Immunological observations in patients with active Mycobacterium tuberculosis disease. The signifcance of the negative tuberculin skin test.

Ph.D.Thesis

Zoltán Balikó, M.D.

Institute of Respiratory Medicine, Pécs,
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Tutor: Julia Szekeres-Bartho

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List of abbreviations

CMI:	**cell-mediated immune**
DTH :	**Delayed type hypersensitivity**
hsp:	**heat shock protein**
IFN:	**Interferon**
IL :	**Interleukin**
MDR:	**multi-drug resistant**
NK:	**Natural Killer**
PBL:	**Peripheral blood lymphocytes**
TCR:	**T cell receptor**
TH:	**T helper**
TNF:	**Tumor necrosis factor**

PROLOGUE

Forty years after the introduction of tuberculosis chemotherapy the number of new infectious cases (8 million per year) is higher than ever. Mycobacterium tuberculosis (M. tuberculosis) kills 2.9 million people each year - more than any other infectious pathogen (WHO,1993, Houston, S. , Fanning, A. ,1994). It is estimated that approximately 90 million new cases of tuberculosis will occur worldwide between 1990 and 1999. In many developing countries, tuberculosis has emerged as the most common opportunistic disease associated with HIV infection (Raviglione, M. C. et al.,1995).

The incidence of both primary and acquired drug-resistant tuberculosis is becoming increasingly alarming in industrialized countries. (Primary resistance is defined as the presence of drug resistance to one or more anti-tuberculosis drugs in a tuberculosis patient who had never received prior treatment. Acquired resistance is defined as resistance to one or more anti-tuberculosis drugs which occurs during the course of the treatment, usually as a result of non-adherence to the recommended regimen or incorrect prescribing).

Because of the increasing incidence of multi-drug resistant M. tuberculosis infections, an essential line of defense must be the development of new, effective anti-tuberculosis **drugs**. It has been more than a quarter of century since the last major first-line drug - rifampin - was developed. Resistance to fluoroquinolones, an initially effective group of second-line drugs, has over the past five years been witnessed (Bloch, A. B. et al.,1996).

A well-functioning cell-mediated **immunity** is needed for protection to tuberculosis, yet this may also be responsible for much of the tissue damage. Though immune protection has for long been the focus of study, there still remain fundamental questions about the nature of immune responses recquired for protectiom, the *antigenic* determinants involved, and the *extent* to which

tissue damage is the price we pay for protection (Bloom, B.R. and Murray, Ch. J. L. , 1992). Tuberculous infection and disease are still not well understood; our methods of investigations, such as the traditional skin test, are clumsy and approximate despite the enormous care and rigour observed in their application and reading (Chretien, J.,1995).

Recovery from active disease, that is, the elimination of disease symptoms and clearance of visible mycobacteria from sputa and other tissue samples, is dependent on effective antibiotic therapy acting **in concert** with an adequate cell-mediated immune (CMI) response. In some individuals, even if the organism is sensitive to the usual antituberculotic drugs, the CMI response may be suboptimal, so that the infection cannot be eliminated by the usual antibiotic therapy alone, resulting in chronic active tuberculosis. In individuals infected with multi-drug resistant (MDR) strains of M. tuberculosis, available antibiotic therapy is bacteriostatic at best, and even an adequate CMI response fails to contain the infection, so that progressive, often fulminant, disease may ensue. In this context it is understandable, that besides producing more effective antibiotics, evaluation of the immunologic events during M. tuberculosis infection may be essential for the development of additional therapeutical approaches to combat this potentially life-threatening disease (Kaplan, G., Freedmann V. H. , 1996). As mechanisms of effective CMI in mycobacterial diseases become clear, we should not only be able to evaluate the cause or causes of persistent mycobacterial disease in patients but also, potentially, to develop immunomodulation to boost CMI. Once mycobacterial antigens are characterized, effective vaccines may be developed (Edwards, D., Kirkpatrick, Ch. H. ,1986).

INTRODUCTION

1.Importance of cellular immunity in cases with active Mycobacterium tuberculosis infection

Following exposure to M. tuberculosis, some individuals (about 10%) develop active disease, while others do not. It is not clear what factors determine resistance to this infection.

Infected host cells usually present microbial antigens on their surface in association with host structures encoded by the major histocompatibility (MHC) gene complex; this feature allows the identification of infected cells by T lymphocytes rather than by B lymphocytes or antibodies. Upon antigenspecifc interaction with infected host cells, T lymphocytes are activated to become the central mediators and coordinators of immunity to intracellular microbes. Both protection from and the pathogenesis of intracellular bacteria are therefore intimately conmected with T lymphocytes (Kaufimmann, S. H. E., 1988).

During the infection activated specific T cells induce local accumulation of mononuclear phagocytes that are responsible both for restricting mycobacterial growth inside a granuloma and for establishing a hypersensitive reaction recognizable by tuberculin testing. Mononuclear phagocytes act not only as habitat but also as effectors of protection (Kaufimann, S. H. E., Flesch, E. A., I988). As intracellular pathogens, mycobacteria can replicate within those phagocytic cells that constitute the major effector arm of the cellular immune system.The major task of the mononuclear phagocytes is phagocytosis, the killing and degradation of microorganisms.

Protection against tuberculosis depends on immune T cells of the CD 4 and CD 8 phenotype. Probably the former ones activate macrophages, thus restoring an

effective immune status against the microorganism (Munk, M. E., Kaufimann, S. H. E.,1991).

In several cases, primary infection with M. tuberculosis bacilli, though effectively controlled by T-cell mediated immune responses, is not totally eradicated, and reactivation of endogenous infection or superinfection may lead to manifestation of tuberculosis several years after the primary infection occurred.

2.T cell subsets in the defence mechanisms of Mycobacterium tuberculosis infection

A major function of the immune system is to maintain the host free of infective agents. In order to combat an infection, specific T-cells constantly recognize and respond to several microbial components.

Peripheral T-lymphocytes express on their surface a T-cell receptor (TCR) noncovalently linked to the CD3 complex, which is responsible for the transduction of the antigenic signal to the cytoplasm. The TCR is a disulphidelinked heterodimer, either composed of an α and a β chain, or of a γ and a δ chain. The α/β T-cells expressing the CD4 surface marker recognize small fragments of phagocytosed protein that are processed and presented together with MHC class II molecules. The α/β TCR CD8+ cells recognize peptides derived from proteins of intracellular origin. These peptides are thought to be transported to the endoplasmic reticulum and subsequently bound to and presented by MHC class I molecules. Functionally, CD4+ T-cells (T-helper cells, Th) are involved mainly in the amplification of the immune response through the secretion of several lymphokines, while some CD4+ and the majority of CD8+ T cells (cytotoxic T lymphocytes (CTL)) are capable of directly lysing target cells.

In the mmouse, CD4+ T cells were shown to be subdivided into two more specialized populations, depending upon their cytokine production pattern: CD4+ Thl cells produce interleukin-2 (IL-2) and interferon γ (IFN γ), which support their function as regulatory and effector cells in the cellular- immune response; CD4+ Th2 cells produce interleukins-4, 5, 6, and 10 (IL-4, IL-5, IL-6, and IL-10), and provide help to B cells in the production of different immunoglobulins.

Human lymphocytes that express the T3 glycoprotein but not the T-cell receptor (TCR) α and β subunits have been identified, with the novel T3 associated polypeptides expressed by these lymphocytes designated as a third (Tγ) and a fourth (Tδ) TCR subunit (Brenner, M. B. et al.,1986). The strategic locations of dendritic epidermal cells and intestinal intraepithelial lymphocytes have led to the suggestion that γ/δ T lymphocytes could constitute a first line of defence near large surfaces in contact with the environment. γ/δ T lymphocytes are a minor population among peripheral blood T cells of normal individuals (Gatrill, A. J. et al.,1990). About 95% of peripheral blood T cells of patients with M. tuberculosis proved to be α/β^+ and 5 % of them γ/β^+ (Barnes, P. F. et al. ,1992). Several questions concerning γδ T cells are still unsolved, e.g. the γ/δ T cell MHC restriction. Although the majority of the γ/δ TCR cells do not express the CD4 or CD8 markers, these cells have been shown to recognize antigen in the context of MHC class I or class II. However, antigens for γ/δ cells are poorly defined. Like α/β TCR cells, the γ/δ TCR cells are able to produce lymphokines and induce the lysis of target cells.

Acquired cellular immunity to tuberculosis infection in experimental mouse models has been shown in classical experiments to involve the interaction of specifically sensitized mediator T lymphocytes and mononuclear phagocytes as effector cells (Orme, I. M. et al.,1992).

T-cells, of all subsets, play a dominant role both in protective and in pathogenic host responses to mycobacterial infections. Since γlδ cells have only recently been described, most of the studies on acquisition of resistance against mycobacterial infection are based on previously described α/β TCR cell functions (Munk, M. E., Emoto, M.,1995).

T cells with specificity for mycobacterial antigens have been isolated both from patients and from healthy individuals without clinical signs of disease. These CD4+ T cells, similar to the experimental mouse model, produce IL-2 and IFN_{γ} upon appropriate stimulation and express cytolytic activity. These biological features indicate that mycobacteria-reactive CD4 T cells belong to the Thl set. In contrast to the murine system, CD8 T cells have been isolated only occasionally, and it appears that both cytolytic effector function and interleukin secretion primarily rest in the CD4 T cell population (Kaufmanm, S.H.E. et al.,1992).

Activated T cells have been classically described as controlling mycobacterial infection through two mechanisms: by leasing intracellularly infected macrophages leading to the delivery of bacilli consequently digested
by freshly accumulated, and therefore more active, mononuclear macrophages from the bone marrow, and by secreting cytokines that augment macrophage killing mechanisms.

The γ/δ T cell subset appears to be roughly ten times as active as the α/β T cell subset in the primary response to M. tuberculosis (Janis, E. M. et al., 1989). Mamy studies have reported that cell-wall-associated antigens of mycobacteria can specifically activate T cells expressing γ/δ T cell-receptors (Wang, M. H. et al.,1993).

Both CD4+ and γ/δ T cells from healthy tuberculin-positive donors can respond to intact M. tuberculosis and its cytosolic antigens. However, the range of cytosolic antigens recognized by the two types of lymphocytes are not the same. Despite differences in the antigens recognized, both T cell subsets have similar effector functions in response to M. Tuberculosis-infected macrophages (Tsukaguchi, K. et al.,1995).

Recent data suggest, that particularly in situations in which bacterial loads may be very high, γ/δ T cells play a protective role. In these situations, triggering of

γ/δ T cells by mycobacterial antigens during the early and progressive stages of the infection may provide an early warning mechanism which, though not directly anti-mycobacterial itself, may serve a dual role by helping to create the necessary local cytokine/chemokine signal conditions necessary to promote a protective lymphocytic/monocytic granulomatous response (D'Souza, C. D. et al. ,1997).

3.The role of cytokines in human tuberculosis infection

The critical role of IFNy as a macrophage activating factor in mycobacterial diseases can be clearly demonstrated in murine models of tuberculosis. IFNγ inhibits intracellular growth of M. tuberculosis in murine macrophages.

However, irm human studies, IFNγ fails to inhibit mycobacterial replication consistently in monocytes or monocyte-derived macrophages unless combined with other cytokines or monocyte products.

The role of cytokines in control of intracellular mycobacterial growth thus appears to be complex, with T cell products capable of both macrophage activation and deactivation. In some respects, this parallels the characterization of Thl and Th2 subsets of CD4+ lymphocytes in mice, which are defined on the basis of phenotype and cytokine products. Thl responses are characterized by production of IL-2 and IFNγ, whereas Th2 responses lead to production of IL-4, IL-5, IL-6, and IL-10. Th2 responses may be described as crossregulatory, in that they promote antibody production and inhibit DTH (Wallis, R. S. , Ellner, J. J.,1994).

The overall host acquired immune response to tuberculosis infection is characterized by an early Thl response, in which secreted proteins of the bacilli are the targets, followed a few week later by a Th2 response (Orme, I. M. et al., 1993). The secreted cytokines of Thl and Th2 cell types can mutually regulate and inhibit each other's function. Therefore, a fine balance between the secreted cytokines is crucial for the resulting nature of host resistance against the pathogen.

The cytotoxic effector cells are differentiated and activated in response to IL-2 amd IL-12. IL-2 is a central regulator of the Thl-type T-cell response and stimulates leukocyte proliferation and differentiation (Kaplan, G., Freedman,V. H. ,1996).

IL-2 and IFNγ are produced in response to antigen activation of T-cell subsets including CD4 T cells, CD8+ T cells, CD4-CD8-CD-1 restricted α/β TCR+ cells, γδTCR cells, lymphokine activated killer (LAK) cells and, probably, natural killer (NK) cells.

IL-12, a product of macrophages and B cells, acts indirectly by inducing IFNγ production by CD4+ T cells and NK cells.

Defective proliferative responsiveness and deficient IL-2 and IFNγ have previously been reported in patients with severe tuberculosis (Toossi, Z. et al., 1986).

IL-4 together with other cytokines may act as an influential down-regulator of Thl-type responsiveness in infections (Sourcel, H. M. et al., 1994). Other cytokines, like IL-1 and transforming growth factor (TGF-β), may act in a way similar to IL-4 and may enhance its effect (Mustafa, A. S., 1996). Besides these, interleukin-10, interleukin-6 (Van Heyningen, T. K. et al., 1997), and, probably, natural killer (NK) cells (Benmudez, L. E., Champsi, J., 1993) can suppress Thl cell responses.

Infection with M. tuberculosis can result in a down regulation of cellular immune response in humans. Clarification of the mechanisms resulting in an inappropriate response would be useful in identifying ways both to prevent and to treat its occurrence (Cooper, A. M. , Flymn, J. I. ,1995).

4.Values and limitations of the tuberculin skin test (Paper 1)

The tuberculin skin test is the only in vivo immunologic reaction which is used for diagnostic purpose throughout the world. After a person becomes infected with mycobacteria, T lymphocytes proliferate and become sensitized. Within weeks these sensitized T cells appear in the bloodstream. The injection of tuberculin into the skin stimulates the lymphocytes and activates the mechanisms leading to a delayed type hypersensitivity response (DTH). The previously sensitized T lymphocytes are stimulated, proliferate, and produce lymphokines, which induce the accumulation of other cells at the site of the tuberculin injection (Huebner, R. R. et al.,1993; Platt, J. L. et al.,1983; Lefford,M.J.,1975). The T lymphocytes which take part in the DTH reaction as effector cells (Pithie, A. D. et al.,1992) have been shown to be THl type lymphocytes (Tsicopoulos, A. et al.,1992).

