In vivo effect of Rituximab on regulatory T cells and apoptosis in patients with rheumatoid arthritis

M. Vigna-Pérez1, MSc, C. Abud-Mendoza2, MD, E. Cuevas-Orta2, MD, L. Baranda1,2, MD, O. Paredes-Saharopoulos1,2, MD, R. Moreno2, MD, R. González-Amaro1, MD, PhD

1Department of Immunology, Facultad de Medicina, UASLP. 2Regional Unit of Rheumatology and Osteoporosis, Hospital Central Dr. Ignacio Morones Prieto, San Luis Potosí, S.L.P., México.

ABSTRACT

The aim of this study was to assess the in vivo effect of anti-CD20 therapy with Rituximab on regulatory T lymphocytes, and apoptosis of immune cells in patients with rheumatoid arthritis (RA). In an open and pilot clinical trial, Rituximab was administered (1.0 g at days 1 and 30) to seven patients with RA that were refractory to conventional therapy. Peripheral blood samples were obtained at days 0, 15, and 180 of Rituximab therapy, and the number and function of regulatory T lymphocytes, as well as the presence of apoptotic cells were analyzed. Five out of seven patients showed significant clinical improvement upon Rituximab therapy (ACR response 50-70), and a significant increase in the number and function of regulatory T cells. In addition, an increased percent of apoptotic cells was detected in the peripheral blood after the onset of Rituximab therapy. These data suggest that Rituximab seems to exert an interesting effect on regulatory T cells in patients with RA; this phenomenon may contribute to the therapeutic effect of this anti-CD20 agent in RA.

KEY WORDS: Regulatory T cells/ Anti-CD20/Therapy/ Apoptosis.
INTRODUCTION

There is growing evidence that B cells participate in the pathogenesis of rheumatoid arthritis (RA)\(^3\)\(^2\). In this regard, it has been proposed that, in addition to its role in the synthesis of rheumatoid factor, in this condition B cells may function as potent antigen presenting cells (APCs), favoring the activation of CD4\(^+\) T cells\(^1\)\(^2\).

Rituximab is a chimeric monoclonal antibody (mAb) specific for the CD20 surface antigen expressed by mature B cells and their precursors\(^3\). This biological agent is very effective in depleting normal and malignant B lymphocytes in vivo\(^4\). For this reason, this chimeric mAb seems to be an important tool for the therapy of B cell-mediated autoimmune diseases\(^5\). In this regard, it has been suggested that Rituximab, alone or in combination with disease modifying anti-rheumatic drugs (DMARDs), induces a significant improvement in patients with RA\(^6\). Rituximab can induce the killing of CD20\(^+\) cells via multiple mechanisms, including antibody-dependent cytotoxicity, complement-mediated lysis, and induction of apoptosis\(^7\). However, the clinical and immunological relevance of this effect has not been utterly elucidated in RA.

Several subsets of CD4\(^+\) cells with immunomodulatory properties have been described, including regulatory T lymphocytes (T\(_{reg}\)) and regulatory type-1 T cells (Tr1)\(^8\)\(^9\). T\(_{reg}\) lymphocytes express CD4, CTLA-4, high levels of CD25, and are anergic\(^8\). These CD4\(^+\)CD25\(^{high}\) cells arise from the thymus as natural regulatory cells and exert their activity by cell-to-cell contact as well as by inducing the differentiation of CD4\(^+\)CD25\(^{low}\) lymphocytes into regulatory cells\(^9\). On the other hand, Tr1 lymphocytes synthesize IL-10, and are antigen specific cells derived from conventional CD4\(^+\)CD25\(^{low}\) naive precursors\(^9\). Different animal models of autoimmune disease, including collagen-induced arthritis, support the important role of regulatory T cells in the pathogenesis of these conditions\(^8\)\(^9\). In addition, the number and function of T\(_{reg}\) lymphocytes in patients with RA has been studied\(^3\)\(^1\)\(^2\). In this work, we investigated the possible in vivo effect of Rituximab on the function and/or number of regulatory T cells in the peripheral blood of patients with RA.

