THE production of interferon- γ and tumour necrosis factor- α was evaluated in the peripheral blood mononuclear cells (PBMCs) from healthy donors and AIDS patients after *Rhodococcus equi* infection *in vitro*. PBMCs from healthy donors secreted elevated levels of IFN- γ and TNF- α when challenged *in vitro* with killed *R. equi*, whereas the release of both cytokines was impaired in supernatant cultures from AIDS patients. We conclude that the failure of IFN- γ generation in AIDS patients in response to *R. equi* is not antigen-specific but it may reflect the global impairment of T-cell function. In such patients, however, the infection with *R. equi*, a facultative intracellular pathogen which survives and replicates within macrophages, may be responsible for the impairment in the TNF- α release, possibly enhancing the HIV-induced macrophage dysfunction.

Key words: AIDS, IFN- γ , *In vitro* defective production, *Rhodococcus equi*, TNF- α .

Defective production of interferon- γ and tumour necrosis factor- α by AIDS mononuclear cells after *in vitro* exposure to *Rhodococcus equi*

S. Delia, C. M. Mastroianni,^{CA} M. Lichtner, F. Mengoni, S. Moretti and V. Vullo

Department of Infectious and Tropical Diseases, La Sapienza University, Rome, Italy

^{CA}Corresponding Author

Introduction

Rhodococcus equi, a well-known animal pathogen, has been reported to cause increasingly frequent life-threatening opportunistic infections in patients with AIDS.¹ Like the mycobacteria, *R. equi* can invade and replicate within infected macrophages.² Several hypotheses have been put forward concerning the survival of *R. equi* within phagocytes and one major pathogenetic mechanism might be its ability to block phagolysosome fusion.

Cell-mediated immunity is considered to be of major importance in resistance to infection.³ Because of the intracellular nature of the organism, the immunologic resistance to *R. equi* infection is thought to be mediated by co-operative interaction between T lymphocytes and macrophages. This interaction probably depends on the interplay of cytokines secreted by lymphocytes and other mononuclear cells. A recent investigation in euthymic mice suggests a role for endogenously generated interferon- γ (IFN- γ) and tumour necrosis factor- α (TNF- α) in host defence against *R. equi* infection.⁴ The cytokines that mediate immunologic resistance to *R. equi* in humans remain undefined.

To gain insight into the pattern of the primarily T cell- and macrophage-secreted cytokines that contribute to human anti-rhodococcal defences, we evaluated IFN- γ and TNF- α production by peripheral blood mononuclear cells

(PBMCs) from healthy donors and AIDS patients in response to *R. equi* stimulation *in vitro*.

Materials and Methods

Subjects: Five AIDS patients, hospitalized at the Department of Infectious and Tropical Diseases of La Sapienza University, and six healthy volunteer donors were considered in this study. None of the AIDS patients had evidence of overt opportunistic infections at enrolment.

Bacteria: *R. equi* reference strain ATCC 33701 was obtained from American Type Culture Collection (Rockville, MD). Bacteria were grown at 38°C for 48 h in brain heart infusion broth, and then suspended in RPMI 1640 (GIBCO, Bio Cult., Paisley, UK). Killed bacteria were obtained by heat-inactivation at 65°C for 30 min.

Stimulation of PBMCs with *R. equi*: PBMCs were obtained from venous blood using anticoagulant acid citrate dextrose blood collection tubes (Becton-Dickinson, San Jose, CA, USA) and isolated by centrifugation with Ficoll-Hypaque (Sigma Chemical Co., St. Louis, MO, USA).⁵ The isolated PBMCs were resuspended in RPMI medium supplemented with 5% fetal calf serum (FCS) (GIBCO), 2 mM glutamine (GIBCO), penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells were seeded into 24-well microtitre plates at a concentration of 1×10^6 cells/well

and suspensions of killed bacteria at a final concentration of $10^4 - 2 \times 10^6$ cells/ml were added. Control wells contained no bacteria. Plates were then incubated at 37°C with 5% CO_2 in a humidified atmosphere and supernatants were harvested after 24 h for measurement of $\text{TNF-}\alpha$ concentrations and after 48 h for measurement of $\text{IFN-}\gamma$ levels. The concentrations of cytokine induced by *R. equi* were calculated as that measured in the appropriate supernatant, minus the cytokine concentration in control wells containing media alone. All supernatants were stored at -80°C before determination of cytokine levels.

Cytokine measurement: $\text{TNF-}\alpha$ concentrations were measured by a quantitative immunoenzymatic sandwich assay (Quantikine, R & D Systems, Minneapolis, MN, USA). Unknown values of $\text{TNF-}\alpha$ in the samples were determined by referring to the standard curve and expressed as pg/ml. The detection limit of the assay was 4.8 pg/ml.

