CONCISE REPORT

Immunological consequences of thalidomide treatment in Sjögren's syndrome

N M Moutsopoulos, N Angelov, V Sankar, R A Leakan, S Pillemer, S M Wahl

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Objective: To study the immunological consequences of systemic thalidomide treatment in patients with Sjögren's syndrome.

Methods: Cytokine (tumour necrosis factor α (TNFα), interleukin (IL) 6) and soluble receptor (sIL2R) levels were measured in patient and control plasma (n = 7), before and after thalidomide treatment. Peripheral blood mononuclear cells were examined by FACS analysis for potential changes in specific cell populations (T cells, B cells, monocytes), and for the expression of activation markers (CD25, HLA-DR), costimulatory molecules (CD40, CD40L), TNF receptors, chemokine receptors, and adhesion molecules (L-selectin (L-sel)).

Results: Owing to adverse effects of thalidomide, the treatment interval was limited. None the less, statistically significant changes in markers of cell activation were recorded in the four treated patients. Before treatment, HLA-DR, TNFRI, CXCRI, and CXCRII were raised in the patients compared with healthy controls ($p<0.05$) and their expression was down regulated after treatment. B cell numbers and expression of the adhesion molecule L-sel also declined with thalidomide.

Conclusion: Significant changes in measures of cell activation were detected during thalidomide treatment within this limited study, which upon further investigation may offer insight into the underlying immunoregulatory pathways of thalidomide.

In the past decade, thalidomide has been rediscovered as a potential anti-inflammatory, immunomodulatory, and antiangiogenic agent for the treatment of a variety of conditions, ranging from dermatological disorders and autoimmune diseases to cancer.[1] The beneficial effects on conditions such as Behçet's disease,[2] systemic lupus erythematosus, and rheumatoid arthritis[1] have been attributed to the multiple immune regulatory actions of thalidomide. Thalidomide has been shown to suppress chemotaxis and phagocytosis of polymorphonuclear cells without producing cytotoxic cell effects and to inhibit selectively tumour necrosis factor α (TNFα) production by human monocytes.[1] Given the central role of TNFα in immune and inflammatory conditions,[3] the ability of thalidomide to down regulate TNFα may, in large part, explain its clinical usefulness in immune mediated disorders.

The successful use of thalidomide in several autoimmune conditions led to the suggestion that this immunomodulator might be beneficial in the treatment of Sjögren's syndrome (SS), a systemic autoimmune disorder that is characterised by a lymphocytic infiltration of the lachrymal and salivary glands[4] which compromises secretory functions. To date, the treatment of SS remains symptomatic[5] and extensive research into developing new treatments is in progress. To this end, thalidomide was tested as a potential treatment for SS, and its clinical efficacy[6] was monitored together with the evaluation of systemic immunological measures, which may serve as potential prognostic markers of treatment outcome.

PATIENTS AND METHODS

Study design

A 12 week randomised, double blind, placebo controlled pilot clinical trial was designed to evaluate efficacy, safety, and potential side effects of thalidomide in patients with primary SS.[6] According to the original study design, patients were to receive 300 mg/day or placebo orally, and the group randomisation was to be accomplished by a standard method using a random number table a priori. In the event of adverse effects dosing was to be reduced to 200 mg/day, 100 mg/day, 50 mg/day. Thalidomide and placebo capsules were kindly donated by the Celgene Corporation (Warren, NJ), and the clinical protocol was approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research.

Patient selection and clinical evaluation

Entry criteria included the following: diagnosis of primary SS according to Fox *et al*,[7] ability to provide informed consent, and 6 weeks or more without other treatment with modifying agents (such as antimalarial drugs or steroids). Exclusion criteria included: women with childbearing potential, known hypersensitivity to thalidomide, current peripheral neuropathy by history or physical examination, confounding medical illness or abnormal laboratory test results, male sex.

Control subjects were women, of a similar age to the matched patient ($\pm$10 years), with no history of autoimmune disease, confounding illness, or oral and ocular symptoms.

Baseline evaluations included medical history and physical examination, laboratory values, and assessments of disease activity (ocular dye test, Schirmer's I test, total stimulated/unstimulated salivary flow, and autoantibodies SSA/SSB).

