B cells and B cell directed therapies in rheumatoid arthritis: towards personalized medicine
Thurlings, R.M.

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B CELLS AND B CELL DIRECTED THERAPIES IN RHEUMATOID ARTHRITIS

TOWARDS PERSONALIZED MEDICINE

R.M. Thurlings
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TOWARDS PERSONALIZED MEDICINE

R.M. Thurlings
The studies performed in this thesis were performed at the Department of Clinical Immunology & Rheumatology, Academic Medical Center, University of Amsterdam, the Netherlands.
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1

GENERAL INTRODUCTION

B Cells and B Cell-directed therapies in Rheumatoid Arthritis
INTRODUCTION

RHEUMATOID ARTHRITIS (RA) is a chronic inflammatory condition of unknown origin which affects around 1% of the population worldwide. Patients experience swelling, pain and limited motion of joints due to inflammation of the synovial tissue lining the inside of joints. Characteristically, RA manifests itself as a symmetric polyarthritis that involves the metacarpophalangeal joints. Some patients may experience a mild illness, but the majority of patients suffer from an invalidating condition that during its course leads to development of joint destruction, progressive invalidity and associated morbidity and mortality.

In this thesis we investigate B cells, key players in the pathogenesis of RA, and B cell directed therapy to further improve the treatment of RA.

DEVELOPMENTS IN THE DIAGNOSIS OF RA There is no specific diagnostic test to differentiate RA from other types of arthritis. However, around 70-80% of patients have elevated serum levels of rheumatoid factor (RF), autoantibodies directed against antibodies of the IgG class. The classification criteria for RA, designed for epidemiological studies, were first defined in 1958 and revised in 1987. They include the presence of a symmetric polyarthritis and/or rheumatoid factor, extra-articular manifestations and bone erosions.

In the 1990s it was found that around 70-80% of RA patients also have antibodies against citrullinated peptides. The presence of anti-citrullinated peptide antibodies (ACPA) is more specific for RA than the presence of RF. ACPA and RF were also shown to be present before the onset of manifest arthritis. The presence of ACPA has therefore been incorporated in the recently revised diagnostic criteria for RA. These criteria have been adapted to increase the sensitivity to detect RA at an early disease stage. The emphasis has been shifted from features present at a late disease stage, such as erosive joint damage, to features present at an early stage that predict the development of persistent and destructive disease. These features include the presence of RF or ACPA, the extent and pattern of joint involvement and the presence of an acute phase response.

DEVELOPMENTS IN THE TREATMENT OF RHEUMATOID ARTHRITIS During the last fifteen years the treatment of RA has markedly improved. First, as mentioned above, better diagnostic markers have been developed, resulting in recognition of the disease in an earlier stage. Second, RA is treated more aggressively. Disease-modifying antirheumatic drugs (DMARDs), especially methotrexate, have replaced non-steroidal anti-inflammatory drugs (NSAIDs) as first-line treatment. Third, increasing knowledge of the underlying pathogenetic process has resulted in a growing armoury of new treatments. These new, targeted, treatments have supplemented and in part replaced conventional DMARDs. They have been designed using a biotechnological approach and are therefore called ‘biologics’. The first biologicals registered for RA block the function of the cytokine TNF, which is abundantly present in the synovial tissue of RA patients. These TNF blockers are infliximab (a chimeric antibody), adalimumab (a humanized antibody) and etanercept (a soluble receptor). More recently, certolizumab (a pegylated antibody fragment) and golimumab (a fully human monoclonal antibody) were registered. In patients who fail initial treatment with methotrexate or other DMARDs, treatment with a combination of a TNF blocker and methotrexate is effective in a subset of RA patients. In randomized controlled trials a 20% improvement in disease activity according to the American College of Rheumatology (ACR) response criteria was found in around 50-80% of the patients, a 50% improvement in 20-50% of patients and a 70% improvement in 10-25% of patients, which was statistically significant when compared to placebo treatment.