$\text{IFN-}\gamma$ concentrations were measured by a commercial sandwich ELISA (human $\text{IFN-}\gamma$ ELISA kit; Central Laboratory of Netherlands Red Cross Blood Transfusion Service, Amsterdam). Levels of $\text{IFN-}\gamma$ were determined by comparison with a standard curve generated from specimens of known $\text{IFN-}\gamma$ concentrations. The detection limit of the assay was 20 pg/ml.

Statistical analysis: Statistical evaluation was performed by one-way analysis of variance.

Results

The results for $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$ release by human mononuclear cells in response to stimulation with *R. equi* are summarized in Fig. 1. The levels of $\text{IFN-}\gamma$ produced by the cells from healthy donors were significantly greater than that in the AIDS patients (mean $\pm$ S.E.M., 778 ± 129 pg/ml and 57.4 ± 28.6 pg/ml, respectively; $p < 0.001$). Moreover, *R. equi* was able to induce the production of $\text{IFN-}\gamma$ in the supernatants of cell cultures from healthy donors in a dose-dependent manner (Fig. 2). When cells of AIDS patients were stimulated with killed bacteria at a final concentration of $10^4 - 10^6$ cells/ml, no *in vitro* secretion of $\text{IFN-}\gamma$ in supernatants was obtained. In response to *R. equi* at a maximum dose of 2×10^6 cells/ml, $\text{IFN-}\gamma$ was still undetectable in culture supernatants from two AIDS patients.

With regard to $\text{TNF-}\alpha$ production, detectable levels of this cytokine were found in all culture supernatants from AIDS patients. However, healthy donors produced three- to fourfold more $\text{TNF-}\alpha$, on average, in response to *R. equi* than did the AIDS patients (mean $\pm$ S.E.M., 144 ± 31 and 39.7 ± 15.2 , respectively; $p < 0.05$) (Fig. 1).

FIG. 1. Concentrations of $\text{IFN-}\gamma$ and $\text{TNF-}\alpha$ in the supernatants of cell cultures from AIDS patients and healthy controls after stimulation with killed *R. equi* (2×10^6 cells/ml). Vertical bars represent the mean values $\pm$ S.E.M.

FIG. 2. Dose responses of IFN- γ secretion by PBMCs from the six healthy donors after stimulation with different concentrations of killed *R. equi*. Each symbol corresponds to a different individual.

Moreover, no dose-dependent effect on TNF- α release was observed when PBMCs were incubated with different concentrations of bacteria (Fig. 3).

Discussion

R. equi infections in humans are related to the impairment of cell-mediated immune mechanisms involving the T-cell-macrophage system. IFN- γ and TNF- α are two cytokines which have been reported to be involved in human cell-mediated immunity to infection with intracellular pathogens.^{6,7} The results of the present study indicate that PBMCs from healthy human donors that were stimulated *in vitro* with killed *R. equi* secreted high levels of IFN- γ . Moreover, IFN- γ release was dose-dependent with respect to the concentration of the bacterial suspension in the culture medium. In contrast, the production of IFN- γ was strongly impaired in supernatants of *R. equi*-stimulated cultures from AIDS patients. These findings are

FIG. 3. TNF- α production by PBMCs from the six healthy donors after stimulation with different concentrations of killed *R. equi*. Each symbol corresponds to a different individual.

consistent with previous investigations which have demonstrated that the mononuclear cells from patients with AIDS are defective in their ability to release IFN- γ in response to various microbial antigens.⁸ Since IFN- γ plays an essential role in the activation of macrophages contributing to enhancing their activity against microbial challenge, the defect in IFN- γ release represents a critical immunologic alteration that predisposes AIDS patients to infections with opportunistic intracellular pathogens. In such patients, the failure of IFN- γ generation reflects the global impairment of T-cell function. On the other hand, in HIV-seronegative patients with certain infectious diseases, such as leprosy, leishmaniasis, tuberculosis and filariasis, the *in vitro* defect in antigen-induced IFN- γ production is not generalized but is restricted to the infecting pathogen alone and probably renders these subjects deficient in their individual T-cell responsiveness to their own progressive intracellular infection.⁹ To date, there is no evidence that in human rhodococcal disease the impairment of *in vitro* *R. equi*-induced IFN- γ production is antigen-specific and may be limited to the inability of T-cells to respond to *R. equi* alone.

Our *in vitro* experiments demonstrated also that TNF- α , a primarily macrophage-secreted cytokine, seems to be involved in the immunologic response to *R. equi* infection. Indeed, PBMCs from healthy donors produced elevated levels of TNF- α when challenged *in vitro* with *R. equi*. These findings are in agreement with the results of Nordmann *et al.*⁴ who have demonstrated that, in a murine model, splenic cells of uninfected euthymic mice produced, *in vitro*, greater amounts of TNF- α when incubated with *R. equi*.

