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# Recombinant tumour necrosis factor-alpha decreases whereas recombinant interleukin-6 increases growth of a virulent strain of *Mycobacterium avium* in human macrophages

M. DENIS* & E. O. GREGG *Bioscience 1, ICI Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire, U.K. and* **Unite de Recherche, Centre de Pneumologie, Hospital Laval, Sainte Foy, Canada*

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## SUMMARY

The ability of a virulent strain of *Mycobacterium avium* to infect and replicate within human monocyte-derived macrophages of normal donors was assessed. Moreover, the ability of selected cytokines to modulate the intracellular growth of *M. avium* was investigated. Our virulent strain of *M. avium* grew progressively in human macrophages. Treatment of macrophage monolayers with interferon-gamma (IFN-$\gamma$) did not lead to any significant change in the infection pattern. Conversely, treatment with tumour necrosis factor-alpha (TNF-$\alpha$) led to a significant reduction in the growth of *M. avium* in the macrophages. In contrast, treatment of macrophages with interleukin-6 (IL-6) enhanced their susceptibility to *M. avium* significantly. This finding was substantiated by other results which showed that IL-6 increased the growth of *M. avium* in tissue culture medium. These results suggest that cytokines may influence the *M. avium*–macrophage interaction, in a positive or negative manner.

Infections with *Mycobacterium avium* constitute an important health problem in immunosuppressed patients (Schnittman *et al.*, 1988), recent evidence also suggests that otherwise normal individuals may develop infections with this pathogen (Prince *et al.*, 1989). These infections may be life-threatening in as much as many strains of *M. avium* are resistant to first line anti-tuberculosis drugs (Snider *et al.*, 1987). Often, treatment consists of very aggressive combinations of different agents, which sometimes prove to be very toxic. Recent evidence suggests that virulent strains of *M. avium* infect and multiply within human monocytes–macrophages (Crowle *et al.*, 1986). This allows *in vitro* studies of the modulation of this interaction. Cytokines may thus be studied for their ability to modulate the *M. avium*–macrophage interaction. We set out to investigate the ability of selected cytokines to modify the growth of a virulent *M. avium* inside human macrophages. To this end, human mononuclear cells from normal donors were isolated as described in detail elsewhere (Douvas *et al.*, 1985). Cells were washed in Hanks' balanced salt solution (HBSS) without $Ca^{2+}$ and $Mg^{2+}$ (Gibco, Paisley Renfrewshire, U.K.), centrifuged and suspended in RPMI-1640 (Gibco) with 10% autologous serum at $10^7$/ml. One-millilitre aliquots were distributed in 24-well tissue culture plates (Nunc, Roskilde, Denmark). Plates were incubated for 2 hr at 37° in 5% $CO_2$/95 air in moist air. Non-adherent cells were then removed by vigorous washings with HBSS. Adherent monocytes (c. $10^6$/well), as determined by the method of Nakagawara & Nathan (1983), were kept in RPMI-1640 with 2% autologous serum and 2 mM glutamine for 7 days. *M. avium* TMC 7497 (Serovar 4) was obtained from the Trudeau Mycobacterial collection (Saranac Lake, NY). It was grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI) for 21 days and filtered through a 1·2 $\mu$m filter and aliquots were frozen at −70°. For *in vitro* infections, bacteria were thawed, sonicated briefly for 5 seconds and diluted in complete tissue culture medium. For infection, $10^7$ colony-forming units (CFU) of *M. avium*/ml were added to monolayers and the plates cultured at 37° in 5% $CO_2$ in moist air for 18 hr in complete tissue culture medium. After 18 hr, extracellular bacteria were removed by vigorous washing with HBSS. At time zero (18 hr phagocytosis) and at various predetermined times, selected monolayers were lysed by adding 0·2 ml distilled water. After 15 min, 0·5 ml of 0·25% SDS was added to each well. Wells were then scraped and lysates suspended in 1·0 ml of distilled water. The lysate was then sonicated for 30 seconds, and then plated on 7H10 agar (Difco). Bacterial numbers were determined after 21 days. Bacterial numbers were also determined in the supernatants of infected cells at various times. Results are reported as mean CFU per ml of lysate. Macrophage numbers were determined in all experimental groups at various times post-infection. Loss of cells occurred in all groups at the same rate during infection (15–20% loss of initial cells at Day 4 and 30–40% at Day 7). There was no selective loss in any experimental group. In selected experiments, macrophage monolayers were

Correspondence: Dr E. O. Gregg, Bioscience 1, ICI Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K.

