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LAVORO ORIGINALE

Blocking TNF in vitro with infliximab determines the inhibition of expansion and interferon gamma production of Vγ9/Vδ2 T lymphocytes from patients with active rheumatoid arthritis. A role in the susceptibility to tuberculosis?*

Il blocco del TNF in vitro determina inibizione della espansione e della produzione di interferone gamma da parte di linfociti Vγ9/Vδ2 di pazienti con artrite rumatoide. Un ruolo nella suscettibilità alla tubercolosi?

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RIASSUNTO

Tra gli effetti collaterali della neutralizzazione del TNF, per la maggior parte di tipo infettivo, il più importante appare la riattivazione di un processo tubercolare. Il subset di linfociti γ/δ sembra giocare un ruolo importante nella protezione contro il bacillo tubercolare.
Oggetto dello studio è stato quello di valutare la capacità di espansione di tale subset linfocitario in pazienti con artrite reumatoide (AR) e di studiare l'effetto in vitro di infliximab.
Sono stati inclusi nello studio 42 pazienti affetti da AR (28 PPD positivi, 14 PPD negativi) e 155 controlli (110 PPD positivi e 45 PPD negativi) ed è stata valutata la capacità di espansione, l'espressione del recettore per il TNF e il contenuto di Interferone dei linfociti γ/δ.
Il fattore di espansione dei linfociti γ/δ risultò più elevato nei soggetti PPD positivi, con i livelli più alti nei pazienti affetti da AR. L'aggiunta di infliximab nelle culture di linfociti γ/δ dei pazienti PPD positivi determinò una significativa inibizione dell'espansione, della espressione del recettore del TNF e del contenuto di Interferone γ.
Conclusioni: *I soggetti con artrite reumatoide PPD positiva hanno una elevata capacità proliferativa del subset γ/δ in risposta ai fosfoantigeni che è inibita in vitro dalla esposizione a infliximab. È ipotizzabile che il blocco del TNF può svolgere un ruolo nella emergenza dell'infezione tubercolare attraverso l'inibizione di tale subset.*

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INTRODUCTION

Biological therapeutic agents neutralising tumour necrosis factor (TNF) are highly active in treating chronic inflammatory diseases, such as Crohn's disease, rheumatoid arthritis, ankylosing spondylitis, uveitis, and psoriasis (1-3). From the beginning, side effects of TNF neutralisation - mostly infectious complications - were recognized, the most important being, however, pulmonary tuberculosis infections (4).
TNF is a cytokine produced primarily by macrophages in response to stimuli activating toll-like receptors, but can also be expressed by activated T cells, B cells, and NK cells (5, 6). An effective host response against mycobacterium tuberculosis (Mtb) involves the differentiation of specific T cells to secrete an appropriate Th1 cytokine pro-

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file and the development of granulomas in which activated epithelioid macrophages restrict mycobacterial growth and TNF is necessary for optimal coordination of both aspects of Mtb immunity (7).
The peripheral blood of patients with rheumatoid arthritis (RA) contains oligoclonal gamma/delta T cell populations which may contribute to the pathogenesis of the disease (8, 9).
Gamma/delta T cells and dendritic cells are quickly recruited to the lungs shortly after intranasal vaccination with BCG and can contribute to protective immune response against Mycobacterium tuberculosis (10). It ha been also previously reported that Vγ9/Vδ2 T cells from tuberculin purified protein derivative (PPD)-positive children, either healthy or affected by different clinical forms of tuberculosis, strongly proliferate to different phosphoantigens in vitro, whereas Vγ9/Vδ2 T cells from PPD-negative healthy subjects proliferate very poorly (11, 12). Disease-associated changes in Vγ9/Vδ2 T cell effector functions in patients with tuberculosis are consistent with the possibility that these T cells may play a protective role in immune response against M. tuberculosis infection.
Aim of the present study was to assess the expansion capacity of Vγ9/Vδ2 T cells from (PPD positive and PPD negative) patients with active rheumatoid arthritis, and to examine the in vitro effect of infliximab on this lymphocyte subset.