Baseline assessments of disease activity were performed at study entry and subsequent visits. Concurrently, drug safety and adverse effects were monitored. Improvement was required in two of three SS disease criteria (oral, ocular, or laboratory tests) to consider treatment successful.

Isolation of plasma and peripheral blood mononuclear cells (PBMC)

Peripheral blood (60 ml) was drawn from patients at baseline and subsequent visits and from healthy untreated subjects matched for age and sex. Plasma was removed from whole blood of patients and controls after centrifugation. PBMC

Abbreviations: IL, interleukin; IL2R, interleukin 2 receptor; L-sel, L-selectin; PBMC, peripheral blood mononuclear cells; SS, Sjögren's syndrome; TNFα, tumour necrosis factor α

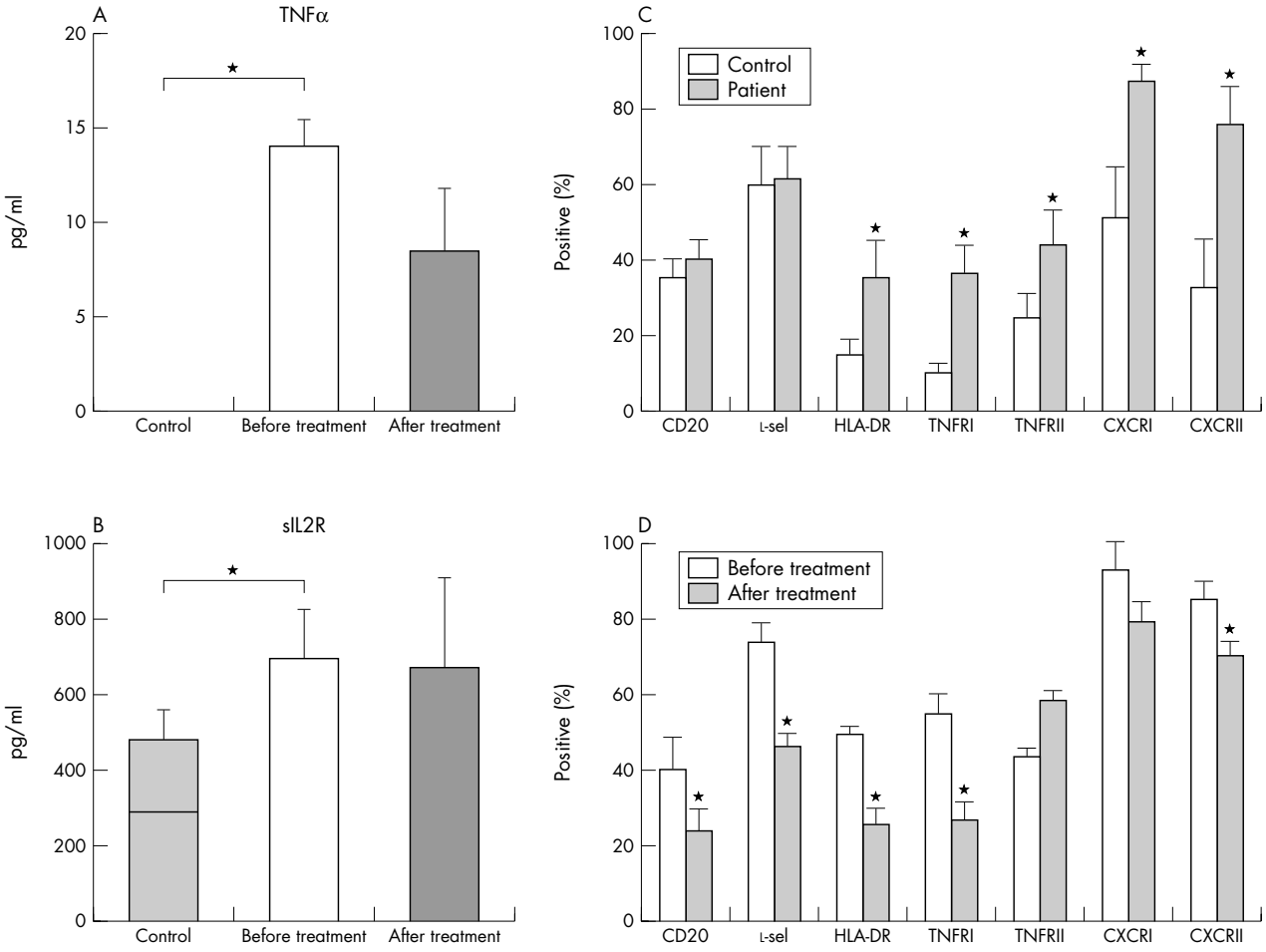

Figure 1 (A) TNFα levels in the plasma of patients and controls. TNFα was not detected in healthy controls but reached a mean (SEM) of 13.8 (1.5) pg/ml in untreated patients with SS (*$p<0.05$). After treatment TNFα levels were decreased, but not significantly. (B) sIL2R levels in the plasma of patients and controls. IL2R levels were higher in patients with SS than in healthy controls (*$p<0.05$). (C) Phenotypic comparison of PBMC from patients with SS and healthy controls. Fluorescent antibody (fluorescein isothiocyanate, phycoerythrin) staining for indicated surface markers expressed on the PBMC of patients with SS ($n=7$) before thalidomide treatment and on PBMC of age and sex matched controls ($n=7$), showed significantly increased (*$p<0.05$) expression of the indicated antigens in the patients. (D) Phenotypic analysis of PBMC before and after thalidomide treatment. After thalidomide treatment, cell surface staining for the indicated markers ($n=4$), showed a significant decrease of CD20, L-sel, HLA-DR, TNFRI, and CXCRII expression (*$p<0.05$).

were isolated from patient blood, and whole blood buffy coats obtained from healthy volunteers (NIH Blood Bank, Department of Transfusion Medicine, Bethesda, MD, USA) by Ficoll-LSM (Lymphocyte Separation Medium, ICN Biomedicals, Aurora, OH, USA) gradient centrifugation.

Cytokine and soluble receptor ELISA

Levels of TNFα, interleukin (IL) 6, and soluble IL2 receptor (sIL2R) were monitored in the plasma of seven patients and seven healthy volunteers, by enzyme linked immunosorbent assay (ELISA; Biosource, Camarillo, CA).