Besides adhesion molecules, interleukin 8, as a potent T lymphocytic chemotactic factor, plays an important role in recruiting lymphocytes, monocytes, and neutrophil granulocytes (Patarroyo, M., 1994; Larsen, C. G. et al.,1995; Friedland, J. S. et al.,1992). The sensitisation after the infection must last between two and ten weeks to produce a positive tuberculin reaction. This sensitisation may persist for years, although reactivity can lessen with increasing age (Huebner, R.E. et al.,1993).

To evaluate the positivity of the skin reaction, the size of the induration is crucial. In the case of HIV infection, a reaction of 3 mm should be seen as positive, with preventive therapy started. In instances of silicosis, in populations with a high rate of tuberculosis infection, or among health workers who are in contact with patients with M. tuberculosis or who work with contaminated materials in laboratories, the lowest measure of positive reaction should be

defined as 10 mm. In communities where tuberculosis infection is rare, the lowest positive skin reaction should be defined as 15 mm. It is known that in populations where the rate of infection with M. tuberculosis is less than 10%, the tuberculin skin test has a very low predictive value, and in populations with a high rate of infection (25 - 50 %), the diagnostic value of the tuberculin skin test is high (Huebner, R. E. et al. ,1993 ; Pesanti, E. L. ,1994).

It has often been asserted that there is no association between the DTH reaction and protective immunity, they are distinct and separate events. We now know that protective immunity can develop in the case of a negative tuberculin skin test after BCG vaccination (Huebner, R. E. et al.,1993); conversely, rapidly spreading tuberculosis can evolve in the presence of positive tuberculin skin reaction. Consequently, a positive tuberculin skin test administered after BCG vaccination indicates only the success of the vaccination; it cannot measure the degree of immunologic protection (Fine, P. E. M. et al. I 994).

The discrepancy between the strengths of the DTH reaction and of the protective immunity can be explained in that different T lymphocyte populations are responsible for the skin test reaction and for the immunoprotection. It may also be that protective immunity is induced by specific antigens secreted by M. tuberculosis (Platt, J. L. et al.,1983; Orme, I. M.,1988).

Possible ways to differentiate between reactions to infection with M. tuberculosis and reactions to BCG vaccination include larger induration size; known contact with cases of M. tuberculosis; high level of infection with M. tuberculosis in the population; and a time interval of more than 10 years after BCG vaccination. Neither the absence of induration nor a minimal reaction to

the tuberculin skin test completely excludes ongoing infection or disease with M. tuberculosis.

The tuberculin reaction can also be false positive as a result of nonspecific immunologic events such as infection with other types of Mycobacterium.

The causes of false negative reactions include current or recent viral (especially measles), bacterial, or fungal infections; metabolic disturbances (e.g., uraemia); malnutrition; lymphoid organ disease (e.g., Hodgkin's, non-Hodgkin's diseases, CLL); M. Boeck; treatment with corticosteroids or with immunosuppressants; age (e.g., newborn, elderly) ; stress (e.g., surgery, burns); or advanced M. tuberculosis disease.' Technical causes of negative reactions can be inappropriate storing of the test material, incorrect administration or dosage of the test material, or an inexperienced reader (Huebner, R. E. et al. ,1993).

More than 20 % of cases with progressive pulmonary tuberculosis produce negative tuberculin reaction (Pithie, A. D. et al., 1992), though there are publications citing lower percentages of tuberculin anergy (Friedland, J. S. et al., 1992). Anergy occurs when despite known hypersensitivity to a specific antigen, the individual cannot produce a positive reaction to a skin test of the same antigen, e.g. the reaction remains negative despite repeating the test with 250 TU (the erythema developing during the reaction is the consequence of vasodilatation and in itself cannot be regarded as positive reaction). Using this rigourous criterion, the number of cases with true tuberculin anergy is much less, only about 8 -10 % (McMurray, D. N., Echeverri, A.,1978).

In the case of tuberculin anergy it is worth checking the cellular immunity of the patient with the so-called anergy panel, involving, among others, the candida antigen. If these tests are also negative, then immunodeficiency must be considered, in particular, an HIV infection. In the latter case, induration of more than 3 mm supports the fiding of an ongoing M. tuberculosis infection (Pesanti,

E. L. ,1994). It was noted previously that in cases with ongoing infection showing tuberculin anergy and negative reaction to the anergy panel antigens and DNCB, the in vitro lymphocyte tests using PPD as specific stimulation for lymphoblastic reaction are also negative. These fidings had no negative impact on the follow up; the success rate of therapy in these patients did not differ from patients with positive tuberculin reaction, and in most cases the negative results, including the in vitro tests, converted positive during the first weeks of treatment. An increase in the level of immunoglobulins in the peripheral blood paralleling the tuberculin anergy has also been observed, representing the altered function of the T and B lymphocytes (McMurray, D.N., Echeverri, A.,1978).

A negative tuberculin skin test in cases of M. tuberculosis calls attention to the patient's possible immuno-compromised state, and the consequent need for further consideration.

IV. Aims and results of the present study

Since the tuberculin skin test is a simple and widely used tool in the hands of the clinician, we were interested if it could provide additional information to that normally expected from it.

1. We planned to examine the rate of the Tγ/δ lymphocytes of the peripheral blood of patients with active Mycobacterium tuberculosis. Because the exact role of these cells in tuberculosis is not precisely defined, we chose to evaluate their role in the disease in correlation with the tuberculin reactivity of the patients. It was presumed that the intensity of the tuberculin reactivity might provide additional information about the significance of Tγδ lymphocytes.

2. It is known that during Mycobacterium tuberculosis infections, interferon gamma and interleukin 2 play important roles in the immunologic events. Therefore, we intended to look for specific cytokine patterns that might characterize the different manifestations of the disease.

1. Tγ/δ lymphocytes in the peripheral blood of patients with active Mycobacterium tuberculosis in context with tuberculin reaction (Paper 2)

The function of γδ cells is still elusive. During the late 1980s it became clear that γδ T cells can recognize mycobacterial antigens (Holoshitz et al., 1992; Pfeffer, K. et al., 1991). In PPD preparations, a major antigenic component was derived from hsp65, a heat shock protein with ability to stimulate γ/δ hybridomas (O'Brien, R. L. et al., 1989). Stimulation of peripheral blood mononuclear cells with M. tuberculosis leads to the selective expansion of Vγ9/Vδ2 cells representing a fraction of human γ/δ cells (De Libero, G. et al.,

1991; Bender, A. et al. ,1993), whereas the Vδ2+ T cell subset of γδ T cells is increased in peripheral blood of tuberculotic patients (Balbi, B. et al. ,1993). In vitro studies show that purified peripheral blood γδ T cells proliferate vigorously in response to killed M. tuberculosis. The proliferatiom required Th1 type CD4+ T cells and was strongly inhibited by IL-10 (Pechhold, K. et al. , 1994).

The nature of antigens recognized by γδ T cells remains unclear. In mice, γδ T cells derived from lung and newborn thymus respond to tuberculin and to hsp65-derived peptides. In humans, though some mycobacterium reactive γδ T lymphocytes recognize hsp65, others appear to have different, unidentified ligands. There are findings which support the hypothesis that some γδ T cells recognize nonpeptidic ligands (Constant, P. et al., 1994). Boom et al. characterized a 10- to 14-kDa, cell associated, heat stable antigen of M. tuberculosis as acting as a major stimulus for human γδ T cells (Boom, W. H. et al. , 1994). It has been recently shown that human Vγ9 Vδ2 T cells are activated by nonpeptidic phosphorylated molecules of mycobacterium origin; these cells are cytotoxic and produce TNF (Lang, F. et al.,1995). There is also evidence that a small subset of the memory helper T-cell population is exclusively responsible for the peripheral expansion of these Vγ9/Vδ2 cells (Vila, L. M. et al.,1995).

Clinical observations evaluating the role of Tγ/δ lymphocytes in M. tuberculosis infection show contradictory results.

It was found that T-cells involved in immune granulomatous reactions in tuberculosis express predominantly αβ TCR; this finding does not support the concept that γδ T-lymphocytes play an important role in granuloma formation (Tazi, A. et al., 1991).

Another study clearly demonstrated an increased proportion of γδ T cells in peripheral blood of patients with pulmonary tuberculosis, though among the 20 tuberculotic patients only 11 were smear positive and 2 were culture positive for M. tuberculosis (Ito, M. et al., 1992). Based on data of only two anergic patients Tazi, A. et al. (1992) claimed that tuberculous infection is not associated with an increase in the number and in the state of activation of circulating γδ cells in humans. They argue that the immune reaction in pulmonary tuberculosis represents a secondary response, and evaluation of the initial processes in the immune response in this disease is essentially impossible.

Concerning the role of γδ T lymphocytes residing in the epithelial lining as relevant in the context of the infective route of Mycobacterium tuberculosis, it should be mentioned that after stimulation by M. tuberculosis, the alveolar macrophages favored γδ cell activation (Balaji, K. N. et al., 1995). However, γδ T cells depleted Mycobacterium bovis infected mice did not show any exaggerated bacterial multiplication compared with control mice (Nabeshima, S. et al., 1995). There are data showing that the percentage of γδ T cells in fresh peripheral blood obtained from tuberculin skin test positive health care workers who had been in constant contact with tuberculotic patients was significantly higher than that in controls. The same study showed that patients with active pulmonary tuberculosis had as low levels of γδ T cells as healthy controls who had no contacts with such patients. (Ueta, Ch. et al.,1994). This unique set of data demonstrates that upon primary infection by virulent M. tuberculosis, the establishment of protective immunity involves an episode of the γδ T-cell response.

Taken together, presently available data support the view that human γδ T-cell mediated responses probably contribute to the establishment of antituberculosis protective immunity (Poquet, Y. et al. ,1997).

To investigate the reason for the contradictory data on the percentage of γδ T cells in peripheral blood of patients with active M. tuberculosis, we conducted a clinical study on patients during the early course of their disease. As we described in paper 2, we found no difference in the rate of γδ T cells in peripheral blood lymphocytes of patients with active tuberculosis and of healthy controls. However, when data were evaluated in the context of tuberculin reactivity, the differences appeared as highly significant. Tuberculin anergic patients had a significantly higher rate of γ/δ positive cells than tuberculin positive patients and healthy volunteers ($p < 0.001$). This implies that tuberculin negative patients with active M. tuberculosis should be regarded as a separate group of patients characterized by specific immune alterations.

2. Significance of the heat shock proteins in effective immune response to *Mycobacterium tuberculosis* antigens (Paper 3).

Most publications suggest that heat shock proteins might be candidate antigens for M. tuberculosis reactive lymphocytes. Heat shock proteins are highly conserved groups of proteins found in all organs. Increased synthesis of these proteins occurs in response to stress, including temperature changes, inflammation and fever, irradiation, viral infection, malignant transformation, exposure to oxydizing agents, heavy metal ions, ethanol, and anoxia (Born, W. et al., 1990). Reports indicate that immune responses against stress proteins can be highly crossreactive and can even involve antiself reactivity.

Phagocytosis of mycobacteria by monocytes is followed by presentation of the mycobacterial 65-kD heat shock protein (hsp) on the monocyte surface in association with MHC class II molecules. Mycobacterial 65 kD hsp is recognized by αβ T cells and γδ T cells (Haregewoin, A. et al.,1989; Ab, B. K. et al.,1990; Friedland, J. S. et al.,1993; Mustafa, A. S. et al.,1993).

Rheumatoid arthritis shares the autoimmune antigen with M. tuberculosis. Four or five T cell clones isolated from human rheumatoid synovia that respond to M. tuberculosis antigens were shown to be CD4-CD8- cells with γδ T cell receptors (Young, R. A., Elliott, T. J.,1989; Billingham, M. E. J. et al., 1990; Van den Broek, M. F. et al.,1989).

These observations suggest a model of immune surveillance in which self reactive T cells provide a first line of defence against infection and transformation by recognizing and helping to eliminate both stressed autologous cells and cells infected with intracellular bacteria (Young, R. A., Elliott, T. J.,1989). This idea that γδ T cells directed against autologous hsps

can detect and subsequently eliminate host cells stressed by a variety of insults seems rather attractive (Kaufmann, S. H. E.,1990).

Though it has been proven that the recombinant 65-kD heat shock protein of Mycobacterium bovis/M. tuberculosis is a target molecule for CD4+ cytotoxic T lymphocytes that lyse human monocytes (Ottenhoff, T. H. M. et al.,1988), now it seems that specifc antigens for reliable skin test reaction, as well as antigens useful in generating more effective vaccines than BCG, may be produced by a mixture of low molecular peptides secreted by M. tuberculosis bacilli (Friscia, G. et al.,1995; Vordermeier, H. M.,1995; Jurcevic, S. et al., 1996).

3. Thl/Th2 type cytokine responses in relation to tuberculin reactivity (Papers 4 and 5)

An effective immune response to intracellular parasites is always of a Thl type. Shifting the response towards Th2 is inadequate and not protective. Mycobacteria preferentially induce Thl responses as reflected by the production of high titers of IFNγ and TNFα and low levels of IL-4. It is known that tuberculin anergy accompanies advanced disease. We conducted a study to evaluate the possible role of Thl/ Th2 type cytokine pattern in the generation of anergic tuberculin reaction and progressive disease.

We examined the ratio of IL-4, IL-10, IL-12 in CD4, and CD8 positive peripheral lymphocytes in patients with active M. tuberculosis (Paper4 and 5). Our results showed no significant difference between patients and healthy volunteers. However, after classifying the patients according to their tuberculin skin test reaction, we obtained significant differences between reactive and anergic patients. The ratio of the IL-4 and IL-10 positive lymphocytes in peripheral blood of patients with tuberculin anergy was signifcantly higher

($p<0.01$) than that of patients with tuberculin positivity or of healthy volunteers, whereas the ratio of IL 12 positive lymphocytes in peripheral blood of patients with tuberculin anergy was significantly lower ($p<0.01$) than of patients with tuberculin positivity or of healthy volunteers. Assuming that cases with tuberculin anergy generally show an advanced pulmonary involvement, we analysed our data in view of the extent and type of pulmonary disease. Patients were classified according to the extent and type of X-ray finding into three groups following the classification of Dlugovitzky et al (1977). Ninty percent of tuberculin negative patients were classifed as grade III. whereas in the tuberculin positive group only thirty percent fell in this category. Evaluating the ratio of IL-4, IL-10, IL-12 positive lymphocytes of peripheral blood we found that there was no significant correlation between the radiological grade of the patients and the examined cytokine expression unless tuberculin reactivity was also considered. Based on these findings, we suggest that the cytokine pattern correlates with the degree of the tuberculin reactivity and consequently with the pulmonary manifestation of the disease. Therefore it can not be ruled out that, icreased production of IL-4 and IL-1o together with decreased IL-12 production are involved in the development of tuberculin anergy and also partly in disease progression.