MATERIALS AND METHODS

Patients

Forty-two patients with rheumatoid arthritis classified according to the 1987 revised ACR criteria (13) were studied. Twenty-eight were PPD-negative (8 males and 20 females, mean age 57±12 years), 14 were PPD-positive (4 males, 10 females, mean age 64±7 years). At time of sampling, the disease was active in all patients. Treatments included methotrexate (10-20 mg per week) with or without NSAID and/or steroids (prednisone <10 mg/day). Forty-five PPD-negative (mean age 34±8 years) and 110 PPD-positive (mean age 39±12 years) healthy volunteers were enrolled as controls. Human studies committee approval and individual informed consent from each subject were obtained.

Monoclonal antibodies and flow cytometry

mAbs specific for human surface antigens anti-CD3 phycoerythrin-labelled (PE) and anti-T-cell receptor (TCR) Vδ2 fluorescein isothiocyanate (FITC; PharMigen, San Diego, CA, USA) were used as follows. Peripheral blood mononuclear cells (PBMCs; 10^6 in 100 1 phosphate buffered saline with 1% heat-inactivated foetal calf serum and 0.02% Na-azide) were incubated at 4°C for 30 min with anti-CD3-PE conjugated mAb and anti-TCR Vδ2 FITC conjugated mAb simultaneously. After washing, the cells were suspended in PBS with 1% foetal calf serum and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) by using forward scatter/side scatter gating to select the lymphocyte population for analysis.

Cell separation and expansion in vitro of Vγ9/Vδ2 T lymphocytes

PBMCs were obtained from each individual by separating heparinized venous blood on Ficoll (Euroclone, Wetherby, Yorkshire, UK). The cells were washed in RPMI-1640 medium (Euroclone), and cultured in 24-well plates (Costar, Cambridge, MA, USA) at a concentration of 5×10^5 cells/ml in RPMI 1640 supplemented with 10% foetal calf serum (Euroclone), hepes 20 mmol/l (Euro-clone), 2 mmol/l L-glutamine (Euroclone) and penicillin/streptomycin 100 U/ml (Sigma, St Louis, USA), at 37°C and at 0.5% CO_2.
For the expansion of Vγ9/Vδ2 T cells, PBMCs were cultured for 10 days in medium alone or in the presence of the follow phosphoantigens: xylose 1-P (Sigma; 0.5 mmol/l final concentration); ribose 1-P (Sigma; 0.5 mmol/l final concentration); dimethylallyl pyrophosphate (DMAPP; Sigma; 0.5 mmol/l final concentration).
After 72 hours, cultures were supplemented with a 0.5 ml medium containing 20 U/ml recombinant human interleukin (IL-2; Genzyme, Cambridge, MA, USA). Every 72 hours, 0.5 medium was replaced with a 0.5 ml fresh medium containing 20 U/ml IL-2.
After 10 days, cells were washed three times in medium, and expansion of Vγ9/Vδ2 T cells was assessed using FACScan, as described above.
The absolute number of Vγ9/Vδ2 T cells in each culture was calculated according to the following formula: % Vγ9/Vδ2 positive cells before culture × total cell count/100. The Vγ9/Vδ2 expansion factor (EF) was then calculated by dividing the absolute number of Vγ9/Vδ2 T cells in specifically stimulated cultures by the absolute number of Vγ9/Vδ2 T cells cultured in the absence of any antigen.

In vitro effect of infliximab on Vγ9/Vδ2 T lymphocyte cultures

In order to examine the effects on Vγ9/Vδ2 T lymphocyte expansion, TNF-RII expression and IFN-gamma content, infliximab (Remicade; Centocor Inc., Malvern, PA; Shering Plough SpA, Italy) was added in the medium at a final concentration of 10, 50 (for 3 days) and 100 µg/ml (for 10 days). The surface expression of TNF-RII expression was studied using anti-TNF receptor II PE (R&D system, Minneapolis, MN; USA) and anti Vδ2 simultaneously. The number of TNF-RII molecules (MESF; molecular equivalents of soluble fluorochrome) was calculated by FACS analysis of cells stained with saturating amounts of PE labelled anti-TNF-RII of known PE/protein ratio and comparing the staining with a standard curve of microbeads labelled with defined numbers of PE molecules (Quantum Fluorescence Kit, Sigma). The analysis was done using Quickal Program for MESF Units for Windows.

For the evaluation of intracytoplasmic content of IFNγ, $3x10^5$ cells were stained with anti- Vδ2 TCR FITC and, after washing, fixed with 4% paraphormaldeyde (Sigma) for 30 minutes at 4°C. After two washes with permeabilization buffer (saponine containing) the cells were incubated at 4°C for 45 minutes with anti-IFN-gamma PE (Euroclone). After washing, the cells were suspended in PBS with 1% foetal calf serum and data were acquired on a FACScan instrument and analyzed using WinMDI version 2.8 software.