PATIENTS AND METHODS

**Patients.** Seven female patients with RA\(^3\)\(^3\) refractory to the conventional therapy with DMARD’s were studied. The main clinical data of the patients studied are shown in table I. Patients received 1.0 g of Rituximab at days 1 and 30 in addition to their therapy with DMARD’s (Table I). No modifications in the therapy were made during the study. None of these patients had received anti-TNF-\(\alpha\) agents nor received them during the study. In all cases, an informed consent was obtained, and this study was approved by the local hospital ethics committee.

At day 180 of Rituximab therapy, the seven patients, who had long time of evolution with a disease refractory to the conventional immunosuppressive therapy, were...
evaluated, and in five of them, a marked clinical improvement was observed (ACR 50-70, Table I).

**Blood samples and cell isolation.** Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque from all patients before (day 0) and at days 15 and 180 of Rituximab therapy. CD4+ and CD25+ lymphocytes were purified using MACS LS separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany).

**Quantification of TREG cells.** PBMNC were double immunostained with anti-CD4 and anti-CD25 mAbs (BD Pharmingen, San Diego, CA) or with anti-CD4 and anti-CTLA-4 mAbs (BD Pharmingen), and then analyzed by flow cytometry, as described\(^ {14}\). CD4+CD25\(^{bright}\) cells were defined as CD4+ lymphocytes that showed a higher expression of CD25 than autologous CD8+ lymphocytes activated in vitro with PHA\(^ {14}\).

**Cytokine secretion assays.** The percent of Tr1 lymphocytes synthesizing IL-10 was obtained by using the appropriate commercial kits (Miltenyi), according to the manufacturer instructions, and flow cytometry analysis\(^ {14}\).

**Detection of apoptosis.** Fresh isolated PBMNC were fixed and stained by the TUNEL technique using the ApoDirect kit (BD Pharmingen). In additional experiments, PBMNC from the same sample were stained with Annexin V labeled with FITC and propidium iodide and analyzed by flow cytometry.

**Cell proliferation assays.** In experiments run by triplicate, non-regulatory CD4+CD25– T cells (1x10\(^5\)) were mixed with CD4+CD25– regulatory T cells (1x10\(^5\)) in the presence of phytohemagglutinin (PHA, 5.0 \(\mu\)g/ml) and cultured for 72 h in 96 well plates. \(^{3}\)H-TdR (1 \(\mu\)Ci/well, New England Nuclear, Boston, MA) was added for the last 12 h of culture, and at the end of incubation cells were harvested and proliferation was determined using a liquid scintillation counter.

**Statistical analysis.** Data were compared with the Sigma STAT software (SPSS Inc., Chicago, IL) using Wilcoxon, and T paired tests with a level of significance of p<0.05.

** RESULTS**

We first determined the percent of regulatory T cells in the peripheral blood of RA patients after and before of Rituximab therapy. A significant increase of the percent of CD4+CD25\(^{bright}\) T lymphocytes was observed at days 15 and 180 of Rituximab therapy (p<0.05, Fig. 1A). However, this enhancement was not observed in one patient, and in another one, the increase in TREG cells was very modest (Fig. 1A). Similar results were found, but only at day 15, in the case of CD4+CTLA-4+ and CD4+TGF-\(\beta\)1+ cells (p<0.05, Fig. 1A). In contrast, no significant changes in the levels of CD4+IL-10+ cells were observed (Fig. 1A).

We then studied the function of TREG cells in RA patients. We observed a significant increase in the regulatory function of TREG cells at days 15 and 180 of Rituximab therapy (p<0.05 in both cases, Fig. 1B). However, this phenomenon was not apparent in one case at day 15, and in two additional patients, only a very modest enhancement in the function of TREG cells was observed (Fig. 1B).