Paper 1

Medicina Thoracalis /közlésre elfogadva/

Baranya Megyei Kórház Tüdőgyógyintézete, Pécs

A tuberkulin reakció értéke és korlátai

BALIKÓ ZOLTÁN DR.

Összefoglalás

A tuberkulin bőrtesztnek a Mycobacterium tuberculosis infekció megállapitásában fontos szerepe van. A reakció értékelésénél egyéb szempontokat is mérlegelnünk kell, pl. az egyén foglalkozását, a környezeti tuberkulózis jelenlétét, a tuberkulózisra hajlamositó betegségek társulását, a Mycobacterium tuberkulosis infekció népesség szintű elterjedését. A teszt specificitásának növelése érdekében a Mycobacterium tuberculosisra specifikus peptidek keveréke és a Mycobacterium tuberculosis által szecernált antigének alkalmazása igéretes módszernek látszik.
Tuberkulin anergia esetén a betegség hátterében addig rejtett, egyéb immunhiányos állapot irányában további vizsgálatok végzése javasolt.

A tuberkulin bőrteszt az egyetlen in vivo immunológiai reakció, melyet széles körben alkalmaznak diagnosztikus célra. A tuberkulin reakció elhúzódó tipusú túlérzékenységi reakció (DTH = delayed type hypersensitivity antigén specifikus sejtmediálta immuno-inflammatorikus válasz). A Mycobacterium tuberculosis bacilussal történt találkozás után a kórokozó antigénjével kapcsolatba került T lymphocyták progrediálnak és heteken belül megjelennek a keringő vérben. Tuberkulin injekcióra a korábban érzékenyitett T lymphocyták stimulálódnak, proliferálnak, lymphokineket termelnek, melyek serkentik más sejtek helyi akkumulóládását [1, 2, 3].
A rekcióban résztvevő T lymphocyták, melyek a DTH-ban effektor sejtekként szerepelnek [4], immunhisztológiai vizsgálattal Th_1 lymphocytáknak bizonyultak [5].
A lymphocyták, monocyták és neutrophil leukocyták rekrutálásában adhézios molekulák mellett az IL-8-nak, mint potens T lymphocyta kemotaktikus faktornak is jelentős szerepe van [6, 7, 8].
Az infekciok következtében kialakuló érzékenyitődés 2-10 hét alatt ér el olyan mértéket, hogy a tuberkulin reakció pozitivvá válik. Ez az érzékenyitődés évekig perzisztál, idősebb korban azonban csökkenhet [1].

A tuberkulin bőrteszt kivitelezése

A vizsgálat során elölt baktériumok szürletének tisztitott kivonatából 5 TU (tuberkulin egység)-et adunk intracután 0,1 ml volumenben [1].

Kétféle módszer terjedt el világszerte:
1. Az Old Tuberculin vagy OT Koch eredeti módszeréhez hasonlóan, de attól eltérően, szintetikus médiummal készül. Egy nemzetközi egysége (IU) az a biológiai aktivitás, ami a nemzetközi standard 0,011111 mikrolitere, azaz a nemzetközi standard mililiterenként 90 000 IU-t tartalmaz.
2. A másik módszer a PPD, amit vagy ammoniumszulfáttal vagy triklórecetsavval vagy a kettő együttes felhasználásával, precipitációval állitanak elő. Standardja a PPD 49608 sorszámú készitmény, más néven a PPD-S. A PPD nemzetközi egysége az a biológiai aktivitás, ami 0,000028 mg PPD-S-ben van (a PPD-S összetétele:0,00002 mg PPD és 0,000008 mg só), a liofilizált ampulla 500 000 IU-t tartalmaz. Az összes készitményt ehhez a standardhoz mérik. Azért, hogy a hatóanyag ne tapadjon ki az üveghez, vagy a műanyaghoz, Tween 80 detergenst adagolnak hozzá [1].
A reakció kiértékelése a beadást követően 72 óra múlva történik. Az indurácio mértékének megállapitására két módszer terjedt el. Az egyik, amikor ujjbeggyel a reakció felszinét áttapintva állapitjuk meg az indurácio határait. A másik az un. ballpoint pen módszer, amikor gólyostoll szerü eszközzel haladva a szöveti reakció felszinén, az emelkedés kezdetét megjelöljük. Standardnak a tapintási módszert tekintik, de újabban többen ajánlják a pen módszert [9, 10].
Az indurácio mértéke az alkar hosszával párhuzamosan mért átmérő mm-ben.

A tuberkulin teszt értékelése

Számos vizsgálat megállapitotta, hogy a DTH és a protektiv immunitás között nincs összefüggés. Mig BCG-t követően negativ tuberkulin teszt dacára is kialakulhat védettség [1], addig pozitiv tuberkulin reakció mellett is felléphet disszeminált tuberkulózis. Igy a BCG eredményességét illetően a pozitiv tuberkulin reakció csupán azt jelenti, hogy a vakcináció sikeres volt, de az immunologiai védelem mértékét nem mutatja ki [11].
Van olyan megfigyelés is, hogy éppen hyperergiás tuberkulin reakció mellett lép fel később gyakrabban tuberkulózis. Azt is feltételezik, hogy hyperergiás tuberkulin teszt esetén a tüdő infekciójában a bőrben zajló folyamatokhoz hasonló módon dominál a hypoxia és hiányoznak az immunkompetens sejtek, ami sajtosodáshoz és üregképződéshez vezet [12].
A DTH mértéke és a protektiv immunitás közötti eltérés magyarázatául feltételezik, hogy két eltérő T lymphocyta csoport felelős a bőrreakcióért és az immunvédettségért. Az is lehet, hogy a protektiv immunitás kiváltásában inkább a Mycobacterium tuberculosis baktérium által szecernált antigéneknek van szerepük [2, 13].
Aki a közelmúltban szoros kapcsolatban volt tuberkulózisban szenvedő egyénnel és a mellkas röntgen felvételén látható eltérés felveti a tuberkulózis lehetőségét, annál a pozitiv tuberkulin bőrteszt valószinüsiti az infekció fennállását.

A pozitiv reakció kiértékeléséhez meg kell szabni az indurációmértékét. HIV infekcióban a tuberkulin reakció már 3 mm felett pozitivnak tekinthető, mely legtöbbször egyúttal a preventiv terápia megkezdését is jelenti. Szilikozisban, továbbá olyan közösségben élő egyénnél, ahol a tuberkulózis infekció elterjedt, tuberkulotikus betegeket gyógyító kórházak dolgozói esetében és a Mycobacterium tuberculosis kórokozóval dolgozó laboratoriumok alkalmazottjainál az induráció alsó határértékét 10 mm-ben célszerű megszabni. Azokban a közösségekben, ahol igen valószinütlen a tuberkulózis infekció, ott 15 mm feletti indurációt ajánlatos pozitivnak tekinteni. Ismeretes, hogy azokban a közösségekben, ahol az infekció gyakorisága ≤ 10%, a pozitiv tuberkulin reakció prediktiv értéke igen alacsony, ezzel szemben azokban a populációkban, ahol az infekció előfordulása 25-50%, ott a tuberkulin bőrteszt diagnosztikus értéke magas [1, 14]. Hazánkban *Böszörményi K.* végzett vizsgálatot a tuberkulin bőrteszt értékelésére, és kiemelte, hogy hazai körülményeink között még a hyperergiás tuberkulin reakció is az aktiv tuberkulózisban szenvedő betegekhez hasonló arányban fordult elő a kontroll csoportban [15].

Szenzitivitás és specificitás

A tuberkulin bőrteszt az egyetlen olyan módszer, ami kimutatja a Mycobacterium tuberculosissal történt inficiálódást, de sem az érzékenysége, sem a specificitása nem 100 százalékos. (Specificitás alatt azt értjük, hogy a betegség nélküli egyének hány százaléka ad negativ reakciót).
Már az 1930-as években rájöttek arra, hogy pozitiv lehet a tuberkulin teszt Mycobacterium tuberculosissal történt infekció nélkül is [1]. Az 5 mm alatti induráció magyarázható egyéb Mycobacteriummal történt fertőződéssel. A vizsgálat specificitása növelhető azzal, ha pozitivnak csak az 5 mm feletti reakciót tekintjük, mely ugyanakkor a szenzitivitás mérsékelt csökkenését eredményezi. Tovább növelhető a specificitás, ha olyan antigénnel végezzük a vizsgálatot, ami eltér az összes többi Mycobacterium tipustól. Ilyen antigén a Mycobacterium tuberculosis 16 kD protein, melyet a PPD-nél érzékenyebbnek tartanak [16]. Újabban olyan peptid keveréket alkalmaznak, melynek összetevői specifikusak Mycobacterium tuberculosisra, ilyen a 16 kD, 19 kD és a 38 kD protein. A több protein keverék alkalmazásának elméleti alapja az, hogy ezáltal több egyénnél kapunk pozitiv reakciót, azaz szélesebb az antigénekkel lefedhető T lymphocyta populáció. A sokféle peptid számos MHC molekulához kötődik, de egyik sem kötődik univerzálisan. Ezen kívül, ha csak egy peptidet alkalmazunk, a reakció mértéke is várhatóan sokkal kisebb lesz [17]. További lehetőség a specificitás növelésére a Mycobacterium tuberculosis baktérium által szecernált antigének alkalmazása, mint amilyen az MPT64 jelzésü 24 kD protein [18].

A tuberkulin teszttel tehát **fals pozitiv** választ kaphatunk akkor, ha egyéb Mycobacteriummal került kontaktusba az egyén vagy BCG vakcinációban részesült. A BCG hatásától elkülönithető az infekcióra adott reakció akkor, ha nagyobb az induráció mértéke; ha az egyén környezetében tuberkulózisban szenvedő beteg él; ha a populációban a tuberkulózis infekció gyakori és ha több, mint 10 év telt el a BCG vakcináció óta. Sem az induráció hiánya, sem a kismértékü induráció nem zárja ki azonban fennálló tuberkulózis betegség vagy infekció lehetőségét.

Fals negativ reakció okai lehetnek: virus (különösen kanyaró), baktérium, gomba infekció alatt és azt követően rövid ideig; metabolikus zavar (pld. uraemia); alultápláltság; lymphoid rendszer betegsége (Hodgkin, non Hodgkin lymphoma, CLL); sarcoidosis; gyógyszerek (corticosteroidok, immunszupresszív szerek) hatása; bizonyos életkor (újszülöttkor, idős kor), stressz (mütét, égés); előrehaladott Mycobacterium tuberculosis betegség. A negativ eredménynek technikai okai is lehetnek, úgymint a vizsgálati anyag rossz tárolása, rosszul és keveset beadott szer, a leolvasó gyakorlatlansága [1].

A "booster" jelenség

Ha a tuberkulin tesztet 1 hó-1 éven belül megismételjük, akkor, elsősorban idősebb egyénekben (>55 év) u.n. tuberkulin "konverziót" kaphatunk. Ennek az a magyarázata, hogy a korábbi Mycobacterium tuberculosis infekció vagy a BCG hatása az immun memóriában lecsökkent, az ismétlés viszont elégséges antigén inger ahhoz, hogy a reakció a vizsgálat megismétlésekor pozitivvá váljon.

Fontos tudni, hogy korábban nem fertőződött és BCG vakcinációban sem részesült egyénnél önmagában a tuberkulin reakció nem elégséges inger ahhoz, hogy ismételt alkalmazáskor pozitiv választ kapjunk.

Értékelés: ha az első 5 TU hatására a teszt negativ és 1-3 hét múlva megismételve is negativ, akkor valódi negativ esetről van szó,és ha ez később pozitiv lesz, akkor valódi tuberkulin konverzióról beszélünk. Ha azonban negativ teszt után 1-3 hét múlva pozitiv lesz a reakció, akkor "booster" fenoménről van szó, ezeket az egyéneket nem konvertálóknak, hanem tuberkulin reaktoroknak helyes nevezni.

Meg kell jegyezni, hogy a teszt terhességben is elvégezhető és a korábbi véleményekkel szemben, a terhességnek nincs hatása a tesztre [1, 19].

A tuberkulin anergia

Progresziv pulmonális tuberkulozisban a betegek több mint 20%-ánál várható **negativ** tuberkulin reakció [4], mások ennél kisebb %-ban találtak tuberkulin

negativitást [19], mely részletesen a fals negativ tuberkulin reakcióval kapcsolatban került tárgyalásra.
Anergia az az állapot, amikor az egyén adott antigénnel szembeni ismert túlérzékenysége ellenére az antigénnel végzett bőrteszt során nem képes pozitiv reakcióra. Van, aki szerint tuberkulin anergia az, amikor 5TU-val végzett teszt negativ eredménye után 250TU-val megismételve a vizsgálatot sem kapunk reakciót. (A reakció során fellépő erythema értágulat következménye, önmagában nem jelent pozitiv reakciót). Ilyen feltételek mellett már kisebb az esetszám, 8-10% közöttre tehető [20].
Tuberkulin anergia esetén érdemes az un. anergia panellel tovább vizsgálni a celluláris immunreaktivitást, ide tartozik többek között a candida antigén is [14] Ha ezekkel a mintákkal is negativ eredményt kapunk, akkor immunszupresszív állapotra, elsősorban HIV infekcióra kell gondolni. Ilyenkor, ha a tuberkulin reakció meghaladja a 3 mm-t, akkor már a beteg fertőzőttségéről beszélünk [14].
Zajló tuberkulozisban tuberkulin anergia mellett egyéb bőr tesztekre és DNCB-re is negativ választ találtak, amelyet in vitro PPD-re csökkent lymphocyta blasztosodás kisért. Ez prognosztikailag nem bizonyult rossz jelnek, gyógyszeres kezelésre a betegek éppúgy reagáltak, mint a tuberkulin pozitiv betegek és a legtöbb esetben néhány hét alatt a tuberkulin teszt és a többi bőr teszt is pozitivvá vált és az in vitro eredmények is normalizálódtak [20].
Csökkent tuberkulin reakció mellett gyakran a keringő immunglobulinok szintje megemelkedik, ami a betegek megváltozott T és B lymphocyta státuszára utal [20]. Vizsgálatainkkal tuberkulin anergiában emelkedettnek találtuk a betegek keringő vérében az IL-4 és IL-10 pozitiv lymphocyták arányát [21] és a Tγ/δ lymphocyták arányát is [22].
Mycobacterium tuberculosis betegségben a negatív tuberkulin reakció jelentősége az, hogy a háttérben meghúzodó esetleges immunszuppresszált állapotra hívja fel a figyelmet.

Summary

The tuberculin skin test can be valuable in the diagnosis of Mycobacterium tuberculosis.To assess the result of the tuberculin skin test we must consider the circumstances which might impact the development of the reaction. The size of the reaction is influenced by the immune state of the patient; in the case of tuberculin anergy HIV infection should be considered. To increase the specificity of the tuberculin skin test a mixture of specific proteins of the Mycobacterium tuberculosis bacilli as well as secreted antigens of the bacilli were implemented. Both procedures might be promising tools in this regard in the near future. The significance of the tuberculin anergy is that it may call attention to an eventual immunocompromised state of the patient.