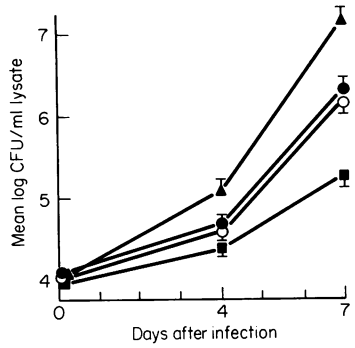


**Figure 1.** Growth of *M. avium* in human macrophages treated with various cytokines. Macrophages were left untreated (○), or were pulsed overnight with $10^3$ U/ml of IFN-γ (●), TNF-α (■) or IL-6 (▲); they were infected with *M. avium* and their ability to affect growth was assessed as described. Lymphokines were added to the culture medium 24 hr before infection and were also present throughout the incubation. Each datum point is the mean of five separate determinations ± SEM.

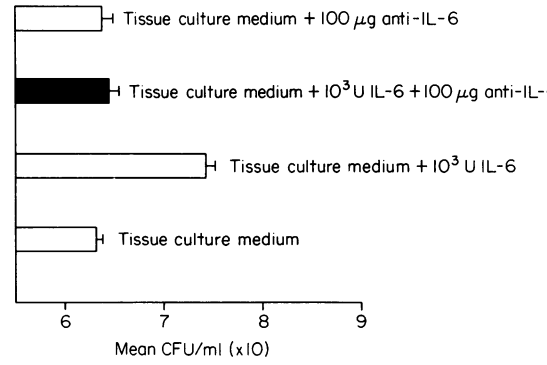


**Figure 2.** Growth of *M. avium* in tissue culture medium (RPMI-1640, 2 mM glutamine with 10% FCS): selected tubes were supplemented with $10^3$U IL-6 and/or 100 μg of a polyclonal rabbit antiserum against IL-6. Two × $10^6$ CFU of *M. avium* were seeded in the different conditions in 2 ml and the bacterial numbers determined at 7 days by plating serial dilutions. Each column is the mean of five separate determinations ± SEM.

treated overnight with cytokines; recombinant human tumour necrosis factor-alpha (TNF-α) and recombinant interferon-gamma IFN-γ were obtained from Genentech Inc. (San Francisco, CA); recombinant interleukin-6 was obtained from British Biotechnology (Oxford, U.K.). A polyclonal rabbit anti-human IL-6 was obtained from Genzyme (Boston, MA); 1 mg of this antiserum neutralizes approximately $10^4$ U of recombinant IL-6 in a CESS cell immunoglobulin production assay (Helle, Bolije & Aarden, 1988). All cytokines were diluted in pyrogen-free saline.

In the first set of experiments we evaluated the ability of *M. avium* 7497 to grow in untreated human macrophages. As Fig. 1 shows, strain 7497 grew progressively in normal human macrophages, with a mean generation time of approximately 24–28 hr in normal human macrophages. In untreated macrophage populations, bacterial numbers in the supernatants were less than 15% of the numbers which were macrophage associated at all times (Day 4 and Day 7). We treated selected macrophage monolayers with TNF-α and IL-6; both of these cytokines have been shown to endow macrophages with substantial anti-mycobacterial activity against *M. avium* and *M. bovis* (Bermudez & Young, 1988; Flesch & Kaufmann, 1990). Macrophages were treated with 1000 U IL-6, TNF-α or IFN-γ/ml, and their ability to phagocytose and harbour *M. avium* was monitored. As Fig. 1 suggests, these cytokines had quite varied effects on *M. avium* growth in human macrophages. Macrophages that were pulsed with IFN-γ were no more resistant or susceptible to *M. avium* than their control counterparts at all times after infection. Increasing the doses of IFN-γ up to $10^5$ U/ml, in an attempt to reveal an effect, did not modify this growth pattern, although very high doses of rIFN-γ led to macrophage death (data not shown). In contrast, treatment of macrophages with 1000 U TNF-α/ml generated a significant reduction in the growth of *M. avium*. This led to significantly fewer CFU recovered at Day 4 and Day 7 after phagocytosis in TNF-α-treated monolayers ($P < 0{\cdot}0001$ by Student's *t*-test).