On the other hand, PBMCs from AIDS patients released very little TNF- α in the supernatants in response to *R. equi* infection *in vitro*. The production of TNF- α in AIDS patients has been widely investigated with different results. Amman *et al.*¹⁰ reported that PBMCs from AIDS or AIDS related complex patients were deficient in their inability to produce TNF- α in comparison to normal controls. In contrast, Molina *et al.*¹¹ have demonstrated that, after stimulation with endotoxin, HIV-infected mononuclear cells produce amounts of TNF- α comparable to that of uninfected cells. Similar results were reported by Chehimi and co-workers¹² who showed that when PBMCs from HIV-infected individuals and uninfected control donors are stimulated *in vitro*, they produce equivalent levels of TNF- α .

In the present study, the TNF- α released by

R. equi-infected PBMCs from AIDS patients was significantly less than that seen in normal controls, suggesting that *in vitro* infection with *R. equi* may affect the production of this cytokine in mononuclear cells harvested from patients with AIDS. As reported for disseminated *Mycobacterium avium-intracellulare* complex disease, it is conceivable that *R. equi* infection in AIDS patients may contribute to the aggravation of HIV-induced macrophage dysfunction which leads to the impairment of TNF- α release.¹³

On the other hand, it cannot be excluded that the reduced secretion of TNF- α may depend on differences in the virulence of *R. equi*. In our experiments, the production of TNF- α by *R. equi*-infected mononuclear cells was evaluated after stimulation with the strain ATCC 33701, a *R. equi* strain which was previously demonstrated to be virulent *in vivo* in both mice and horses.¹⁴ Moreover, it has been reported that this virulent strain had the capacity to survive and replicate within macrophages *in vitro*, whereas avirulent strains were unable to efficiently replicate within macrophages.¹⁵ In this respect, the intracellular growth of virulent *R. equi* may decrease the viability of HIV-infected macrophages and cause them to become unable to produce TNF- α *in vitro*. Further studies will be done to assess the effect of TNF- α in rhodococcal growth in macrophages from AIDS patients with and without *R. equi* infection.

References

- Drancourt M, Bonnet E, Gallais H, Peloux Y, Raoult D. *Rhodococcus equi* infection in patients with AIDS. *J Infection* 1992; **24**: 123–131.
- Prescott JF. *Rhodococcus equi*: an animal and human pathogen. *Clin Microbiol Rev* 1991; **4**: 20–34.
- Nordmann P, Ronco E, Nauciel C. Role of T-lymphocyte subsets in *Rhodococcus equi* infection. *Infect Immun* 1992; **60**: 2748–2752.
- Nordmann P, Ronco E, Guenounou M. Involvement of interferon- γ and tumour necrosis factor- α in host defense against *Rhodococcus equi*. *J Infect Dis* 1993; **167**: 1456–1459.
- Boyum A. Ficoll-Hypaque method for separating mononuclear cells from human blood. *Clin Lab Invest* 1968; **21**(suppl 97): 77–89.
- Murray EW. Gamma-interferon, cytokine-induced macrophage activation, and antimicrobial host defense: *in vitro*, in animal models, and in humans. *Diagn Microbiol Infect Dis* 1990; **13**: 411–421.
- Murray HW. Interferon-gamma and host antimicrobial defense: current and future clinical applications. *Am J Med* 1994; **97**: 459–467.
- Murray EW, Rubin BY, Masur H, Roberts RB. Impaired production of lymphokines and immune (gamma) interferon in the acquired immunodeficiency syndrome. *N Engl J Med* 1984; **310**: 883–889.
- Murray EW. Interferon-gamma, the activated macrophage, and host defense against microbial challenge. *Ann Intern Med* 1988; **108**: 595–608.
- Amman AJ, Palladino MA, Volberding P, Abram D, Martin NL, Connant M. Tumor necrosis factor α and β in acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. *J Clin Immunol* 1987; **7**: 481–485.
- Molina JM, Schindler R, Ferrari R, et al. Production of cytokines by peripheral blood monocytes/macrophages infected with human immunodeficiency virus type 1 (HIV-1). *J Infect Dis* 1990; **161**: 888–893.
- Chehimi BJ, Starr SE, Frank I, et al. Impaired interleukin 12 production in human immunodeficiency virus-infected patients. *J Exp Med* 1994; **179**: 1361–1366.
- Sathe SS, Sarai A, Tsigler D, Nedunchezian D. Pentoxifylline aggravates impairment in tumor necrosis factor- α secretion and increases mycobacterial load in macrophages from AIDS patients with disseminated *Mycobacterium avium-intracellulare* complex infection. *J Infect Dis* 1994; **170**: 484–487.
- Takai S, Sekizaki T, Ozawa T, Suguwara T, Watanabe Y, Tsubaki S. Association between a large plasmid and 15- to 17-kilodalton antigens in virulent *Rhodococcus equi*. *Infect Immun* 1991; **59**: 4056–4060.
- Hondalus MY, Mosser DM. Survival and replication of *Rhodococcus equi* in macrophages. *Infect Immun* 1994; **62**: 4167–4175.

Received 5 April 1995;

accepted in revised form 22 May 1995