Other biologicals that have been registered as treatment for RA are rituximab, which depletes CD20 positive B cells, abatacept, which blocks the interaction between CD80 and CD86 on T cells and antigen presenting cells, and tocilizumab, which blocks the IL-6 receptor. These biologicals induce on average a decrease in disease activity in a similar percentage of patients compared to TNF blockers. Despite the advent of these new treatments early disease remission is only achieved in a proportion of patients and patients need to be treated with often relatively expensive treatments. There is therefore a continued need to better understand the disease pathophysiology to further improve treatment of RA.

RA PATHOPHYSIOLOGY The synovial tissue normally consists of an intimal lining layer, comprising a few cell layers of fibroblast-like synoviocytes, above a loose tissue, called the synovial sublining layer, which consists of a network of collagen fibres and scattered fibroblasts and blood vessels. In RA patients the synovial tissue mass is increased due to influx of inflammatory cells and proliferation of synoviocytes. The hyperplastic synovial tissue invades adjacent cartilage and bone, ultimately resulting in joint destruction. The inflammatory cell infiltrate consists of macrophages, mast cells, natural killer cells, dendritic cells, T cells, B cells, plasma cells, and neutrophils. These cells secrete diverse cytokines, chemokines and other inflammatory mediators.
The etiology of RA is currently unknown. A body of evidence indicates that genetic predisposition, environmental factors and immune mechanisms are involved in its pathophysiology. The strongest genetic link is that between RA and the presence of a polymorphism in HLA-DRB1, encoding the ‘shared epitope’. Recent genome-wide association studies have identified weaker associations between RA and polymorphisms in other risk loci. The causal genetic mutations still have to be determined for the majority of these risk loci. Up till now, genetic data suggest a link between RA, inflammatory pathways and defective antigen presentation. Furthermore, epidemiologic studies have found an association between RA and smoking. Supporting evidence for other environmental risk factors is weak.

With regard to immunological mechanisms the different inflammatory cells and mediators that are present in the inflamed synovial tissue have shown to play a role in RA pathophysiology.

**HETEROGENEITY IN RA PATHOPHYSIOLOGY** Multiple lines of evidence suggest that RA is a shared clinical manifestation of different pathogenic conditions. On the clinical level this is suggested by the fact that the severity and course of arthritis differ between RA patients and that bone erosions and extra-articular manifestations do not always occur. Furthermore, certain genetic and environmental factors, such as polymorphisms in HLA-DRB1 and smoking, predispose to RA, but do not occur in all patients. On the biological level, different immunological mediators, such as B cells, T cells, macrophages, diverse cytokines and chemokines, have been shown to play a role in RA, but the variable response to targeted treatments suggests that the role of immunological mediators, such as IL6 and TNFα, differs between patients. In line with this hypothesis, detailed immunological analyses have shown considerable variability in immune responses between different patients. For instance, the extent and pattern of lymphocyte infiltration in the synovial tissue varies widely between patients. In some patients a diffuse or scarce infiltrate is found, while in other lymphocyte aggregates are found with characteristics resembling those of germinal centers of lymphoid tissue, a process which is called lymphoid neogenesis. In several small patient cohorts the presence of synovial lymphoid neogenesis was correlated with the presence of RF and erosive disease. In peripheral blood, microarray analysis of gene expression in peripheral blood mononuclear cells has shown a signature consistent with activation of different inflammatory cells that regulate lymphoid tissue architecture and ectopic lymphoid neogenesis.

In line with this hypothesis, detailed immunological analyses have shown considerable variability in immune responses between different patients. For instance, the extent and pattern of lymphocyte infiltration in the synovial tissue varies widely between patients. In some patients a diffuse or scarce infiltrate is found, while in other lymphocyte aggregates are found with characteristics resembling those of germinal centers of lymphoid tissue, a process which is called lymphoid neogenesis. In several small patient cohorts the presence of synovial lymphoid neogenesis was correlated with the presence of RF and erosive disease. In peripheral blood, microarray analysis of gene expression in peripheral blood mononuclear cells has shown a signature consistent with activation of different inflammatory cells that regulate lymphoid tissue architecture and ectopic lymphoid neogenesis.