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Paper 2

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γ/δ T lymphocytes in *Mycobacterium tuberculosis* infection

Zoltán Balikó, László Szereday, Júlia Szekeres-Bartho

Department of Pulmonology, County Hospital, Pécs, Hungary
Z Balikó

Department of Microbiology, University Medical School of Pécs, Hungary
L Szereday
J Szekeres-Bartho

Correspondence to: Dr Z Balikó

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Abstract

Background **– Data on the percentage of γ/δ T lymphocytes in the peripheral blood of patients infected with *Mycobacterium tuberculosis* are few and contradictory. The percentage of γ/δ T lymphocytes in the peripheral blood of tuberculin positive and tuberculin negative patients with *Mycobacterium tuberculosis* infection and healthy controls was compared.**

Methods **– Thirty six patients infected with *Mycobacterium tuberculosis* and 11 healthy controls were studied. Lymphocytes were separated, cytocentrifuged onto glass microscope slides, and reacted with anti-γ/δ monoclonal antibody. The percentage of γ/δ positive cells was determined by microscopic counting of 300 lymphocytes.**

Results **– No difference was found in the percentage of γ/δ T lymphocytes between patients and controls. However, when the patients were divided into two groups according to reactivity or non-reactivity in the Mantoux skin reaction a higher percentage of γ/δ T lymphocytes was found in the peripheral blood of patients with tuberculin anergy than in tuberculin positive patients or controls.**

Conclusions **– Higher γ/δ T cell counts are found in tuberculin negative patients with tuberculosis than in tuberculin positive patients or tuberculin positive controls. The high γ/δ T cell counts in tuberculin anergic patients may reflect a shift in the immune response in a Th2 direction characterised by increased antibody production and decreased cell mediated responses.**

(*Thorax* 1997;52:375–377)

Keywords: γ/δ T lymphocytes, *Mycobacterium tuberculosis*, tuberculin anergy

Tuberculosis is the leading cause of death due to a single infectious agent in adults. In the last 10 years the incidence of *Mycobacterium tuberculosis* infection has increased slightly even in the developed countries and there has been a considerable increase in the number of infections caused by multidrug resistant *M tuberculosis*. The development of novel anti- [illegible]

Most T lymphocyte receptors consist of alpha and beta chains, and about 10% of the peripheral blood T lymphocytes possess receptors containing gamma and delta chains. The exact role of this population of T lymphocytes is not yet clear. There have been reports of an increased percentage of γ/δ T lymphocytes in the peripheral blood of patients with *M tuberculosis* infection, though others have found no difference between patients and healthy controls.

The aim of this study was to measure the percentage of γ/δ T lymphocytes in the peripheral blood of patients with tuberculosis who had a positive or negative tuberculin reaction.

Methods

Thirty six patients (23 men) of mean age 46.0 years (range 20–71) infected with *M tuberculosis* were included in the study. Twenty four had a positive sputum culture for *M tuberculosis*, 10 of whom were also sputum smear positive. Patients with negative sputum culture were identified by the typical chest radiographic features and the course of the disease during antituberculous therapy. Tuberculin testing was performed according to the original Mantoux test – that is, 5 TU PPD (Human Rt, Gödöllő, Hungary) were given intradermally in the forearm and the results were evaluated 72 hours later. The skin test was considered positive if there was an induration of 10 mm or more and negative if there was no reaction. Positive reactions of more than 10 mm in diameter were seen in 19 cases (more than 15 mm in six cases) and 17 patients had no tuberculin skin reaction at all. Eleven nurses who had worked in our department for longer than six months (10 women) of mean age 34.2 years (range 19–49) acted as healthy controls. Each had been vaccinated with BCG as part of the required Hungarian national vaccination programme and all were tuberculin positive.

γ/δ T cell counts

Ten ml of venous blood was taken before starting antimycobacterial treatment. Lymphocytes were separated from heparinised venous blood on a Ficoll-Hypaque gradient. The purity of the isolated population was periodically checked by reactivity to anti-CD3 antibody and was found [illegible]

Table 1 Details of patients and controls

	Men	Women	Mean age (range)	Tuberculin test		Sputum M tuberculosis	
				[illegible]	[illegible]	[illegible]	[illegible]
Patients	[illegible]	13	[illegible] years ([illegible])	[illegible]	[illegible]	10	[illegible]
Healthy volunteers	[illegible]	10	[illegible] years ([illegible])		[illegible]		

were fixed with acetone at 4°C for 10 minutes and the cells were reacted with a pan anti γ δ monoclonal antibody (T Cells, Sciences, Cambridge, Massachusetts, USA) in a dilution of 1:50. The reacting cells were identified by peroxidase labelled antimouse IgG (Dako) in a dilution of 1:100 using aminoethyl carbasol as chromogen. The percentage of γ/δ T cells was determined by microscopic counting of 300 lymphocytes. Counting was done blind. The patients were divided into two groups according to positivity or anergy of tuberculin reaction. Statistical analyses were performed using the Mann-Whitney U test.

Results

The percentage of γ/δ positive cells in peripheral lymphocytes of all the patients with tuberculosis did not differ significantly from the values for healthy individuals; however, tuberculin anergic patients had a significantly higher rate of γ/δ positive cells than tuberculin positive patients ($p<0.001$). The γ δ cell count in healthy tuberculin positive subjects was low, similar to that in tuberculin positive patients and significantly different from the values in tuberculin negative patients (fig 1).

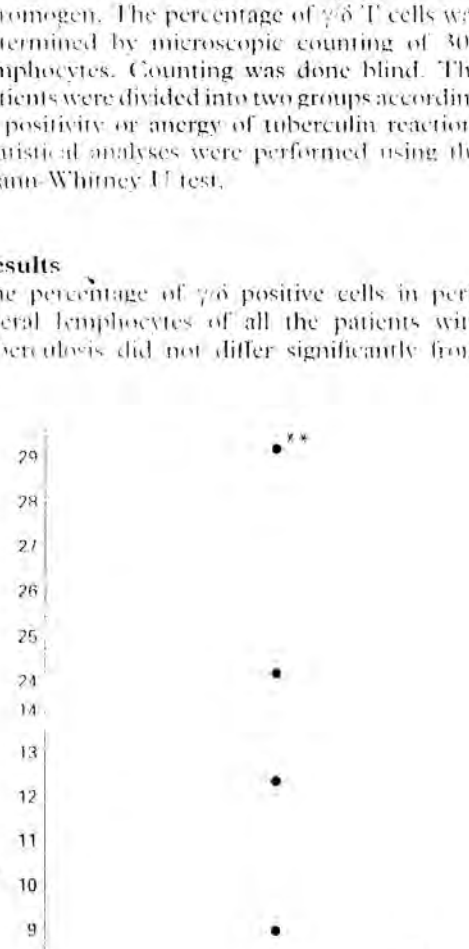

*Figure 1 Percentage of γ/δ positive lymphocytes in peripheral blood of tuberculin positive and tuberculin negative patients and healthy individuals. The horizontal lines represent the mean (SE) of 19, 17, and 11 determinations, respectively. **$p<0.001$ compared with healthy controls.*

Discussion

Several reports have suggested a protective role for γ/δ T lymphocytes in human *M tuberculosis* infection but the exact role of these cells is still not clear.[2-9] Some authors found no difference in the percentage of peripheral γ/δ T lymphocytes in patients with *M tuberculosis* infection and healthy individuals[4-6] while others observed an increase in the number of γ/δ T lymphocytes in the peripheral blood of patients with tuberculosis.[15] The percentage of γ/δ T lymphocytes in our patients with tuberculosis was not significantly higher than that in healthy individuals. However, there was a significantly increased number of γ/δ T lymphocytes in tuberculin negative patients compared with tuberculin positive patients and healthy individuals.

There are no data on the relationship between tuberculin reactivity and γ/δ T cell positivity except for one report in which all patients showed positive tuberculin test reactivity and the percentage of γ/δ T cells did not differ from that of the healthy controls.[6]

The significance of the association between increased numbers of γ/δ T lymphocytes and tuberculin negativity is not clear. These patients did not differ in the course of the disease during chemotherapy or the time needed for recovery from those with positive tuberculin tests and normal γ/δ T lymphocyte numbers.

Isolated γ/δ T lymphocytes from patients with protective immunity to *M tuberculosis* show a higher rate of proliferation when stimulated with the bacterium than those obtained from anergic patients.[7] We found a higher number of γ/δ T cells in tuberculin negative patients than in those positive for *M tuberculosis*; however, the function of these lymphocytes has not been tested. Since γ/δ T cells are known to react with conserved sequences, their role might be one of protection from a potentially harmful autoimmune reaction.

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Bronchoalveolar lavage cell profile in methotrexate induced pneumonitis

A Schnabel, C Richter, S Bauerfeind, W L Gross

Abstract

Background **– Pneumonitis is a rare but potentially life threatening side effect of methotrexate treatment for rheumatoid arthritis which needs to be distinguished from interstitial lung disease due to rheumatoid arthritis.**

Methods **– To examine the value of bronchoalveolar lavage (BAL) in diagnosing methotrexate pneumonitis, the BAL cell profile of four patients with methotrexate pneumonitis was compared with findings in 16 patients with rheumatoid arthritis treated with methotrexate without clinical or radiological evidence of lung disease and eight patients with interstitial lung disease secondary to rheumatoid arthritis treated with methotrexate.**

Results **– Methotrexate pneumonitis was associated with an increase in the lymphocytes in the BAL fluid to 33–68% of total BAL cells. BAL lymphocytosis was also found in five patients in each of the two control groups. The four patients with methotrexate pneumonitis had a disproportionate increase in CD4 + cells to 72–84% of total lymphocytes and in the CD4/CD8 ratio to 17.0, 6.6, 8.7, and 4.0, respectively, figures which exceeded those of the two control groups.**

Conclusions **– Methotrexate pneumonitis was associated with lymphocytic alveolitis with a preferential increase in CD4 + cells. This pattern differs from that in interstitial lung disease due to rheumatoid arthritis and may therefore assist in making an early diagnosis of methotrexate pneumonitis.**

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Keywords: rheumatoid arthritis; methotrexate; lung pneumonitis

Rheumatologische Poliklinik, Medizinische Universität Lübeck, Germany
A Schnabel
C Richter
W L Gross

Medizinische Krankenhausabteilung
A Schnabel
C Richter
W L Gross

Abteilung für Labormedizin
S Bauerfeind

Rheumaklinik Bad Bramstedt, Oskar-Alexander-Straße 26, D-24576 Bad Bramstedt, Germany

Corresponding author:
Dr A Schnabel, Rheumaklinik Bad Bramstedt, Oskar-Alexander-Straße 26, D-24576 Bad Bramstedt, Germany.

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Pneumonitis is a potentially life threatening side effect of treatment with methotrexate that requires immediate discontinuation of the drug.[1] Characteristically, patients experience a prodromal phase with progressive cough, dyspnoea, and malaise which can last from a few days up to several weeks. At this stage incipient methotrexate pneumonitis needs to be distinguished from interstitial lung disease due to rheumatoid arthritis.[2] This is usually made on clinical grounds such as the presence or absence of constitutional symptoms, the rate of progression, and the response to withdrawal of the drug. While interstitial lung disease due to rheumatoid arthritis is usually a chronic disorder which takes a slowly progressive course and is associated with minor constitutional complaints,[3] methotrexate pneumonitis is an acute and rapidly progressive disorder accompanied by prominent constitutional symptoms.[1]

The value of bronchoalveolar lavage (BAL) in this situation is unclear. We have therefore performed a study of the BAL cell profile and the immunophenotype of BAL lymphocytes in patients with rheumatoid arthritis with methotrexate pneumonitis and compared our findings with those of methotrexate treated patients with rheumatoid arthritis with and without interstitial lung disease, to see whether characteristics of the BAL fluid help in distinguishing between these disorders.

Methods

Three women and one man aged 59, 66, 60 and 57 years, respectively, with an established diagnosis of seropositive rheumatoid arthritis were diagnosed as having methotrexate-induced pneumonitis. Three of the patients were diagnosed according to the criteria of Carson *et al*,[4] comprising a clinical course consistent with a hypersensitivity reaction, resolving infiltrate on the chest radiograph after discontinuing methotrexate, exclusion of infection or other pulmonary disease, and pathology consistent with drug induced injury. The presence of any three of these criteria was

Paper 3

Medicina Thoracalis 48. 373–376. 1995.

I. Mezeyi Kórház Tüdőgyógyintézete (Pécs) közleménye

Hő shock proteinek szerepe a Mycobacterium tuberculosis elleni védekezésben

BALIKÓ ZOLTÁN DR.

Összefoglalás

A szerző irodalmi adatok alapján tárgyalja a hő shock proteinek szerepét az emberi sejtekben. Részletesen kitér a Mycobacterium tuberculosis elleni immunológiai folyamatokban betöltött szerepükre. Az eddigi kísérletes adatok alapján arra a következtetésre jut, hogy a kezdeti reményekkel szemben a mycobacteriális hő shock proteinek önmagukban vakcina készítésére eljárás alkalmatlanok. Megállapítja, hogy ma még nincs hatékonyabb vakcina a tuberkulózis vonatkozásában, mint a polivalens BCG.

A hő shock proteineket (továbbiakban hsp) először *Ritossa* írta le Drosophila esetében [15]. Kezdetben azt gondolták, hogy ezek a fehérjék elsősorban a hőhatás ellen védik a sejtet később kiderült, hogy a sejtet ért egyéb stressz hatásokban is protektív szerepük van. Mindegyik, a későbbiekben megvizsgált sejttípusban megtalálták és kimutatták, hogy a filogenezis során a struktúrájuk alig változott, azaz a szerkezetükben legjobban megőrzött fehérjék közé tartoznak. Szerkezeti konzervativizmusuk miatt autoimmun betegségekben etiológiai szerepüket vetették fel [12]. Osztályozásuk molekulasúly szerint történik. Mind növényi, mind baktérium és állati sejtekben vizsgálva az egyes hsp-k aminosav szekvenciája több, mint 50 %-ban megegyezik.

Sejten belüli feladatuk elsősorban ún. „háztartási" funkció, más fehérjékhez kötődnek, részt vesznek fehérjék szétválasztásában („unfolding"), tönkrement fehérjéket degradálnak, molekuláris chaperonnak is nevezik őket (chaperon = kísérő fehérje), mely a fehérjék aktív konformációját alakítja ki [18]. Fagocitózis során a bekebelezett baktériumban megnő a mennyiségük, ezáltal védik a kórokozót a fagocita ellen. Ezzel egyidőben a fagocitában is megnő a hsp szint, ami a sejt védelmét szolgálja a saját maga által termelt anyagoktól, elsősorban a reaktív oxigén metabolitoktól.