A one-log reduction was monitored in three separate experiments using two different donors. In supernatants from both IFN-γ- and TNF-α-treated macrophage monolayers, the number of bacteria never exceeded 15% of the number associated with cells at Day 0, 4 and 7 (data not shown). In sharp contrast, treatment of macrophages with IL-6 led to a significant increase in *M. avium* growth in human macrophages at 4 and 7 days after infection ($P < 0{\cdot}0002$). A one-log increase was seen at 7 days post-infection in three separate experiments. Boiled IL-6 and boiled TNF-α failed to modify *M. avium* growth in macrophages, suggesting that residual endotoxin was not mediating the effects observed. Counts of CFU in the supernatant of IL-6-treated monolayers were higher than in supernatants of untreated macrophages at Day 4 and Day 7 (30–40% of the macrophage-associated CFU, data not shown). There was little indication that this was due to lysis of infected macrophages and release of intracellular bacilli. This suggested that IL-6 itself or a factor-secreted by IL-6-treated cells promoted extracellular multiplication of *M. avium* in tissue culture medium. We tested the possibility that IL-6 was promoting *M. avium* growth directly by inoculating 2 × $10^6$ CFU of *M. avium* in tissue culture medium supplemented with 10% foetal calf serum (FCS). Selected tubes were supplemented with $10^3$ U IL-6/ml and/or a polyclonal rabbit antiserum against IL-6 (Genzyme, Boston, MA). As Fig. 2 shows, IL-6 promoted the growth of *M. avium* in tissue culture medium (c. 0·8-log increase in CFU counts in five separate experiments, $P < 0{\cdot}00003$). This increase in growth was abrogated by inclusion of the antiserum, which itself did not modify *M. avium* growth. Our study has thus uncovered a number of phenomena: (i) IFN-γ appears to be inactive in enhancing bacteriostatic ability of macrophages against *M. avium*; (ii) TNF-α endows macrophages with a significant ability to restrict *M. avium* growth; (iii) IL-6 enhances *M. avium* growth in macrophages; (iv) IL-6 promotes the extracellular growth of *M. avium*. The first point confirms previous studies in as much as most investigators find that IFN-γ does not activate human macrophages/monocytes to an anti-mycobacterial state (Douvas *et al.*, 1985; Squires *et al.*, 1989; Toba, Crawford & Ellner, 1989), whereas it does in murine cells (Flesch & Kaufmann, 1987). The reasons for this species difference are still not clear. The role of TNF-α in resistance to mycobacteria remains uncertain but recent studies suggest TNF-α may be an important entity in resistance against mycobacterial infections. Treatment of mice infected with *M. bovis* BCG and depleted of endogenous TNF-α, by administration of an antibody against TNF-α was shown

to significantly increase the susceptibility to mycobacterial infection (Kindler *et al.*, 1989). TNF-α has been shown to enhance killing of an avirulent strain of *M. avium in vitro* by human macrophages (Bermudez & Young, 1988). Our own finding suggests that TNF-α may endow human macrophages with the ability to restrict growth of a virulent *M. avium*. The exact mechanism which is involved remains to be defined.

Further, our data indicate that IL-6 is a potent growth factor for *M. avium* in both macrophages and extracellularly. It is not clear if the mechanism for both observations is the same. These findings would indicate that *M. avium* may utilize IL-6 as a growth factor. Although this finding may appear quite surprising, recent data from the literature suggests that the use of cytokines by infectious agents may be a common phenomenon. IL-2, as well as granulocyte–macrophage colony-stimulating factor (GM-CSF), has been shown to act as a growth factor for *Leishmania* species *in vivo* and *in vitro* (Mazingue *et al.*, 1989). Recent results from our laboratory have shown that IL-2 may act as a growth factor for a virulent strain of *Escherichia coli* (M. Denis, D. Campbell and E. O. Gregg, manuscript submitted for publication). Although the significance of these observations remains to be determined, it may be that use of cytokines by bacterial pathogens represents a virulence factor which is of importance in determining the final outcome of an infection.

The ability of soluble factors from intracellular and extracellular growth of mycobacteria has been alluded to in a number of studies. Human macrophages pulsed with rIFN-γ sponsor increased growth of *M. tuberculosis* (Douvas *et al.*, 1985), and they also secrete a factor which enhances growth of *Mycobacterium* extracellularly. Similarly, IFN-γ-pulsed macrophages are more susceptible *in vitro* to infection with *M. lepraemurium* (Mor, Goren & Crowle, 1989) or *M. avium* (Bermudez & Young, 1988). Moreover, the ability of macrophages to secrete a factor which promotes extracellular growth of *M. avium* has been recognized before (Crowle *et al.*, 1986). It may be suggested that some of these effects were mediated by IL-6. IL-6 is one of the major mediators of the acute reaction to viral and bacterial infections. The major effects of IL-6 have been summarized and include stimulation of immunoglobulin secretion by B cells, growth stimulation of hybridomas, activation of T cells and stimulation of acute-phase proteins (Revel, 1989). Our data suggest a hitherto unappreciated activity for IL-6, that is stimulation of bacterial growth. These findings have a number of strong implications for *M. avium* infections. They suggest that production of IL-6 may be detrimental to the host. Since IL-6 is produced in quite high amounts by HIV infection (Breen *et al.*, 1990), this may partially explain the unusual susceptibility of AIDS patients to *M. avium* infections. Furthermore, recent data showing increased growth of intracellular *M. avium* caused by treatment with serum from AIDS patients may be explained by the fact that these sera were rich in IL-6 (Crowle, Cohn & Poche, 1989).

Overall, our data suggest an important role for cytokines in resistance to *M. avium* infections, with TNF-α having a positive role and IL-6 being largely detrimental.

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