**THE ROLE OF B CELLS IN RA PATHOPHYSIOLOGY** When focussing on B cells a role for B cells has been proven by the effect of rituximab in RA. However, the clinical response to rituximab differs between patients, which suggests that the role of B cells may differ between patients. B cells are important as producers of autoantibodies. RA is related to the presence of diverse autoantibodies. As mentioned, the two most frequently occurring are RF and ACPA, which occur in about 70% of the patients. As mentioned above, RF are autoantibodies directed against autologous antibodies of the IgG class. RF were for long regarded as an epiphenomenon, since IgG is a ubiquitous antigen and IgM-RF-IgG complexes are too large to enter the synovial tissue. However, recent experimental research suggests that certain RF isotypes are capable of entering the synovial tissue and sustaining RA synovial inflammation. Furthermore, it has been shown that RF and other autoantibodies are produced locally in the synovial tissue.

ACPA are directed against citrullinated proteins. Citrullination is a form of post-translational modification of proteins, in which the amino acid arginine is converted into citrulline. This process occurs amongst others in the inflamed synovium, but also in other inflamed tissues. The citrullinated antigens against which ACPA are directed differ between patients. In most cases their target is citrullinated fibrinogen, in others vimentin or α-enolase or type II collagen. Presence of RF and ACPA has been associated with a history of smoking, polymorphisms in HLA-DRB1, a more aggressive disease course and an improved response to rituximab.

B cells may have multiple additional potential roles in RA. After B cells are activated they acquire distinct phenotypes. They differentiate either into antibody secreting plasma cells, central memory B cells or one of the effector B cell types. Effector B cells secrete polarized arrays of cytokines, dependent on the mode in which they are stimulated. Effector B cells can activate T cells and thereby stimulate their proliferation, differentiation and polarization, and enhance/sustain the activation of primed T cells. In line with this, T cell activation in the synovial tissue of RA patients is dependent on the presence of B cells. Furthermore, B cells belong to the cells that regulate lymphoid tissue architecture and ectopic lymphoid neogenesis, which, as mentioned, also occurs in RA synovial tissue. Finally, B cells cross-talk with dendritic cells in the process of T cell activation and can acquire a regulatory phenotype. It is however unknown whether this also relevant for RA.

Taken together, the precise relationship between B cell related immunological mechanisms, genetic and environmental risk factors, clinical characteristics and treatment response has not been elucidated. Analysis of these relationships and the precise mechanisms of B cell related therapies may yield biomarkers to predict response and help to design novel treatments.
B Cells and B Cell directed therapies in Rheumatoid Arthritis

- **IgG RA using atacicept, a fusion molecule of the soluble TACI receptor and**
- **Alternatively, one might interfere with the humoral response in RA using atacicept, a fusion molecule of the soluble TACI receptor and IgG**. The TACI receptor binds the B-cell associated factors B-Lymphocyte Stimulator (BLyS), A Proliferation-inducing Ligand (APRIL) and the heterodimer of these 2 proteins - BLyS and APRIL are involved in B cell survival, differentiation and class-switching during different stages of B cell development. Both BLyS and APRIL levels are elevated in blood and synovial fluid of RA patients. Mice transgenic for BAFF spontaneously develop autoimmune manifestations. Atacicept treatment could perhaps represent an alternative therapeutic approach in RA. Its application may also increase our understanding about the role of BLyS and APRIL in RA pathogenesis.