Immunológiai szerepükre utal többek között az is, hogy a hsp 70 lokusza emberben a komplement komponensek és a tumornekrózis faktor gének között helyezkedik el. Több, mint 50 féle baktériumban a hsp-k struktúrája megegyezik [8], mely a természetes és a specifikus immunitás közötti kapocs szerepükre utal [9].

Normális körülmények között a cytoplasmában rejtetten vannak jelen, a sejtet ért nagyobb stressz esetében mennyiségük megnő, ilyenkor a gazdasejt felszínén is megjelenhetnek, mely autoimmun folyamatok elindulásához vezethet [2, 3, 4]. Stresszhatás lehet a hő, de lehet vírus infekció vagy gamma interferon is, ezért újabban a hő shock protein elnevezés helyett a stressz protein elnevezés is használatos [18]. Emberben a T-gamma/delta lymphocyták esetleges ligandjai között felmerült a hsp k, elsősorban a

Mycobacterium tuberculosis 65 kilodalton hsp antigén szerepe, mely a T gamma delta lymphocyták elsővonalbeli immunológiai folyamatokban játszott szerepét segítette elő [5, 7].

Hsp-k szerepe Mycobacterium tuberculosis infekcióban

A Mycobacteriumok elleni protektív immunitás Th1 lymphocytákkal függ össze, melyek az antigén prezentáló sejtek felszínén megjelenő antigént a nagy hisztokompatibilitási komplexszel együtt ismerik fel. Aktiválódás után makrofág aktiváló faktorokat szekretálnak, ezzel segítik a makrofágokat az intracelluláris kórokozó elpusztításában.

A Mycobacteriumok nemcsak a CD4+ lymphocytákat, hanem a CD8+ T lymphocytákat és az antigénre nem specifikus ölő sejteket is stimulálják [12, 14].

Klinikailag a Mycobacterium fertőzésnek két fontos útja van. Az egyik a Koch jelenség, a szöveti destrukció, melyben elsősorban a nem antigén specifikus ölő sejtek, mint az NK és LAK sejtek (NK = természetes ölő, LAK = lymphokin aktivált ölő sejtek) vesznek részt. A másik út a protektív immunitás, amiben a specifikusan indukált CD4+ T lymphocyták, ezen belül is a Th1 lymphocyták játszanak szerepet [10]. A fertőzést okozó Mycobacteriumra adott immunválasz nagy része a 70 és 65 kilodalton hsp antigénre irányul [17].

Az irodalmi adatok szerint a BCG vakcina preventív hatása változó — beszámolók szerint nulla és 80 % között változik [16], — ezért évek óta hatékonyabb vakcina előállításán fáradoznak. Az antigének elemzése során derült ki, hogy a Mycobacterium bovis BCG és a Mycobacterium leprae 65 kilodalton hsp struktúrája 95 %-ban megegyezik [1, 16]. A Mycobacterium bovis BCG 65 kilodalton hsp-vel történt stimulálásra CD3+CD4+CD56− T lymphocyták proliferálnak in vitro körülmények között, melyek kifejezett HLA DR korlátozást mutatnak, — egyúttal megnő az NK és LAK sejtek száma is. Utóbbiak szöveti károsodáshoz vezetnek (a szöveti károsodás nemcsak a Mycobacterium tuberculosis, de a Mycobacterium leprae fertőzésre is jellemző).

A protektív immunitás kialakulásakor a baktériumot fagocitált sejtekből felszabaduló hsp-k aktiválják a Th1 lymphocytákat, melyek kevés tumornekrózis faktort indukálva segítik a makrofágokat a fagocitált kórokozó elleni védekezésben. Egyidőben aktiválódnak a CD8+ lymphocyták is, melyek a CD4+ T-lymphocytákkal együtt a fertőzött sejt lízisét okozzák. A lízist követően kiszabaduló baktériumokat fiatalabb makrofágok elimináljak. Ha nemcsak a Th1, hanem a Th2 lymphocyták is aktiválódnak, akkor a Koch jelenség részeként nagy mennyiségű tumornekrózis faktor termelődik, mely szöveti nekrózishoz vezet [6].

A Mycobacterium tuberculosis 65 kilodalton hsp molekula ezek szerint kiemelt jelentőségű a tuberkulózis immunitásban. Kimutatták, hogy a rekombinált Mycobacterium tuberculosis 65 kilodalton hsp a Mycobacterium tuberculosisra immunizált egerek immundomináns molekulája [11]. Mivel ez a molekula az emberi sejtekben meglévő hsp-kel nagyfokú homológiát mutat, ezért az ellene képződő antitest diagnosztikus célra nem alkalmas, ugyanezen okból vakcinának sem felel meg (autoimmunitás veszélye).

Újabban vitatják, hogy néhány kitüntetett molekulához lehetne kötni a Mycobacterium tuberculosis elleni protektív immunitást, a hsp-k mellett számos egyéb antigén szerepe is felmerül ezekben a folyamatokban [13].

Ismételten visszatérő kérdés a hsp-k autoimmun kórképekben játszott szerepe. Rheumatoid arthritisben azt találták, hogy a Mycobacterium tuberculosis és a Freud adjuváns által kiváltott kísérletes arthritist a Mycobacterium tuberculosis 65 kilodalton hsp előzetes adás, — még öt nappal korábban adva is, — ki tudta védeni [2, 12, 18].

A *tumorsejt felismerésben* résztvevő, minden tumorféleség esetén hatékony immun mechanizmus a Th1 lymphocytán alapuló antitumor hatás, amit alátámasztanak az IL-12 (elsősorban Th1 és NK sejteket stimuláló lymphokin) kedvező hatásai egyes daganatos betegségekben. Hypotetikusan a Mycobacterium tuberculosis hatására a hsp elleni humorális és celluláris válasz felerősödik és ily módon a tumorsejtekben is meglévő jelentős mennyiségű hsp révén aktiválódó cytolytikus T-lymphocyták hatékonyan eliminál hatják a tumorsejteket. Ezek alapján ismét érdemes megfontolni a BCG nem specifikus aktív immunitást indukáló hatását daganatos betegségekben [6].

Összefoglalva megállapítható, hogy a hsp-k mint ősi, struktúrájukban magasan konzervált fehérjék, összekötő kapcsot jelentenek a természetes és a specifikus immunitás között. Minden eddig megvizsgált sejtben kimutatható a jelenlétük. Kérdés, hogyan tudtak elbújni a saját antigén elleni védekező rendszer elől, mindenesetre úgy látszik, hogy alacsony koncentrációban a szervezet elviseli jelenlétüket. Ismételt infekciók esetén a hsp-k elleni immunválasz felerősödik (booster hatás, melynek alapja a hsp-k számos baktériumban meglévő szerkezeti azonossága). Ez a strukturális egyezés teszi lehetővé, hogy egy újabb bakteriális fertőzésben a baktérium hsp-t ismertnek tekinti az immunrendszer és a nemspecifikus immunválasz korán felerősödik. Ehhez, a természetes immunválasz humorális ágához kapcsolódnak a test felszínén (bőr, nyálkahártyák) otthonos T-gamma/delta lymphocyták, melyek a természetes immunválasz sejtes vonalát képviselik [18].

A különböző eredetű hsp-k közötti homologiák alapján fennáll az autoimmun folyamatok aktiválódásának a veszélye. Kísérletes körülmények között az ellenkezője bizonyosodott be (kísérletes rheumatoid arthritist az előzetesen beadott 65 kilodalton Mycobacterium tuberculosis eredetű hsp kivédte). Fontos a hsp-k „hétköznapi" szerepe, azaz a különböző okból stresszhelyzetbe került sejtekben a károsodott fehérjék eltakarítása. Amennyiben a sejt nagymértékben károsodott, mint pld. vírus vagy intracelluláris baktérium fertőzéskor, a sejtfelszínen megjelenő hsp-k révén aktiválódott cytotoxikus lymphocyták elpusztítják magát a sejtet.

A Mycobacterium család különböző tagjai között a hsp-k tekintetében nagyfokú a hasonlóság, mely hatékony subunit vakcina előállításának reményét vetette fel. Ez a remény mára nagymértékben csökkent. DNS exrepssziós könyvtárak segítségével ma már több, mint 50 féle mycobacteriális antigén különíthető el és ezek mindegyike kiválthat immunválaszt [8]. Megállapítható, hogy ma még mindig nincs jobb vakcina a tuberkulózis prevenciójára, mint a polivalens BCG.

Köszönöm dr. Szekeres Júliának (POTE, Mikrobiológiai Intézet) a kézirat elkészítéséhez nyújtott segítségét.

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Balikó, Z.: *The role of the heat shock proteins played in the protective immunity of Mycobacterial tuberculosis infection*

The author discusses the role of the heat shock proteins played in human cells based on medical literature datas. He evaluates their importance in the immunologic processes of Mycobacterial tuberculosis infection. He concludes that based on experimental datas despite of previous hopes the mycobacterial heat shock proteins are not satisfactory source for preparing socalled subunit vaccine. For preventive indication in the case of Mycobacterial tuberculosis, there is up to now no other effective vaccine as the polyvalent BCG.

Paper 4

MEDICINA
THORACALIS 1997. 51. NOVEMBER 11. SZÁM

Medicina Thoracalis 50, 551–555, 1997.

Baranya Megyei Kórház Tüdőgyógyintézete (Pécs)[1] és a Pécsi Orvostudományi Egyetem Mikrobiológiai Intézete

Interleukin-4 és interleukin-10 pozitív lymphocyták aránya aktív Mycobacterium tuberculosis infekcióban szenvedő betegek perifériás vérében*

BALÁZS ZOLTÁN DR.[1], SZEREDAY LÁSZLÓ DR., SZEKERES BARBARA JULIA DR.

Összefoglalás

Számos adat utal arra, hogy az interleukin-4 és az interleukin-10 lymphokinek szuppresszív szerepet játszanak a Mycobacterium tuberculosis infekcióban. Mivel a betegségben előforduló tuberkulin anergia oka még nem tisztázott, ezért elhatároztuk, hogy megvizsgáljuk aktív Mycobacterium tuberculosis infekcióban szenvedő betegeink esetében a perifériás vérben keringő interleukin-4 és interleukin-10 pozitív lymphocyták arányát és azokat összefüggésbe hozzuk a tuberkulin reakció mértékével. 33 betegnél végzett vizsgálataink alapján megállapítottuk, hogy tuberkulin negatív betegeinknél szignifikánsan magasabb volt a keringő vér interleukin-4 és interleukin-10 pozitív lymphocytáinak az aránya, mint a tuberkulin pozitív betegeknél és az egészséges kontrolloknál, ami a tuberkulin negatív esetekben az immunológiai folyamatok Th2 irányú eltolódását jelzi és a tuberkulin anergia egyik magyarázatául szolgálhat.

Bevezetés

Közel egymilliárd hétszázmillió ember inficiálódott világszerte Mycobacterium tuberculosissal és vált ezáltal kórokozó hordozóvá, mely állapot reaktiválódás útján betegséghez és az infekció további terjedéséhez vezethet [1]. A multirezisztens kórokozók. Korábban a betegség elleni küzdelemben újabb gyógyszerek kifejlesztése mel-

Rövidítések

IL-2	interleukin 2
IL-4	interleukin 4
IL-10	interleukin 10
Th1	T helper 1 lymphocyta
Th2	T helper 2 lymphocyta
Th0	T helper 0 lymphocyta
DTH	delayed type hypersensitivity (késői típusú túlérzékenység)
PPD	purified protein derivate

*A Tüdőgyógyász Társaság 49. Nagygyűlésén (Balatonfüred, 1996. május 9–12.) elhangzott előadás nyomán.

lett fontos a betegség immunológiai történésének vizsgálata is abban a reményben, hogy a hagyományosan gyógyszeres terápiától eltérő eljárásokat dolgozhassunk ki.

Az egér CD4+T sejteket az antigén stimulációra adott citokin profil alapján két alcsoportba sorolták be (Th1 és Th2). A Th1 sejtekre jellemző az interleukin-2 (IL-2) és az interferon-gamma (IFNγ) szekréciója, míg a Th2 lymphocyták típusosan interleukin-4 (IL-4)-et, interleukin-5 (IL-5)-öt és interleukin-10 (IL-10)-et szekretálnak, melyek fokozzák a B lymphocyták antitest termelését és szerepet játszanak az allergiás betegségekben. Leírtak olyan CD4+T sejteket is, melyek intermediaer profilt mutatnak, ezeket Th0 sejteknek nevezték el [2, 3]. A Mycobacteriumok elsősorban és jellegzetesen Th1 típusú immunválaszt indukálnak, ami az IFNγ és a TNFα magas és az IL-4 alig detektálhatóan alacsony szintjében tükröződik [4]. Ugyanakkor tuberkulózisban szenvedő betegekben mycobacteriális antigénekre gyakran mutatható ki csökkent celluláris és fokozott humorális immunválasz [5]. Miután ismeretes, hogy az IL-4 szignifikánsan csökkenti a késői típusú (DTH) immunválaszt egérben [6] és az IL-10 gátolja a Th1 aktivitást (a makrofágok aktivációját visszaszorítja, blokkolja a Th1 lymphocytából az IFNγ kiáramlást) [7], érdemesnek tartottuk megvizsgálni aktív Mycobacterium tuberculosis infekcióban szenvedő betegeinknél a perifériás vér IL-4 és IL-10 pozitív lymphocytáinak arányát a tuberkulin reakcióval összefüggésben.

Betegek és módszer

A tanulmány során 33 beteg esetében végeztünk vizsgálatokat *(1. táblázat)*. 21 betegnél volt Mycobacterium tuberculosisra pozitív tenyésztés, közülük 11 esetben volt a köpet mikroszkóposan is pozitív. A köpettenyésztés során negatívnak bizonyult esetekben a típusos mellkasröntgen eltérések és a betegnek a tuberkulózis gyógykezelésére adott klinikai válasza támasztotta alá a diagnózist. A legsúlyosabb esetekben HIV szerológiai vizsgálatot is végeztettünk, mely minden esetben negatív volt. A tuberkulin bőrtesztet az eredeti Mantoux módszerrel végeztük, azaz 5 TE PPD-t fecskendeztünk intracután és az eredményt 72 óra múlva értékeltük ki. A Mantoux-tesztet mindig ugyanaz a személy végezte és a betegség, valamint az in vitro lymphocyta vizsgálatok ismerete nélkül (vakon) történt a kiértékelés. Pozitívnak értékeltük a bőrtesztet, ha az induráció ≥ 10 mm volt és negatívnak, ha egyáltalán nem volt reakció (betegeink között egy esetben sem találtunk 1 és 10 mm közötti reakciót). 11 önkéntes egészséges egyén szolgált kontrollként, akik tuberkulotikus betegek ellátásában résztvevő nővérek voltak, így valamennyien tuberkulotikus betegek kontaktjai voltak, de egyikükben sem volt korábban tuberkulózis betegsége.