Flow cytometry

Expression of cell surface antigens on PBMC was measured by flow cytometry in an effort to detect a phenotypic shift associated with disease or treatment, or both ($n=7$). Monoclonal antibodies to cell surface antigens included markers of T cell subsets (CD3, CD4, CD8, CD45RO), B cells (CD20, CD21), and monocytes (CD14). Antibodies to costimulatory molecules (CD40, BioSource, Camarillo, CA, USA and CD40L, CD28, B7, CTLA-4), TNF receptors (R&D Systems, Minneapolis, MN, USA), activation markers (CD25, CD69, HLA-DR), chemokine receptors (CXCRI and II), and adhesion molecules (L-selectin (L-sel) were also used (all from Becton Dickinson, San Jose, CA, USA). The antibodies were conjugated with either fluorescein isothiocyanate or phycoerythrin. Cells were analysed by FACScalibur system (Becton Dickinson) and results were expressed as the percentage of positive cells.

Statistics

Mean values and standard errors were calculated, *t* tests and Wilcoxon tests were performed, and significance was reported at a value of $p \leqslant 0.05$.

RESULTS AND DISCUSSION

Clinical outcome

At the end of the study, a total of seven patients were enrolled, four of whom received the study drug (two patients received a final dose of 50 mg/day and two 100 mg/day) and three placebo for 3 weeks. All patients had been diagnosed with primary SS more than 5 years before entry into the study and had extensive glandular manifestations. All patients were positive for Schirmer and van Bijsterveld tests, unstimulated saliva was absent in all patients, and none were autoantibody positive. Extraglandular manifestations

included Raynaud's phenomenon, fatigue, vasculitis, and joint pain.

Because adverse effects were reported, three patients treated with thalidomide had to discontinue the study at 3 weeks and the protocol was modified, thereafter. The adverse effects identified included peripheral neuropathy, sedation, postural hypotension, rashes, vasculitis, and peripheral oedema,[6] and were probably attributable to the high starting dose of the drug (300 mg). Treatment of the remaining patients was started at 50 mg/day and increased by 50 mg each day, to achieve a maximal treatment dose of 300 mg. One patient reached a dose of 150 mg/day, when thalidomide had to be decreased to 50 mg/day and then discontinued at 3 weeks owing to severe adverse effects.