**AIR AND OUTLINE OF THIS THESIS** As a result of the development of new treatments for RA and their application at an early disease stage disease, progressive joint destruction can currently be inhibited in the majority of the patients. Nonetheless, the response to currently registered treatments differs between RA patients and disease remission is only achieved in a proportion of the patients. Furthermore, patients need to be treated chronically with often relatively expensive drugs. There is therefore a need to further improve the treatment of RA. The ultimate goal is to achieve remission in every patient by early intervention based on the treat to target principle. The use of biomarkers may perhaps facilitate the optimal choice of specific therapies in the context of personalised medicine. To achieve this, a number of steps need to be taken: first, biomarkers need to be identified that can predict which patients will benefit most from a specific treatment. Second, we need to understand which immune mechanisms continue to drive the disease in patients do not respond to current therapies. This knowledge can be used to develop novel therapies and to optimize current treatment schedules. Finally, we need to investigate the safety and efficacy of novel treatments.

In this thesis we focus on B cells and B cell-directed therapies. In chapter 2 and 3 we analyse the association between synovial lymphoid neogenesis, clinical and immunological characteristics of RA and clinical response to TNF blockade. In chapter 4 till 7 we investigate the mechanism of action of rituximab in the synovial tissue and peripheral blood in relationship to clinical response. In chapter 8 to 10 we study the current rituximab treatment schedule: in chapter 8 we analyse rituximab levels and formation of anti-rituximab antibodies in relationship to the extent of B cell depletion and the clinical response to rituximab. In chapter 9 and 10 we examine the efficacy of rituximab in a disease activity based schedule, in initial responders versus non-responders to rituximab. In chapter 11 we describe a phase Ib clinical trial to study the safety and efficacy of atacicept for treatment of RA.
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LYMPHOID NEOGENESIS DOES NOT DEFINE A SPECIFIC CLINICAL RHEUMATOID ARTHRITIS PHENOTYPE
Abstract

OBJECTIVE To investigate the relationship between lymphoid neogenesis in the synovium of patients with rheumatoid arthritis (RA) and characteristics of inflammation and disease severity.

METHODS Arthroscopic synovial biopsy was performed in 103 patients with active RA (Disease Activity Score 28-joint assessment ≥ 3.2) who had not received treatment with biologic agents. Sections were stained and assessed by digital image analysis. Lymphocyte aggregates were counted and graded for size (1-3). Synovial lymphoid neogenesis was defined as the presence of grade 2 or 3 aggregates and subclassified based on the presence of follicular dendritic cells (FDCs).

RESULTS Lymphoid neogenesis was present in 31% of the RA synovial tissues, whereas an additional 25% contained only grade 1 aggregates. FDCs were present in 28% of the samples with lymphoid neogenesis, corresponding to 8% of the total RA cohort. Histologically, synovia with lymphoid neogenesis showed increased infiltration by T and B lymphocytes, plasma cells, and macrophages, and increased expression of tumor necrosis factor α and lymphotoxin β compared with samples without lymphoid neogenesis. Patients with lymphoid neogenesis also had higher C-reactive protein levels, erythrocyte sedimentation rates, and leukocyte and thrombocyte counts, but exhibited no increase in the severity of clinical signs and symptoms. Of importance, there was no relationship between the presence of lymphoid neogenesis and IgM rheumatoid factor or anti–citrullinated protein antibodies. The presence of lymphocyte aggregates with FDCs did not define a specific clinical phenotype compared with lymphocyte aggregates without FDCs.

CONCLUSION These findings indicate that synovial lymphoid neogenesis is associated with more severe synovial and systemic inflammation, but this is not confined to a specific clinical subset of RA.