1. táblázat

Betegek és a kontrollok

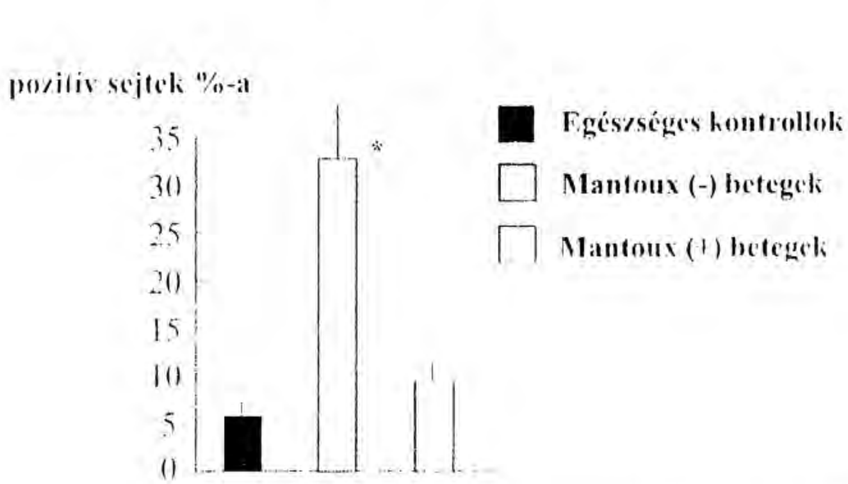
	Férfi	Nő	Életkor	Tuberkulin bőrteszt		Köpet Mycobacterium tuberculosis	
				negatív	pozitív	direkt pozitív	tenyésztés pozitív
Betegek	21	12	42 év (19–74)	11	22	11	21
Egészséges kontrollok		11	33 év (17–58)		11		

10 ml vénás vért vettünk, a betegek esetében az antituberkulotikus gyógykezelés megkezdése előtt. A lymphocytákat heparinizált vérből Ficoll-Hypaque grádiensen szeparáltuk. A sejteket RPMI 1640 médiumban (Gibco) mostuk és mikroszkópos lemezekre centrifugáltuk 1 x 10^6/ml sejtszámban. Az izolált preparátum sejttisztaságát időről időre CD3 ellenes antitesttel ellenőriztük és következetesen állandónak találtuk. Miután mind az interleukin-4 mind az interleukin-10 intracelluláris antigén, ezek az antigének szekretálódnak a sejtekből és így megjelennek a sejtek felszínén is. Ahhoz, hogy az intracelluláris interleukinok is reagáljanak, az immunocytokémiai módszer alkalmazása előtt a sejteket 5 percig acetonban fixáltuk. (Az aceton a sejtmembránt permeábilizálja és ezzel elősegíti az IgG típusú antitest bejutását a sejtbe [8]). Aceton kezelés után a sejteket polyclonális antihumán anti-IL-4 antitesttel (R&D Systems, Minneapolis, USA) 1:100 hígításban, majd a vizsgálat második lépéseként poliklonális antihumán anti IL-10 antitesttel (R&D Systems, Minneapolis, USA) szintén 1:100 hígításban reagáltattuk. Az in vivo stimulálódott és ezért reagáló [9] sejteket peroxidaséval jelölt kecske ellenes IgG (Dako) savóval 1:100 hígításban azonosítottuk. A reakciót diaminobenzidinnel tettük láthatóvá, melyet ezüst festéssel tettünk kifejezettebbé. Az IL-4 és IL-10 pozitív lymphocyták arányát 300 lymphocyta nagy nagyítással történt leszámolása alapján számoltuk ki. A sejtszámolás vakon történt. A statisztikai kiértékelést a Student féle kétmintás t próbával végeztük.

Eredmények

A tuberkulotikus betegek perifériás vérében meghatároztuk az IL-4 és IL-10 pozitív lymphocyták arányát és az eredményeket a betegek tuberkulin reakciójával vetettük össze. Az IL-4 pozitív lymphocyták aránya szignifikánsan magasabb volt a tuberkulin negatív betegeinknél, mint a tuberkulin pozitív eseteknél és az egészséges kontrolloknál (*1. ábra*). Hasonló eredményt kaptunk IL-10 esetében is (*2. ábra*).

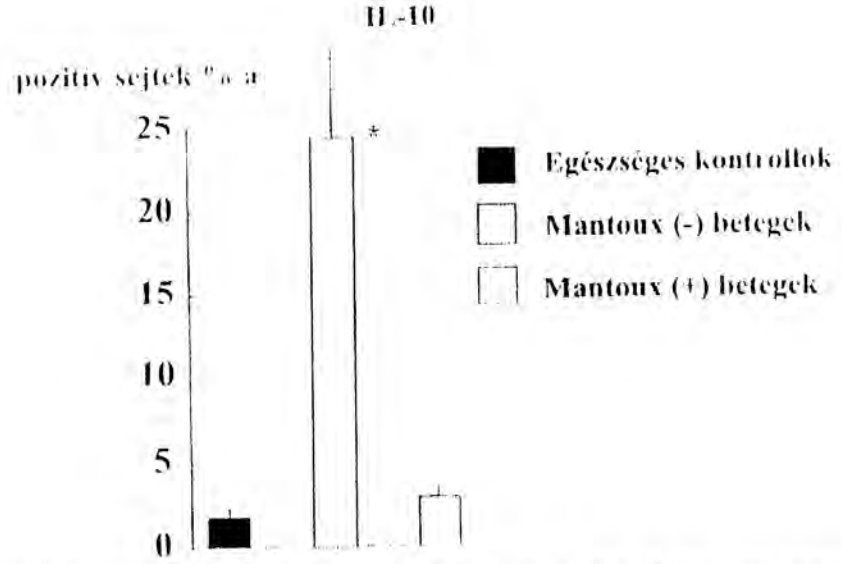
IL-4



1. ábra: A tuberkulin pozitív és negatív betegek és az egészséges kontrollok IL-4 pozitív lymphocytáinak az aránya a perifériás vérben

(Az oszlopok feletti vonalak a ± SEM-et jelzik. 11, 11 és 22 eset vizsgálata alapján

[illegible] 0,05



2. ábra. A tuberkulin pozitív és negatív betegek és az egészséges kontrollok IL-10 pozitív lymphocytáinak az aránya a perifériás vérben.
Az oszlopok feletti vonalak a ± SEM-et jelzik 11, 8 és 22 eset vizsgálata alapján.
**jelzi, ahol a betegek és a kontrollok közötti eltérés szignifikáns, $p < 0,01$*

Megbeszélés

Ismert, hogy a Mycobacterium elsősorban CD4+ Th1 sejteket és Th1 profilt mutató CD8+ cytotoxikus T sejteket indukál, ami az IL-2 és az IFNγ fokozott termelésében is megnyilvánul [1, 11, 12, 13]. Th2 típusú lymphocyta válasz és az IL-4 és az IL-10, mint Th2 típusú citokin jelenléte progresszív megbetegedéssel társul [14], míg a Th1 típusú választ a protektív immunválasz jelének tekintenek [15]. Az ismert makrofág deaktiváló quartett tagjai az IL-4, IL-10, IL-13 és a transzformáló növekedési faktor béta [16, 17]. Mind az IL-4-ről, mind az IL-10-ről azt gondolják, hogy sejt közvetítette immunválasz útján az infekcióra való hajlamot fokozzák [18]. Amíg az IL-4 blokkolja a poliklonálisan stimulált humán T sejtek IL-2 és IFNγ szekrécióját és szelektív potencirozó hatása van a Th2 klónok proliferációjára és citokin szintézisére, addig az IL-10 inkább az antigén kiváltotta immunválaszokat károsítja [21]. Így az IL-4 és az IL-10 a Mycobacterium elleni rezisztencia csökkenésével hozható összefüggésbe.

Közlések szerint Mycobacterium tuberculosis infekcióban a betegek 5–20%-ában találtak csökkent DTH reakciót [24]. Mycobacterium tuberculosisban szenvedő betegeink adatait kiértékelve azt találtuk, hogy tuberkulin anergia esetén a keringő vérben szignifikánsan magasabb volt az IL-4 és IL-10 pozitív lymphocyták aránya, mint a pozitív tuberkulin reakciót mutató betegek és az egészséges kontrollok esetében.

Korábban beszámoltak arról, hogy a tuberkulotikus betegek és az egészséges kontrollok között az IL-4 termelés tekintetében szignifikáns különbség van, mivel a legtöbb tuberkulotikus beteg Th2 típusú immunválaszt mutatott eltérően a tuberkulin pozitív egészséges egyénektől, utóbbiaknál az in vitro vizsgálat Th1 típusú választ jelzett [5]. Hasonló megfigyelést tettek *Sourcel H. M.* és munkatársai is, akik arra a következtetésre jutottak, hogy az IL-4 termelés összefüggésbe hozható a gazdaszervezet védekező képességének csökkenésével és a tuberkulózis patogenezisével [2]. Ha ez így van, akkor még az is felmerülhet, hogy állatkísérletekhez hasonlóan [19, 25, 26] bizonyos

előrehaladott esetek. Kezelése során anti IL-4 és anti IL-10 adását is meg lehetne próbálni, – különösen multirezisztens kórokozó esetén. Lehetséges, hogy a betegség progresszióját elősegítő citokinek antagonizálása jó irányban (Th1 válasz irányában) befolyásolná a betegek immunvédekezését [26, 27].

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Balikó Z., Szeredav L., Szekeres-Bartho J.: *IL-4 and IL-10 positive lymphocytes in the peripheral blood of patients with active Mycobacterium tuberculosis infection*

There is abundant evidence supporting the suppressive role played by IL-4 and IL-10 during Mycobacterium tuberculosis infections. The significance of tuberculin anergy occuring in some cases with Mycobacterium tuberculosis infection is still not clear, therefore we investigated the ratio of IL-4 and IL-10 positive lymphocytes with positivity or negativity of tuberculin skin raction. Thirty-three patients with active tuberculosis infection were included in the study. Eleven healthy volunteers served as controls. According to reactivity or non-reactivity in the Mantoux skin reaction, we found a significantly higher ratio of IL-4 and IL-10 positive lymphocytes in the peripheral blood of patients with tuberculin anergy than in that of tuberculin positive patients or healthy donors. We conclude that the high percentage of IL-4 and IL-10 positive lymphocytes in the peripheral blood of anergic patients suggests a Th2 biased immune response.

Paper 5

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Th2 - biased immune response in cases with active Mycobacterium tuberculosis infection and tuberculin anergy

Z. Balikó[a]*, L. Szereday[b], J. Szekeres-Bartho[b]

[a]Department of Pulmonology, County Hospital, Pécs, Ángyán J. u 2, Hungary, 7635

[b]Department of Microbiology, University Medical School of Pécs, Pécs, Hungary, 7643

*Corresponding author: Tel/Fax: 36-72-227755/100

e-mail:chelonae@mail.matav.hu

Abstract

The significance of tuberculin anergy occuring in some cases with Mycobacterium tuberculosis infection is still not clear. We investigated the ratio of IL-4, IL-10, IL-12, CD-4, CD-8 expressing lymphocytes in the peripheral blood of patients with active Mycobacterium tuberculosis infection and correlated the percentage of the reactive cells with the positivity or negativity of tuberculin skin reactions. Twenty-seven patients were included in the study, with eleven healthy volunteers serving as controls. Ten ml of venous blood was drawn before starting anti-mycobacterial treatment. A tuberculin skin test was performed, introducing intracutaneously 5 TU PPD on the forearm with results evaluated after 72 hours.

Consistent with the reactivity or non-reactivity of the tuberculin skin test, we found a significantly higher ratio of IL-4, IL-10, and CD-4 positive lymphocytes and a significantly lower ratio of IL-12 in the peripheral blood of patients with tuberculin anergy than in that of tuberculin positive patients or healthy donors. There was no difference in the ratio of the CD-8 positive lymphocytes among the three groups. To evaluate whether the differences could be explained by the degree of pulmonary tubercular involvement, we classified the patients into three groups according to the extent and type of X-ray findings. Ninety percent of tuberculin negative patients were classified as grade III, whereas in the tuberculin positive group only thirty percent fell in this category. There were no significant correlation between the radiological grade of the patients and the examined in vitro parameters unless the tuberculin reactivity of each patients was also considered. Tuberculin anergy may reflect an inappropriate immune response to the intracellular pathogen. The high percentage of IL-4 and IL-10 positive lymphocytes together with a low percentage of IL-12 positive lymphocytes in the peripheral blood of anergic patients suggests a Th2 biased immune response during the early course of the disease.

Keywords Interleukin 4, Interleukin 10, Interleukin 12, M. tuberculosis, tuberculin anergy

1. Introduction

Nearly 1 billion 700 million individuals throughout the world are infected with Mycobacterium tuberculosis and thus are carriers of live bacilli able to colonize and disseminate [1]. In the era of multi-drug resistant bacilli, besides producing more effective antibiotics, evaluation of the immunologic events during Mycobacterium tuberculosis infection can be important for the development of additional therapeutic approaches to combat this potentially life-threatening disease.

Murine CD4+ T cells have been divided into at least two different subsets (Th1 and Th2), based on the cytokine profiles that they secrete upon antigenic stimulation. Th1 cells characteristically secrete interleukin-2 (IL-2) and interferon-gamma (IFNγ), whereas Th2 lymphocytes typically produce IL-4, IL-5, and IL-10, which enhance antibody synthesis of B cells and play a role in allergic diseases. CD4+ T cells with intermediate cytokine profile (Th0) have also been described [2]. Mycobacteria preferentially induce Th1-like responses, as reflected by the production of high titers of IFNγ and TNFα with low or undetectable levels of IL-4 [3]. IL-12 favors the development of TH-1 like T-cell responses by enhancing IFNγ and antagonizing IL-4 and IL-10, thereby down-regulating the TH-2 responses[4]. Patients with tuberculosis infection frequently have depressed cellular and increased humoral immune response against mycobacterial antigens [5]. IL-4 and IL-10 are known to significantly inhibit the development of delayed type hypersensitivity (DTH) responses in mice [6]; IL-10 inhibits Th1 activity (through macrophage deactivation and the blocking of IFNγ release by Th1 lymphocytes) [7]. IL-12 is a key cytokine in immune regulation, presumably by inducing commitment from T helper 0 (Th0) to the Th1 phenotype [8], and is held to be a marker of active disease in pulmonary tuberculosis [9]. Consequently, we examined the ratio of the IL-4, IL-10, and IL-12 positive lymphocytes as well as that of CD4+ and CD8+ positive lymphocytes

in the peripheral blood of our patients with active Mycobacterium tuberculosis infection in th context of their DTH reaction.

