such cell types *in vivo*, particularly in view of

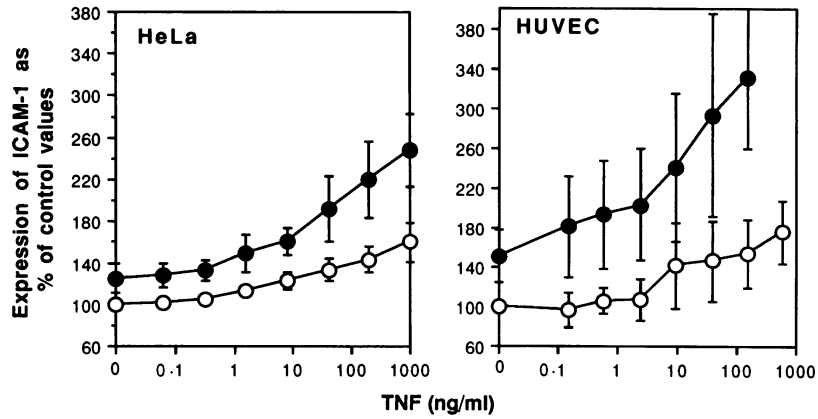

Figure 2. HeLa or HUVEC were cultured alone, or with *M. tuberculosis* as described in Fig 1. ICAM-1 expression was estimated, and corrected for cell numbers as described in Materials and Methods. Results are expressed as mean ± SD of three experiments. Results from the different experiments were normalized by treating the ICAM-1 expression of control cells cultured without *M. tuberculosis* or TNF as 100%.

the fact that *M. leprae* is routinely found inside these cells.[26,27] The present data provide a possible explanation. Cells which take up *M. tuberculosis* may be killed rapidly by TNF-α and other cytokines. This killing takes more than 40 hr *in vitro* (unless emetine is added),[14] but *in vivo* other cytokines such as IFN-γ may further enhance the cytotoxic effect. Similarly the increased expression of adhesion molecules may cause the infected cells to become targets for killer cell activity.[28] These data may also explain the fact that *in vitro* macrophages tolerate intracellular *M. tuberculosis* less well than cell types such as those studied here, and may die if infected with more than five bacilli. Perhaps the macrophages are rendered TNF sensitive, and are then killed by the TNF which they themselves release.

In the mouse it seems that TNF-α plays a major protective role during mycobacterial infection.[29] This suggests that the balance between the protective and immunopathological roles of TNF-α favours the former in this species. This may be attributable to the ability of TNF-α to trigger production of nitric oxide by murine macrophages activated by IFN-γ, resulting in inhibition of *M. tuberculosis*.[30,31] Moreover the immunopathology is much less evident in mice than in man, and in order to evoke necrotic skin-test responses (Koch phenomenon) to mycobacterial antigen in mice it is necessary to supplement the TNF-α levels in the skin-test site.[12,13] The situation is different in man. There is no good evidence that human macrophages can produce nitric oxide. This appears to be attributable to their inability to make tetrahydrobiopterin which is an essential co-factor.[32] Similarly there is only one unconfirmed report that TNF-α together with IFN-γ and calcitriol will cause human monocytes to inhibit growth of *M. tuberculosis*.[33] Thus in human disease the immunopathological role of TNF-α may be dominant, and we suggest that the ability of *M. tuberculosis* to enhance directly both the toxic and proinflammatory effects of TNF-α which we report here, may be one of the mechanisms responsible.

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