Introduction

The extent and pattern of lymphocyte infiltration in the inflamed synovial tissue varies widely among patients with rheumatoid arthritis (RA). In some tissues there is diffuse or scarce infiltration of T cells, while in others B and T cells are organized in perivascular aggregates with surrounding fields of plasma cells. Sometimes there are large aggregates that contain clusters of follicular dendritic cells (FDCs) and exhibit features normally found in germal centers of lymphoid tissue. This suggests that lymphoid neogenesis occurs in rheumatoid synovial tissue.
It has been proposed that synovial tissues with diffuse infiltration and tissues with lymphoid neogenesis represent different pathophysiologic subtypes of RA. A further subdivision, into highly organized "germinal center–like" lymphoid neogenesis and a less organized form of lymphoid neogenesis in which T and B cells form aggregates but exhibit no other germinal center–like features, has been suggested. In contrast, other studies have shown that lymphoid neogenesis in rheumatoid synovial tissue is a more continuous spectrum, with lymphocyte aggregates exhibiting different features of germinal centers in the same patient. A stable presence of synovial lymphoid neogenesis and a relationship with a distinct clinical phenotype have been suggested in some studies. First, it has been demonstrated that lymphoid neogenesis is present even at the onset of clinical arthritis. In accordance with this, it has been suggested to be a "fixed" feature of synovial inflammation within different joints and patients. Furthermore, the presence of lymphoid aggregates in synovial tissues has been shown to be associated with increased expression of cytokines and adhesion molecules locally in the joint as well as in the peripheral blood. Finally, different histologic subtypes of lymphocyte infiltration appeared to be correlated with the presence of rheumatoid factor (RF), rheumatoid nodules, and joint erosiveness in studies of small RA cohorts.

Since previous studies were small and mainly based on analyses of synovial tissue obtained during joint surgery from patients with end-stage, destructive disease, it is still unclear whether lymphoid neogenesis defines a specific subset of RA and whether it is related to the presence of circulating autoantibodies. Therefore, we analyzed synovial tissue samples from a large cohort of patients with active RA for the presence of lymphoid neogenesis in relation to clinical features and autoantibody status.

PATIENTS AND METHODS

PATIENTS. Synovial tissue was obtained from 103 patients with RA fulfilling the 1987 revised criteria of the American College of Rheumatology (formerly, the American Rheumatism Association). All patients were taking methotrexate (25–50 mg/week) and all had active disease, defined as a Disease Activity Score 28-joint assessment (DAS28) of 5.1 or greater at the time of biopsy. Oral corticosteroids (<10 mg/day) and nonsteroidal antiinflammatory drugs were also allowed. Disease activity assessed included disease duration, number of prior disease-modifying antirheumatic drugs (DMARDs) taken, presence of IgM-RF, presence of anti–citrullinated protein antibodies (ACPAs) as measured by anti–cyclic citrullinated peptide 2 enzyme-linked immunosorbent assay (Immunoscan RA, Mark 2 [no. RA-96RT]; Euro-Diagnostica Arnhem, The Netherlands), and radiographic damage as evaluated by the Sharp/van de Heijde score (SHS). Disease activity was assessed based on the DAS28, the 28-joint tender and swollen joint count, the patient’s assessment of global disease activity on a visual analog scale (VAS), the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP) level, hematoglobin level, and thromboctye and leukocyte counts. The study was conducted in compliance with the Helsinki Declaration, and the Medical Ethics Committee of the Academic Medical Center, University of Amsterdam approved the protocol. All patients provided written informed consent.

IMMUNOHISTOCHEMICAL ANALYSIS. Synovial tissue sections were stained using the following monoclonal antibodies: anti-CD35 (67; Serotec, Oxford, UK) to detect fibroblast-like synoviocytes, anti-CD68 (EBM11; Dako, Glostrup, Denmark) to detect macrophages, anti-CD3 (SK7; Becton Dickinson) for T cells, anti-CD21 (CLB-B-H/1.681; Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) for B cells, and anti-CD98 (HB7; Becton Dickinson) for plasma cells. For detection of FDCs, anti–CD21 long isoform (anti-CD21L [16]) (a kind gift from
B Cells and B Cell directed therapies in Rheumatoid Arthritis

Dr. Y. J. Liu, M. D. Anderson Cancer Center, Houston, TX) was used.