STATISTICAL ANALYSIS

Kruskal-Wallis test followed by a Dunnet's post hoc analysis was used to compare continuous variables in the different groups while Wilcoxon rank test was used to evaluate effect of infliximab on cell expansion, TNFRII expression and IFNγ content. A p value <0.05 was considered significant.

RESULTS

Expression of Vγ9/Vδ2 T-cell receptor on lymphocytes in peripheral blood

The percentage of gamma/delta T cells with phenotype Vγ9/Vδ2 was similar in both patients and normal individuals (4.38±1.56% and 3.05±1.34%, respectively).

The number of circulating Vγ9/Vδ2 T cells also was not substantially modified by different therapies or by the presence of PPD positivity.

Expansion in vitro of Vγ9/Vδ2 T cells

The expansion of Vγ9/Vδ2 T lymphocytes was evaluated *in vitro* by incubating the cells with five DMAPP for 10 days or in medium (containing IL-2) alone.

At this time the percentage of expansion was assessed by fluorescence activated cell sorting (FACS) analysis using the anti-TCR Vδ2 mAb. The results were expressed as EF (see materials and

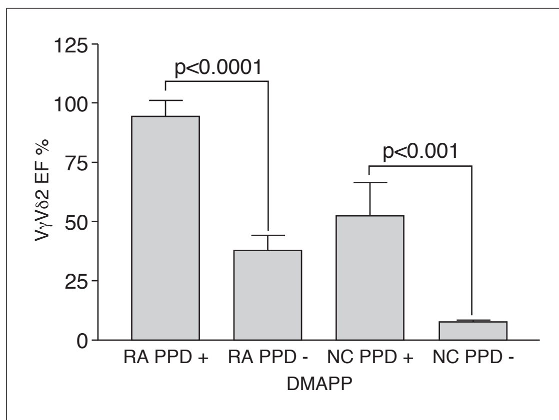

Figure 1 - Expansion of Vγ9/Vδ2 T lymphocytes from patients with PPD positive and negative rheumatoid arthritis and PPD positive and negative controls in response to dimethylallylpyrophosphate (DMAPP). The Vγ9/Vδ2 T cell expansion factor (EF) was calculated by dividing the absolute number of Vγ9/Vδ2 T cell in specifically DMAPP stimulated cultures by the absolute number of Vγ9/Vδ2 T cells cultured in the absence of any antigen. Results are expressed as mean ± SD.

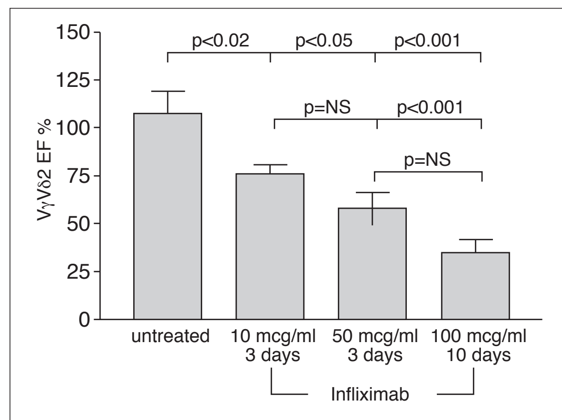

Figure 2 - In vitro effect of infliximab incubation on Vγ9/Vδ2 T lymphocytes expansion (EF) from patients with PPD positive rheumatoid arthritis. Results are expressed as mean ± SD.

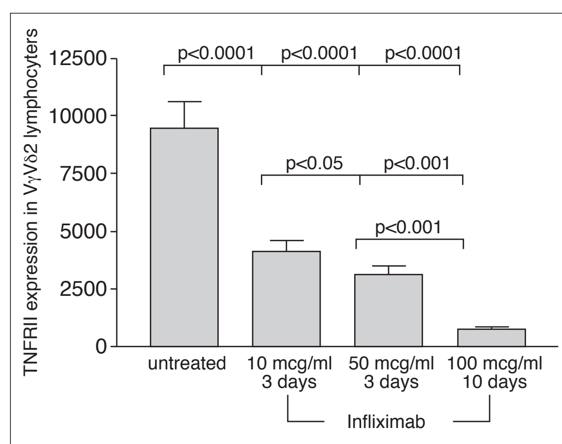

Figure 3 - Effect of infliximab on TNFRII expression of Vγ9/Vδ2 T lymphocytes from patients with PPD positive rheumatoid arthritis. Levels were expressed as MESF (molecular equivalents of soluble fluorochrome). Results are expressed as mean ± SD.