Despite the considerable adverse effects, the possibility that thalidomide may be beneficial and safe at much lower doses cannot be ruled out based on the lack of clinical results of the trial. The immunological response to thalidomide was monitored in the peripheral blood of these patients to identify potential markers associated with their immune status that might not have been evident clinically owing to the short duration of the study and which might provide the basis for monitoring subsequent trials.

Circulating cytokine levels

Although no significant differences were found in plasma IL6 between patients and controls (data not shown), TNFα was detectable in the plasma of the patients with SS before treatment (mean (SEM) 13.8 (1.5) pg/ml), but not in the healthy control group (<2.5 pg/ml, level of detection, $p = 0.001$, fig 1A), consistent with a recent study.[8] Raised circulating TNFα in the peripheral blood of patients with SS supported the suggestion that thalidomide might be effective in the treatment of this disorder and in our study, thalidomide treatment reduced TNFα levels (8.4 (3.2) pg/ml), but not significantly. Whether the lack of significance was due to the small number of patients or to the short duration of treatment, remains to be determined. Moreover, the ability of thalidomide to inhibit TNFα production in vitro has been less consistent in vivo, and in a recent trial, increases rather than decreases, in serum TNFα levels were seen after thalidomide treatment.[9] sIL2R levels were also significantly higher in patients than healthy controls (fig 1B), but were not significantly changed after treatment.

Phenotypic correlates of disease and treatment

Before treatment, no significant differences were noted in the percentages of T cells (CD3), B cells (CD20), or monocytes (CD14) in the patients with SS compared with healthy controls. Furthermore, expression of accessory molecules CD25, CD69, CD40, CD40L, CTLA-4, and B7 was not significantly different between the groups (data not shown). However, evidence of immune activation was seen in the patients. Firstly, cell surface TNF receptors I (TNFRI) and TNFRII were increased in patients with SS (fig 1C, $p = 0.004$ and $p = 0.03$). Secondly, chemokine receptors CXCRI and CXCRII ($p = 0.03$ and $p = 0.023$, respectively) were also up regulated in the patients, indicative of increased cell trafficking in the periphery. Thirdly, enhanced expression of the antigen presentation molecule HLA-DR is also consistent with peripheral immune activation.

After 3 weeks of thalidomide treatment, phenotypic changes began to emerge in the patient PBMC. Although no significant changes were evident in multiple PBMC subsets (CD3, CD4, CD8, CD21, CD14), the number of B cells (CD20) decreased after treatment, which may be attributed to a differential pattern of trafficking of this population, or to an actual population decrease. Selected markers associated with activation or antigen presentation (CD25, CD69, CD40, CD40L, CD28, B7, CTLA-4) remained unchanged, but HLA-DR expression decreased on the PBMC of treated patients. Whether expression is reduced on individual cells or HLA-DR+ cells are removed from the circulation has not yet been established. In addition, cell surface expression of the adhesion molecule L-sel decreased (fig 1D). Interestingly, a reduction in TNFRI ($p = 0.02$) was not accompanied by changes in levels of TNFRII in the periphery (fig 1D), although this may not be the case in the inflammatory lesion of the gland. Finally, expression of CXCRI and II chemokine receptors was reduced in the treated patients, possibly reflecting reduced recruitment potential to areas of disease.

Evidence of multiple systemic immunological consequences within weeks after the onset of thalidomide treatment, despite lack of quantifiable clinical efficacy during this short treatment interval, may offer insight into the immunoregulatory actions of thalidomide. The systemic response in patients with SS may underlie the beneficial role of thalidomide in other disorders and may aid in the understanding of thalidomide's mechanisms of action in vivo, although further studies of the immune response to thalidomide are needed to explain fully the mechanisms of its immunomodulatory action. Moreover, in the case of SS, the raised levels of TNFα and the evidence of systemic immune activation may support the use of thalidomide or other TNF inhibitors in a modified regimen. The use of a low dose treatment or a local delivery system might maximise the benefits of thalidomide, while minimising the possibility of adverse outcome.

Authors' affiliations

N M Moutsopoulos, N Angelov, V Sankar, R A Leakan, S Pillemer, S M Wahl, Oral Infection and Immunity Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bldg 30, Room 323, 30 Convent Dr, MSC4352, Bethesda, MD 20892, USA

Correspondence to: Dr S M Wahl, smwahl@mail.nih.gov

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