Markers used for detection of cytokines were antihuman tumor necrosis factor α (anti-TNF α) (52B83; Monosan, Uden, The Netherlands) and anti–lymphotxin β (anti-LTβ) (C0404; Santa Cruz Biotechnology, Santa Cruz, CA). Staining of cellular markers was performed using a 3-step immunoperoxidase method, as previously described 1. For staining of cytokines, biotinylated tyramine was used for amplification, as previously described 2. As a negative control, irrelevant immunoglobulins were applied to the sections instead of the primary antibody, or the primary antibody was omitted.

ASSESSMENT OF LYMPHOCYTE AGGREGATES. The presence of lymphocyte aggregates was assessed on anti–CD3–stained sections. Aggregates were counted and graded by size according to the method described by Manzo et al 3, with slight modification, as follows: Aggregate size was assessed by counting the number of cells in a radius starting from an estimated center of the aggregate. Aggregate size was then classified as grade 1 (1–5 cells in the radius), grade 2 (5–10 cells in the radius), or grade 3 (>10 cells in the radius). Tissue sections with no lymphocyte aggregates were graded as 0.

The presence of FDCs in lymphoid aggregates observed with CD21L staining was assessed at 3 different tissue levels; the size of lymphoid aggregates and the presence of T–B cell segregation were assessed at 2 different tissue levels at least 50 μm apart on sequential sections stained with CD3 and CD22. Thus, multiple sections representing different levels of a tissue block, and consisting of at least 6 biopsy specimens, were examined to minimize sampling error.

DIGITAL IMAGE ANALYSIS. All sections were analyzed in random order by trained readers who were blinded with regard to the patient’s clinical characteristics. The analysis was performed using a computer-assisted image analysis algorithm, as previously described in detail 4. Images were acquired and analyzed using a Syndia algorithm on a Quin-based analysis system (Leica, Cambridge, UK). Positive staining of cellular markers was expressed as the number of positive cells/mm², and positive staining of cytokines was expressed as integrated optical density/mm². CD68+ macrophages, LTβ expression, and TNFα expression were analyzed separately in the internal lining layer and the synovial sublining.

STATISTICAL ANALYSIS. Independent t-tests or Mann-Whitney U tests were used to compare synovial (CD68+, CD3+, CD22+, and CD38+ cells), serologic (ESR, CRP level, and leukocyte and thrombocyte counts), and clinical (DAS28) parameters of inflammation, and SHS. The chi-square test was used to evaluate specific features of ACPA- and IgM-RF-positive disease and to compare these latter parameters between patients with synovitis and lymphocyte aggregates containing FDCs versus patients in whom lymphocyte aggregates did not contain FDCs. SPSS 12.0.2 for Windows (SPSS, Chicago, IL) was used for analysis.

Results

CHARACTERISTICS OF THE STUDY PATIENTS.

Demographic and clinical features of the 103 patients are shown in Table 1. In addition to the methotrexate treatment received by all patients, 25% were taking oral low-dose corticosteroids. Patients had been treated unsuccessfully with an average of 2.1 DMARDs prior to inclusion in the study. Data on rheumatoid nodules were available for 58 patients, of whom 22 (38%) had nodular RA.

FREQUENCIES OF LYMPHOCYTE AGGREGATES AND FDCS.

Lymphocyte aggregates of different sizes were present in the synovial tissue of 57 patients. In 16 patients (16%), aggregates up to grade 3 were present. In another 15 patients (15%), aggregates up to grade 2 were found, and 26 patients (25%) had only small perivascular infiltrates (grade 1 aggregates). In the remaining 46 patients (45%), no lymphocyte aggregates were detectable (Figure 1).