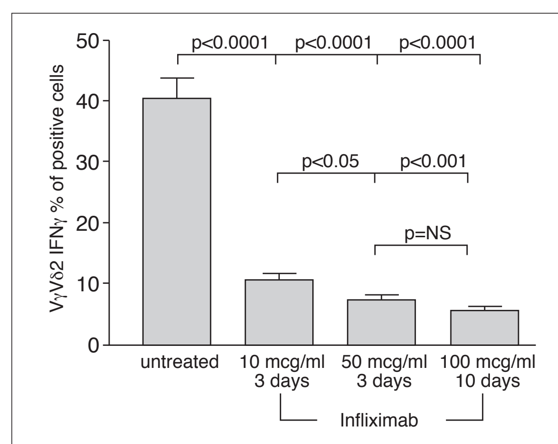

Figure 4 - Effect of infliximab on IFN-gamma content of Vγ9/Vδ2 T lymphocytes from patients with PPD positive rheumatoid arthritis. Results are expressed as mean ± SD of percentage of positive cells.

methods). Significant differences were found in the response to DMAPP in the tested groups (Fig. 1). Specifically, the EF of Vγ9/Vδ2 T cells of RA patients was higher than that of healthy control individuals, and differences were observed between PPD-positive and negative patients and controls (RA PPD-positive 96±38 vs RA PPD-negative 38±26, $p<0.01$; NC PPD-positive 51±98, vs NC PPD-negative 8±5.8, $p<0.01$; RA PPD positive vs NC PPD positive, NS).

In vitro studies with infliximab

The addition of infliximab to the cultures from 7 PPD positive-patients determined a significant inhibition of cell expansion (108±27 in untreated cultures, 76±12 in cultures treated for 3 days with 10 µg/ml infliximab, 58±20 in cultures treated for 3 days with 50 µg/ml and 35 ± 18 in those treated for 10 days with 100 µg/ml infliximab) (Fig. 2) and a significant decrease of TNF-RII expression (Fig. 3).

In particular, the mean TNF-RII MESF was 10.422±1.694 in untreated culture and 4.561±615 (infliximab 10 µg/ml for 3 days), 3.422±584 (infliximab 50 µg/ml for 3 days) and 820±175 (infliximab 100 µg/ml for 10 days) respectively after exposure to infliximab.

IFNγ (Fig. 4) in untreated cultures was 40.4±8.2%. In the presence of infliximab we found values of 10.5±3% (infliximab 10 µg/ml for 3 days), 7.2±2.2% (infliximab 50 µg/ml for 3 days) and 5.5±1.7% (infliximab 100 µg/ml for 10 days) respectively.

DISCUSSION

The use of TNF antagonists is accompanied by an increased risk of active tuberculosis. Although the risk seems to pertain to all 3 available agents, it has been suggested that infliximab and adalimumab carry a higher risk than etanercept (14).

The role of TNF in the human immune response to tuberculosis remains unclear. This cytokine may be responsible for some of the clinical manifestations of the disease, including weight loss, night sweats, and tissue destruction (15). Membrane-bound TNFα seems to provide major protection against Mycobacterium infection in mice models. Yet in animal models, TNFα plays a central part in the host response against tuberculosis, including granuloma formation and containment of disease. Antibodies against TNFα cause a reactivation of tuberculosis in a mouse model of latent infection. Given the key role of TNFα in the innate immune response to tuberculosis, patients receiving treatment with infliximab are probably also susceptible to disease after primary infection and exogenous reinfection with Mtb (16).

In the present study we analyzed the *in vitro* expansion capacity of Vγ9/Vδ2 T cells from patients with rheumatoid arthritis.

Vγ9/Vδ2 T cells represent the majority of gamma/delta T lymphocytes in the peripheral blood (17) and phosphoantigens are known to activate specifically Vγ9/Vδ2 T cells in a major histocompatibility complex unrestricted, but TCR dependent, manner (18).