Patients with tuberculin anergy usually have more advanced disease than those with positive tuberculin reaction. Therefore, it was also necessary to look at whether the severity o pulmonary involvement could be consistently related to the presence of tuberculin anerg and/or any resulting immunological response.

2. Materials and methods

2.1. Subjects

Twenty-eight patients with Mycobacterium tuberculosis were included in the study (table 1). All of them had a positive sputum culture for Mycobacterium tuberculosis and 1 patients showed smear positivity. The patients were checked for HIV infection, all were negative (the incidence of HIV infection in Hungary is still very low). A tuberculin skin test a part of the clinical evaluation of the patients was performed by introducing 5 TU PPD (Human, Hungary) intracutaneously on the forearm,and results were evaluated after 72 hours. All skin tests were administered by the same individual and were read blind to the diagnosis o the patient and to the result of the lymphocyte analysis. The skin test was considered positive i there was an equal or higher than 10 mm induration and negative if there was no reaction (among our patients there were no cases with reactions between 1 and 10 mm).

Patients were classified according to the extent and type of X-ray findings into three groups following the classification of Dlugovitzky et al. [10]: mild (n=8), patients with a single lobe involved and without visible cavities; moderate (n=6), patients presenting unilateral involvement of two or more lobes with cavities, if present, reaching a total diameter no greater than 4 cm; and advanced (n=14), bilateral disease with massive involvement and multiple cavities.

Eleven healthy volunteers serving as controls were all nurses in our department and were therefore in contact with tuberculotic patients, none, however, has had a history of tubeculosis. Permission for the study was obtained from the Clinical Ethics Committee of the Medical University of Pécs, and all subjects gave their consent to participate.

2.2. Isolation of lymphocytes

Ten ml of venous blood was drawn before introducing anti-mycobacterial treatment. Lymphocytes were separated from heparinized venous blood on the Ficoll-Paque (Pharmacia) gradient. The cells were washed in medium RPMI 1640 (Gibco) and centrifuged on microscope slides at a cell count of 1 x 10^6 / ml. The purity of the isolated population was periodically checked for reactivity to anti CD3 antibody and was found to be consistent.

2.3. Identification of IL-4, IL-10, IL-12, CD-8, and CD-4 positive lymphocytes

Interleukin 4, 10, and 12 represent intracellular antigens. These antigens are secreted, thus they also appear on the cell surface. The cells were fixed for 5 minutes in cold acetone. Acetone permeabilizes the cell membrane and lets IgG to penetrate the membrane. Following acetone treatment the cells were reacted with polyclonal antihuman anti-IL-4 antibody (R&D Systems, Minneapolis, USA) or with polyclonal antihuman anti-IL-10 antibody (R&D Systems, Minneapolis, USA), both were diluted 1:500 for 60 minutes at room temperature in a humid atmosphere. As a second antibody we used peroxidase labeled antigoat IgG (Dako) (1:100). In the case of IL-12 we used anti-IL-12 monoclonal antibody (R&D System) in dilution of 1 : 100 and as a second antibody we used peroxidase labeled antimouse IgG (Dako) (1:100). To identify CD8 and CD4 positive lymphocytes we used antihuman monoclonal anti-CD8 antibody (Becton Dickinson) and antihuman monoclonal anti-CD4 antibody (Becton Dickinson) in dilution of 1:50 and as a second antibody we used peroxidase labeled antimouse IgG (Dako) (1:100). Reactions were developed by diaminobenzidine, intensified with silver staining. The percentage of IL-4, IL-10, IL-12, CD-4, and CD-8 positive T cells was

determined by microscopic counting of 300 lymphocytes at high power magnification. The counting was done by a blinded observer.

2.4. Statistics

The two-tailed Student's t-test was used in our statistical analysis of the data.

3. Results

Patients were classified according to the extent and type of X-ray findings into three groups following the classification of Dlugovitzky et al.[10]

The ratio of IL-4, IL-10, IL-12, CD-8, and CD-4 positive lymphocytes was determined in peripheral blood of tuberculotic patients and data were analysed in context of tuberculin reactivity. Because the control group was recruited exclusively from female volunteers, it may be worth mentioning that no differences were found between the sexes in the ratio of IL-4, IL-10, IL-12, CD-8, and CD-4 positive lymphocytes. The percentage of IL-4 positive cells was significantly higher in patients with negative tuberculin reactions than in those with positive tuberculin reactions or in healthy volunteers (Fig.1). Similar results were obtained for IL-10 (Fig.2). In contrast, the ratio of IL-12 positive cells was significantly lower in patients with negative tuberculin reactions than in those with positive tuberculin reactions or healthy volunteers (Fig.3.). There was no significant difference between the ratio of CD-8 positive lymphocytes in the peripheral blood of patients with either anergic or positive tuberculin reactions and of the healthy volunteers, however, the CD-4 lymphocyte rate was significantly increased in the cases with tuberculin anergy.

Following classification of patients into three groups according to the extent and type of X-ray findings, we found that among the 8 tuberculin anergic cases, 7 were in group three (those patients with advanced pulmonary manifestation of the disease), among 19 patients with positive tuberculin skin reaction 7 were also in group three, 6 belonged to group two, and six

group one. Cytokine expression on the lymphocytes showed no relationship with X-ray ıdings.

Discussion

It is known that Mycobacteria predominantly induce CD4+ Th1 cells and CD8+ totoxic T cells with a Th1-like cytokine profile of elevated IL-2 and IFNγ levels [3]. ecently it has been found that the percentage of IL-12 positive cells is more than three times at of the IFNγ-positive cells in the active tuberculosis patients [9]. The presence of a Th2 sponse or of type 2 cytokines IL-4 and IL-10 is associated with progressive disease [10], hereas a Th1 type response has been linked to protective immunity. IL-4, IL-10, IL-13, and nsforming growth factor beta comprise the quartet of defined macrophage deactivating tokines [12]. IL-4 blocks IL-2 and IFNγ secretion by polyclonally stimulated human T cells 3] and has a selective potentiating effect on the proliferation and cytokine synthesis of Th2 nes, while IL-10 might be involved in damaging ongoing antigen-driven immune responses ther than in the selective regulation of Th1 functions [14]. Thus IL-4 and IL-10 could be sociated with diminished resistance to infection by mycobacteria [15].

Evaluating patients with active Mycobacterium tuberculosis infection, we found that tients with tuberculin anergy had a significantly higher ratio of IL-4, IL-10, and CD-4 sitive lymphocytes in peripheral blood than either those with positive tuberculin skin tests or ealthy volunteers. On the other hand, patients with tuberculin anergy had significant lower tio of IL-12 positive lymphocytes than either those with positive tuberculin skin test or ealthy volunteers. There were no difference in the ratio of CD-8 positive lymphocytes.

As noted, patients with tuberculin anergy usually have more advanced disease than tients with positive tuberculin reaction; therefore, it was important to evaluate whether the sults simply correlate with the degree of pulmonary involvement of the disease or the

tuberculin reaction has its own impact on the result independently. Ninty percent of tuberculin negative patients were classified as grade III, whereas in the tuberculin positive group only thirty percent fell in this category. As showed in the Results section, we found that there was no significant correlation between the radiological grade of the patients and the examined in vitro immunologic parameters unless the tuberculin reactivity of each patient was also considered. Based on these findings, we suggest that the cytokine pattern correlates with the degree of the tuberculin skin test and consequently with the pulmonary manifestation of the disease.

We suggest that the increased ratio of IL-4 and IL-10 positive lymphocytes and the decreased ratio of IL-12 positive lymphocytes can be held responsible for the tuberculin skin anergy and, in part, also for the progression of the disease. It should be emphasized that among our patient groups, only four were over 60 years old; thus we believe that the age of those studied had no effect on the observed tuberculin anergy.

It has been reported previously that there is a significant difference in the production of IL-4 among tuberculotic patients and healthy controls, as most tuberculotic patients exhibit a Th2 pattern of immune responsiveness whereas tuberculin positive healthy individuals have a Th1 pattern *in vitro* circumstances [5]. A similar observation was made by H. M. Surcel et al., who concluded that IL-4 production may be involved in the loss of protective host response and thereby be linked to the pathogenesis of tuberculosis [2]. If true, it is tempting to speculate that, as in experimental mouse models [16], anti IL-4 and anti IL-10 therapies might be used in the treatment of selected cases with far advanced disease, particularly in those infected with multi-drug resistant bacilli. It is possible that an antagonist of disease promoting cytokines might be useful in enhancing host resistance following immunologic manipulations which eliminate the Th2 component and boost the type 1 response [16, 17].

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Legends to the Figures

Fig.1

The ratio of IL-4 positive lymphocytes in peripheral blood of tuberculin positive and negative patients as well as healthy individuals.

The bars indicate mean ± SEM of 11, 7 and 13 determinations respectively.

* significantly different from the control and from the Mantoux positive cases at $p < 0.01$

Fig.2.

The ratio of IL-10 positive lymphocytes in peripheral blood of tuberculin positive and negative patients as well as healthy individuals.

The bars indicate mean ± SEM of 11, 4, and 12 determinations respectively.

* significantly different from the control and from the Mantoux positive cases at $p < 0.01$

Fig.3.

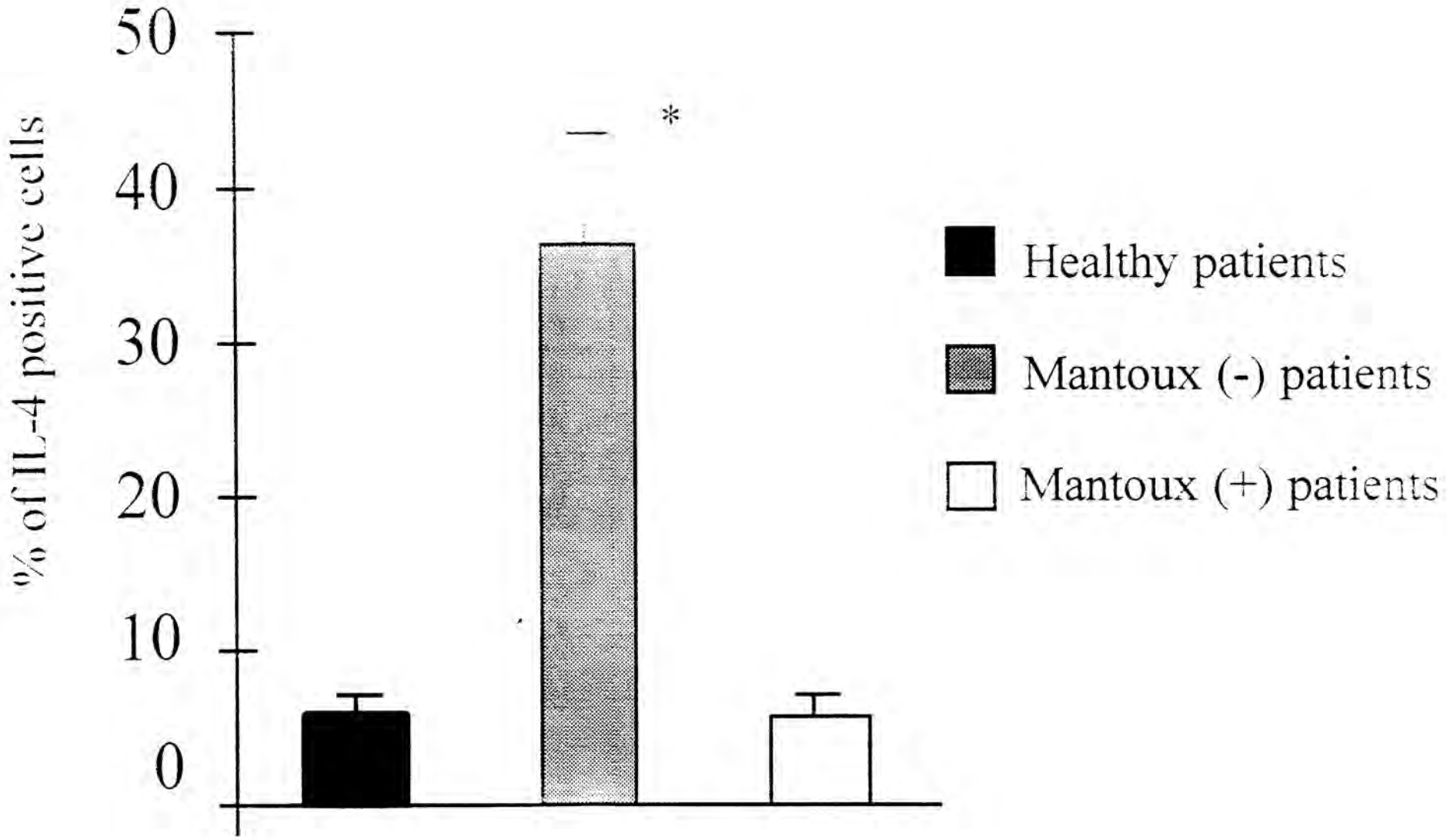
The ratio of IL-12 positive lymphocytes in peripheral blood of tuberculin positive and negative patients as well as healthy individuals.