We also assessed the possible in \(\textit{vivo}\) effect of Rituximab on the apoptosis of PBMC in the patients included in this study. We found an important and significant increase in the percent of TUNEL+ cells at day 15 of Rituximab therapy (p<0.05, Fig. 2). The presence of apoptotic cells in these samples was confirmed by Annexin V staining (data not shown). This effect was also observed at day 180, but with a significant less number of apoptotic cells (p<0.05, Fig. 2).

** DISCUSSION**

There is growing evidence that B cells play an important role in the pathogenesis of RA\(^ {1,2}\), and several reports have suggested that anti-CD20 mAb administration has a beneficial effect in patients with this condition\(^ {10}\). However, the mechanism of action of Rituximab in patients with RA has not been fully elucidated. In this regard, although it is evident that Rituximab is able to induce a long-lasting depletion of B cells, the consequences of this effect on the pathogenesis of the joint destruction characteristic of RA remain to be defined. In this pilot study, we explored the possible in \(\textit{vivo}\) effect of Rituximab on the number and function of regulatory T cells in patients with RA refractory to the conventional immunosuppressive therapy with different combinations of DMRAD’s.

Regulatory T cells play an important role in the inhibition of auto-reactive T cells and the maintenance of self-tolerance\(^ {8}\). The CD4+ TREG lymphocytes are the most extensively characterized cell subset with regulatory function\(^ {10}\). These cells are generated in the thymus («natural» regulatory cells), and express high levels of CD25. The possible role of these cells in the pathogenesis of RA has been addressed both in humans and in animal models\(^ {10,12}\). In addition, the effect of therapeutic biological agents on these regulatory cells has been also explored\(^ {10,11}\). In this work, we have found that the administration of Rituximab to patients with RA is associated with a significant increase in the number and function of TREG cells. Although we studied a small number of patients, our results indicate that those patients with a poor clinical response to Rituximab showed a modest...
or null enhancement of T<sub>REG</sub> cells, suggesting a possible association between the therapeutic effect of Rituximab and the modification of regulatory T cells. In this regard, we, and others, have previously found that anti-TNF-α agents (Infliximab, Adalimumab) enhance the levels and the regulatory function of T<sub>REG</sub> cell in patients with RA<sup>15, 16</sup>. In addition, we have recently reported that the administration of Rituximab to patients with systemic lupus erythematosus
is also associated with a significant and sustained increase in the number and function of CD4+CD25+ regulatory lymphocytes. However, the causal association between Rituximab administration and modification of TREG cells, and in turn, between the enhancement in TREG function and the therapeutic effect of this biological agent remain to be determined. Likewise, the possible mechanisms involved in the stimulation of regulatory cells by therapeutic biological agents remain to be elucidated. However, it is of interest that in the case of TNF-α blocking agents, it has been recently reported that this cytokine downmodulates the function of TREG lymphocytes, a phenomenon that may account for the enhancing effect of anti-TNF-α agents on these cells.

In the case of Rituximab, it is not easy to elucidate the possible link between the effect of this biological agent on B cells and the putative stimulation of TREG cells observed by us. However, we consider that the depletion of B lymphocytes acting as antigen presenting cells induced by Rituximab, would favor lower levels of activated T cells, which bear the ligand of the glucocorticoid-induced tumor necrosis factor receptor (GITRL). This ligand is able to inhibit the function of TREG cells, and therefore, a reduction in the levels of GITRL+ cells would enhance the number and function of TREG lymphocytes, a phenomenon that could account for the enhancing effect of anti-TNF-α agents on these cells.

In summary, our preliminary results suggest that Rituximab therapy is associated with an enhancement in the levels and function of TREG cells. We consider that these data underlines the importance of elucidating the overall effect of anti-B cell therapy in patients with RA, and other autoimmune diseases.

DISCLOSURES

The authors have no financial conflict of interest.

REFERENCES