An increase in γ/δ T cell expansion was observed in RA patients when compared to healthy subjects, our results being not sufficient to hypothesize a role of this subset in the pathogenesis of the disease. Different results, indeed, have previously been reported, but this discrepancy is probably due to inclusion of different populations of patients and/or stages of disease progression (19, 20).

A high proportion of PPD positive RA patients had an increase in Vγ9/Vδ2 T cells expansion suggesting a role in the protection against M. Tuberculosis in these patients. Vγ9/Vδ2 T cells from patients, but not from control individuals, responded to DMAPP *in vitro* with expansion. This phenomenon might be explained by the fact that Vγ9/Vδ2 T cells from PPD positive patients are pre-activated *in vivo*. Alternatively, Vγ9/Vδ2 T cells in PPD positive patients might be less susceptible to apoptosis and account for the increased expansion.

In vitro exposure of gamma/delta T cells to infliximab determined a significant depression of γ/δ T cell expansion and IFN production. The effect of infliximab might explain the increase susceptibility of latent tuberculosis in RA patients to overt infection when exposed to TNF blocking agents.

Studies in humans and animal models have suggested in fact that γ/δ T cells may play an important role in the immune response to Mbt (10-13). In mice, there is a large increase in the number of γ/δ T cells that accumulate in the lungs after intranasal challenge with PPD and, in some experimental conditions, mice lacking gamma/delta T cells suffer a more severe form of tuberculosis and fail to control the infection. In normal healthy individuals, γ/δ T cells contain the highest frequency of Mtb-reactive T cells in the peripheral blood and the predominant subset of Mtb-reactive γ/δ T cells express a TCR encoded by Vγ9 and Vδ2 gene segments (21). Vγ9/Vδ2 T lymphocytes reduce the viability of intracellular Mbt. Participation in early immunity against Mbt is also a feature of a subset of mouse T cells with the antigen receptor variable region encoded by the Vc1 gene. Vc1 cells are rapidly recruited to the lungs of mice infected intranasally with BCG, are able to produce TNFα and IFNγ following in vitro stimulation, and are cytotoxic against BCG-infected macrophages (22). We have also found that Vγ9/Vδ2 T cells from children with tuberculosis have an increased proliferative activity, but decreased IFN-gamma production and granulysin expression (23). After successful chemotherapy, the Vγ9/Vδ2 T cell proliferative response strongly decreased, whereas IFNγ and granulysin production consistently increased (23).

In conclusions in this study we have documented that γ/δ T lymphocytes from patients with PPD positive rheumatoid arthritis have a high capacity to respond in vitro to phosphoantingens with expansion and IFNγ production that is inhibited by the exposure to infliximab. These results might be of relevance in view of the effect of TNF blocking on the pulmonary tuberculosis infection.

SUMMARY

Background: Side effects of TNF neutralisation - mostly infectious complications - were recognized, the most important being pulmonary tuberculosis infections. γ/δ T cells contribute to protective immune response against mycobacterium tuberculosis.
Objectives: The aim of the present study was to assess the expansion capacity of Vγ9/Vδ2 T cells from (tuberculin purified protein derivative (PPD) positive and PPD negative) patients with active rheumatoid arthritis (RA), and to examine the in vitro effect of infliximab on this lymphocyte subset.
Methods: 28 PPD negative RA patients were studied and compared with 14 PPD positive RA patients, 45 PPD-negative and 110 PPD-positive healthy volunteers. Cell separation, expansion in vitro of Vγ9/Vδ2 T lymphocytes (EF) and the expression of tumor necrosis factor receptor II and IFN-gamma content by Vγ9/Vδ2 T lymphocytes were studied before and after infliximab in vitro addiction.
Results: The EF from PPD positive subjects was higher than that from PPD negatives. Patients with RA have the highest levels. The addition of infliximab to the cultures from PPD-positive patients determined a significant inhibition of cell expansion and TNF RII expression and a significant decrease of IFN gamma content.
Conclusion: In this study we have documented that γ/δ T lymphocytes from patients with PPD positive rheumatoid arthritis have a high capacity to respond in vitro to phosphoantingens with expansion TNF-RII expression and IFN-gamma production that is inhibited by the exposure to infliximab. These results might be of relevance in view of the effect of TNF blocking on the pulmonary tuberculosis infection.

Parole chiave - Artrite reumatoide, infliximab, tubercolosi, linfociti γ/δ.
Key words - *Rheumatoid arthritis, tuberculosis infection, infliximab, Vγ9/Vδ2 T lymphocytes.*

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