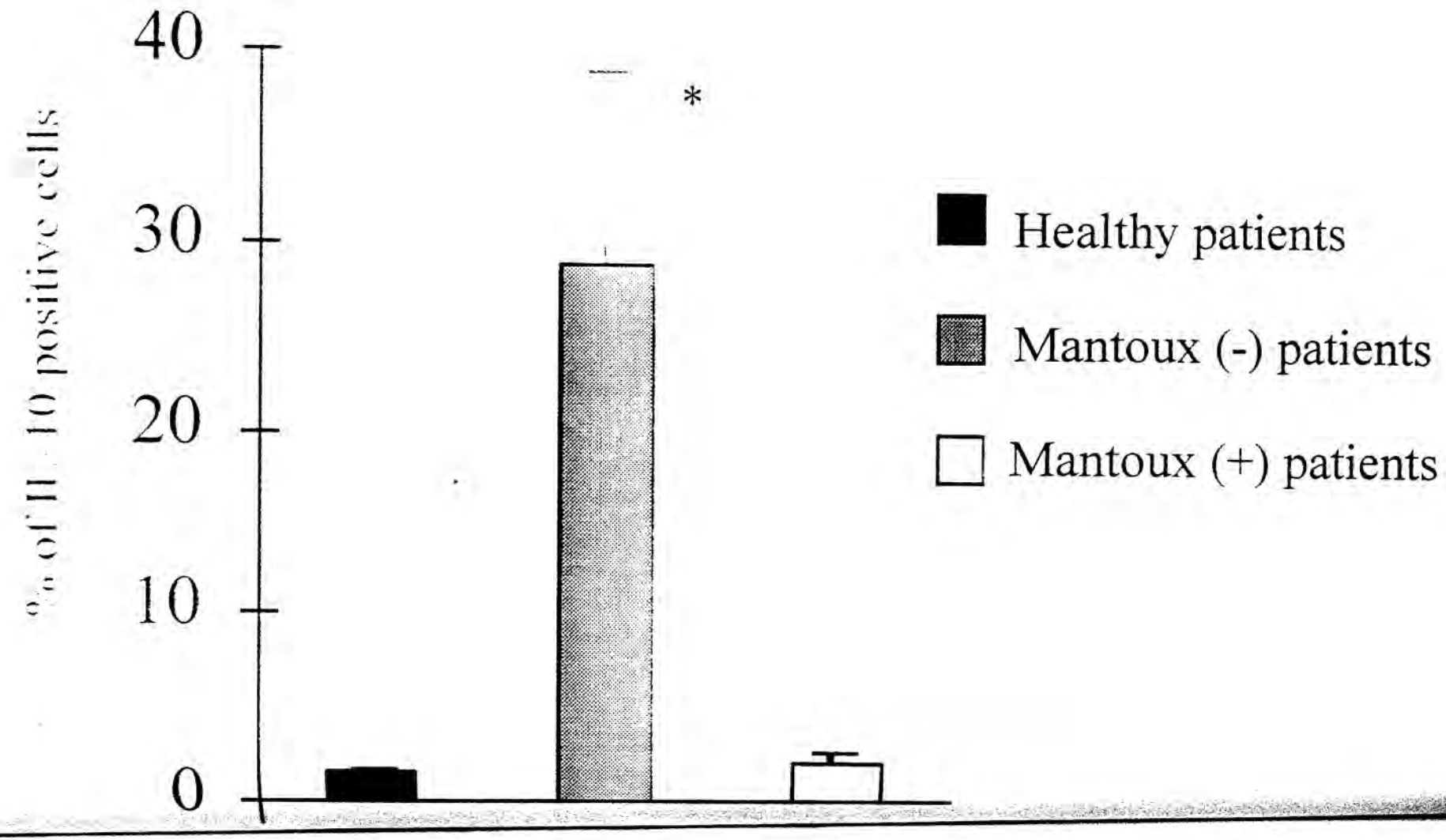
The bars indicate mean ± SEM of 11, 7, 14 determinations respectively

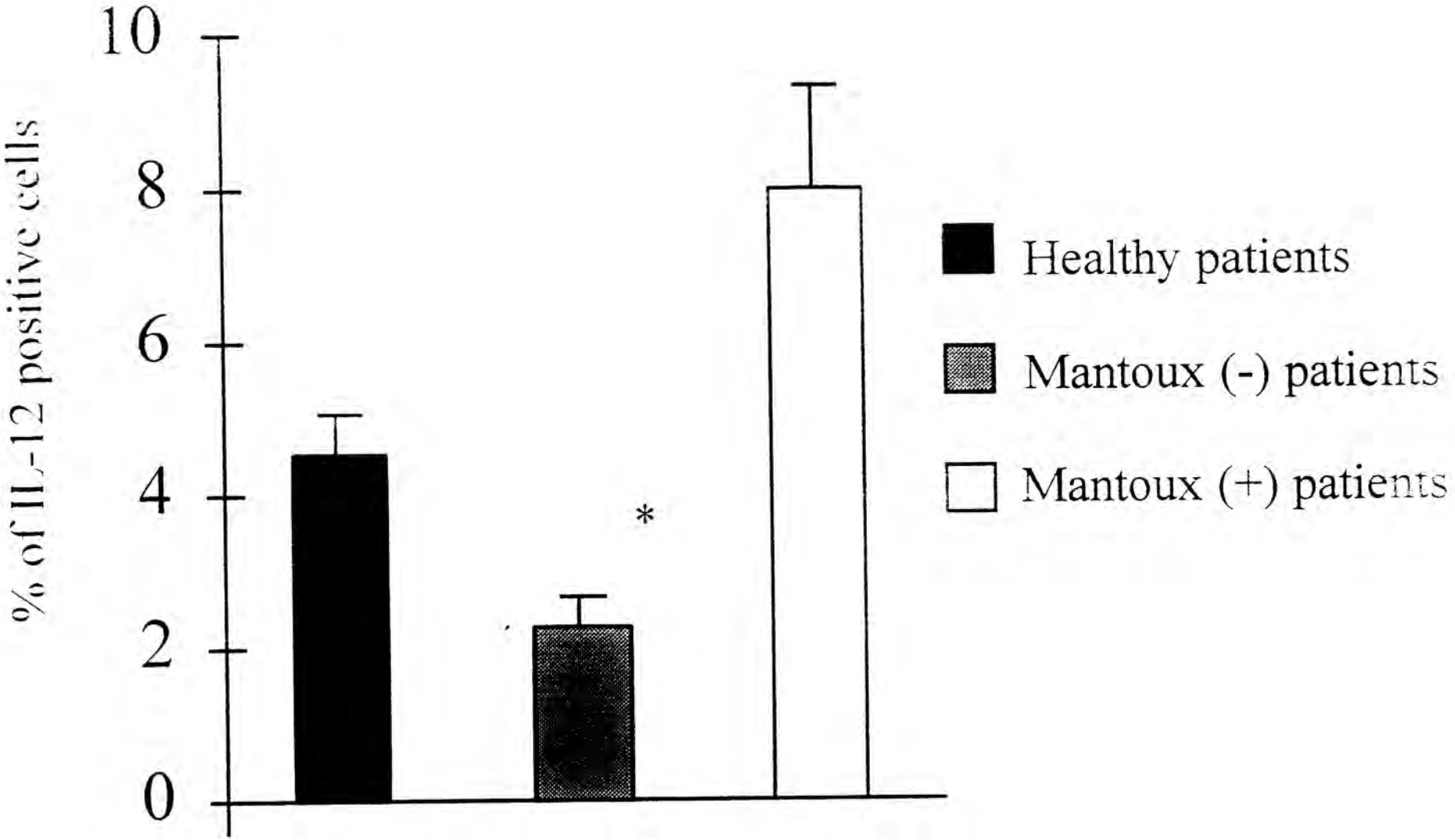
* significantly different from the control and from the Mantoux positive cases at $p < 0.01$

Tabla 1. Data of tuberculotic patients and healthy volunteers.

	Male	Female	Age	Tuberculin skin test		Sputum Myc.tub.	
				negative	positive	smear positive	culture positiv
Patients	21	7	45.8 years (19-83)	8	20	15	28
Healthy volunteers	1	10	34,2 years (19-49)		11		


50
40
30
20
10
0
% of IL-4 positive cells
*
Healthy patients
Mantoux (-) patients
Mantoux (+) patients


40
30
20
10
0
*
Healthy patients
Mantoux (-) patients
Mantoux (+) patients


10
8
6
4
2
0
% of IL-12 positive cells
*
Healthy patients
Mantoux (-) patients
Mantoux (+) patients

VI. Summary and Conclusions

Tuberculin anergy in cases with active M. tuberculosis infections is a specific paradigm of the disease. It has been known for some time that in cases with active M. tuberculosis infection there is no correlation between the rate of immune protection and the size of the tuberculin skin reaction. It had been presumed that different types of T lymphocytes were responsible for the two events (Platt, J.L. et al., 1983).

The occurrence of the tuberculin anergy in cases with active M. tuberculosis infection is about 20 %, and the advanced cases are usually accompanied by reduced tuberculin skin test reactivity. For that reason we decided to study the immunologic background of the tuberculin anergy in correlation with advanced manifestations of active M. tuberculosis disease.

The simplest and most reliable mode of analysing the immunologic events during M. infection is to study immune cells sampled from the affected tissue, i.e., from the pleural cavity in the case of tuberculous pleuritis or from the bronchoalveolar lavage fluid. The analysis of the lymphocytes taken from the peripheral blood can also give reliable information regarding the immune events of ongoing M. tuberculosis infection.

In the first part of our study, we analysed peripheral lymphocytes from patients with active M. tuberculosis disease for Tγ/δ surface receptor positivity, correlating the results with the tuberculin reaction of the patients. During the second part of the study, we defined the rates of the interleukin-10, interleukin - 12, and interleukin- 4 positive lymphocytes in peripheral blood of patients with active M. tuberculosis; these results were correlated with the state of the tuberculin reactivity. In each case 11 healthy volunteers served as controls.

It was found that the rate of the Tγ/δ positive lymphocytes were significantly higher in the cases with tuberculin negativity than in those cases with tuberculin

positivity and in the healthy volunteers. It can be hypothesized that because of the higher bacterial burden in tuberculin negative patients, the Tγ/δ cells, as first line immune protective cells, could contribute to defence the host from the invaders; another of their role might be prevention of the immune events provoked by M. tuberculosis infections.

In the second part of our studies, we found that in cases with tuberculin negativity the immunocompetent cells showed a Th2 biased type immune response. Normally this was accompanied by a more advanced pulmonary process. It seems plausible to suggest that this Th2 biased immune state of the host is sufficient explanation for both the tuberculin anergy and the advanced pulmonary involvement of the disease.

In our opinion this specific paradigm of tuberculous disease deserves further attention, and it is reasonable to study other aspects of the immune defence mechanism during ongoing M. tuberculosis infection.

List of papers:

1.Balikó, Z. A tuberkulin reakció értékei és korlátai. Medicina Thoracalis / in press /.

2.Balikó, Z., Szereday, L., Szekeres-Bartho, J. γδ T lymphocytes in *Mycobacterium tuberculosis* infection. Thorax. 1997, 52, 375-377.

3.Balikó, Z. Hő shock proteinek szerepe a Mycobacterium tuberculosis elleni védekezésben., Medicina Thoracalis. 1995, 48, 373-376.

4.Balikó Z., Szereday, L., Szekeres-Bartho,J. Interleukin-4 és interleukin 10 pozitiv lymphocyták aránya aktiv Mycobacterium tuberculosis infekcióban szenvedő betegek perifériás vérében. Medicina Thoracalis.1997,50,551-555.

5.Balikó Z., mSzereday, L., Szekeres-Bartho,J. Th2-biased immune response in cases with active Mycobacterium tuberculosis infection and tuberculin anergy. Submitted by FEMS Immunology and Medical Microbiology.

Papers presented:

1.Balikó,Z., Szekeres-Bartho,J., Szereday, L. Interleukin-4 és interleukin-10 pozitiv lymphocyták aránya aktiv mycobacterium tuberculosis infekcióban szenvedő betegek perifériás vérében.
Magyar Tüdőgyógyász Társaság 49.Nagygyülése.Balatonfüred.1996, május 9-12. Absztrakt.Medicina Thoracalis XLIX. 17. Suppl. 1996.

2.Balikó,Z., Szereday,L.,Szekeres-Bartho,J. IL-4 and IL-10 positive lymphocytes in the peripheral blood of patients with active mycobacterium tuberculosis infection.
Third International Congress of the Worldwide Hungarian Medical Academy. July 4-6, 1996. Pécs, Hungary .

VIII. APPENDIX

MATERIAL AND METHODS

2.1. Subjects

Patients with M. tuberculosis were included in the study. In the first part of our study there were patients without sputum culture positivity; in the second part of our work only patients with sputum culture positivity were involved in the study. The patients were checked for HIV infection; all were negative (the incidence of HIV infection in Hungary is still very low).

Eleven healthy volunteers served as controls were all nurses in our department and were therefore in contact with tuberculotic patients; none, however, had had a history of tuberculosis.

Permission for the study was obtained from the Clinical Ethical Comittee of the Medical University of Pecs, and all subjects gave their consent to participate.

2.2. Administration of the tuberculin skin test.

A tuberculin skin test as part of the clinical evaluation of the patients was performed by introducing 5 TU PPD (Human, Hungary) intracutaneously on the forearm, with results evaluated after 72 hours. All skin tests were administered by the same individual amd were read blind to the diagnosis of the patient and to the result of the lymphocyte analysis. The skin test was considered positive if there was an equal or higher than 10 mm induration and negative if there was no reaction (among our patients there were no cases with reactions between 1 and 10 mm)

2.3. Classification the extent and type of the pulmonary involvement of the disease

Patients were classified according to the extent and type of X-ray findings into three groups following the classification of Dlugovitzky et al. (1997): mild; patients with a single lobe involved and without visible cavities; moderate; patients presenting unilateral involvement of two or more lobes with cavities, if present, reaching a total diameter no greater than 4 cm; and advanced; bilateral disease with massive involvement and multiple cavities.

2.4. Isolation of lymphocytes

Ten ml of venous blood was drawn before introducing anti-mycobacterial treatment. Lymphocytes were separated from heparinized venous blood on the Ficoll-Paque (Phanmacia) gradient. The cells were washed in medium RPMI 1640 (Gibco) and centrifuged on microscope slides at a cell count of 1×10^6 / ml. The purity of the isolated population was periodically checked for reactivity to anti CD3 antibody and was found to be consistent.

2.5 Identification of IL-4, IL-10, IL-12, CD-8, CD-4, and γδ positive lymphocytes

Interleukin 4, 10, and 12 represent intracellular antigens. These antigens are secreted; thus they also appear on the cell surface. The cells were fixed for 5 minutes in cold acetone. Acetone permeabilizes the cell membrane and lets the IgG antibody penetrate the membrane. Following acetone treatment the cells were reacted with polyclonal antihuman anti-IL-4 antibody (R&D Systems, Minneapolis, USA) or with polyclonal antihuman anti-IL-10 antibody (R&D

Systems, Minneapolis, USA); both were diluted 1:500 for 60 minutes at room temperature in a humid atmosphere. As a second antibody we used peroxidase labeled antigoat IgG (Dako) (1:100). In the case of IL-12 we used anti-IL-12 monoclonal antibody (R&D System) in dilution of 1:100 and as a second antibody we used peroxidase labeled antimouse IgG (Dako) (1:100). To identify CD8 and CD4 positive lymphocytes we used antihuman monoclonal anti-CD8 antibody (Becton Dickinson) and antihuman monoclonal anti-CD4 antibody (Becton Dickinson) in dilution of 1:50 followed by peroxidase labeled amtimouse IgG (Dako) (1:100). In the case of identification of T lymphocytes we used pan anti-γδ antibody monoclonal antibody (T cells, Sciences, Cambridge, Massachusetts, USA) in a dilution of 1:50. As a second antibody we used peroxidase labelled antimouse IgG (Dako) in a dilution of 1:100. Reactions were developed by diaminobenzidine, intensified with silver staining. The percentage of IL-4, IL-10, IL-12, CD-4, CD-8, and γδ positive T cells was determined by microscopic counting of 300 lymphocytes at high power magmification. The cells counts were done by a blinded observer.

2.6 Statistics

The two-tailed Student's t-test and the Mann-Whitney U test were used in our statistical amalysis of the data.

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