Of the 103 synovial samples, 95 could be analyzed for FDC staining. Eight of 16 samples with grade 3 lymphocyte aggregates showed CD21L+ staining (i.e., 50% of tissues containing grade 3 aggregates and 8% of

**FIGURE No.1**

**FIGURE 1** Different patterns of lymphocyte infiltration in representative synovial tissue specimens from patients with rheumatoid arthritis. In some patients, mixed infiltration of aggregates of T and B cells was present (A and C), together with a high number of infiltrating macrophages (C). In other patients, there was diffuse or scarce infiltration of CD3+ T cells (B), and few or no B cells (D), while macrophages were the dominant infiltrating cell population (F). (Original magnification x 20.)
Baseline patient characteristics of the 103 rheumatoid arthritis patients

**DEMOGRAPHICS**

- Age, mean ± SD years: 55 ± 13
- Female, no. (%): 73 (71)

**DISEASE STATUS**

- Duration, mean ± SD months: 130 ± 116
- Erosive disease, no. (%): 79 (77)
- RF positive, no. (%): 76 (74)
- ACPA positive, no. (%): 76 (74)
- DAS28, mean ± SD: 5.9 ± 1.1
- ESR, median (IQR) mm/hour: 33 (18-45)
- CRP, median (IQR) mg/dl: 12 (5-29)

**TREATMENT**

- No. of previous DMARDs, mean ± SD: 2.1 ± 15
- MTX dosage, mean ± SD mg/week: 18.1 ± 8.5
- Receiving corticosteroids, no. (%): 53 (52)
- Receiving NSAIDs, no. (%): 26 (26)

* SD = standard deviation; RF = rheumatoid factor; ACPA = anti–citrullinated peptide protein antibodies; DAS28 = Disease Activity Score 28-joint assessment; ESR = erythrocyte sedimentation rate; IQR = interquartile range; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; NSAIDs = nonsteroidal anti-inflammatory drugs.

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**FIGURE No.2**

**A** Follicular dendritic cells (FDCs) expressing the CD21 long isoform (A), detected in CD22+ B cell–containing lymphocyte aggregates (B). Synovial tissue samples from 8% of the rheumatoid arthritis patients contained lymphocyte aggregates with CD22+ B cells surrounding FDCs. (Original magnification x 20; x 40 in inset.)

**TABLE No.1**

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<td>Female, no. (%)</td>
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<td><strong>DISEASE STATUS</strong></td>
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<td>ACPA positive, no. (%)</td>
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<tr>
<td>MTX dosage, mean ± SD mg/week</td>
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<tr>
<td>Receiving corticosteroids, no. (%)</td>
</tr>
<tr>
<td>Receiving NSAIDs, no. (%)</td>
</tr>
</tbody>
</table>

* SD = standard deviation; RF = rheumatoid factor; ACPA = anti–citrullinated peptide protein antibodies; DAS28 = Disease Activity Score 28-joint assessment; ESR = erythrocyte sedimentation rate; IQR = interquartile range; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; NSAIDs = nonsteroidal anti-inflammatory drugs.
B Cells and B Cell directed therapies in Rheumatoid Arthritis

- Clinical Parameters
  - Data are from 44 patients with diffuse synovitis and 14 with lymphoid neogenesis.
  - When only ACPA-positive patients were analyzed, the trend toward an inverse correlation between lymphoid neogenesis and joint destruction was no longer found (P = 0.178 and P = 0.332, respectively).
  - There was also no relationship between lymphoid neogenesis and the presence of rheumatoid nodules (Table 2).

- Serological Parameters
  - Associations of clinical, serologic, and synovial parameters with lymphoid neogenesis:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Diffuse Synovitis</th>
<th>Lymphoid Neogenesis</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease Activity Index (DAS28, mean ± SD)</td>
<td>5.9 ± 1.1</td>
<td>6.1 ± 1.1</td>
<td>0.330</td>
</tr>
<tr>
<td>Erythrocyte Sedimentation (ESR, mm/hour)</td>
<td>30 (15-41)</td>
<td>36 (23-70)</td>
<td>0.031</td>
</tr>
<tr>
<td>Hemoglobin (mmol/liter)</td>
<td>7.8 (7.2-8.4)</td>
<td>7.8 (6.6-8.5)</td>
<td>0.307</td>
</tr>
<tr>
<td>Leucocytes (10⁹/liter)</td>
<td>7.2 (5.9-8.6)</td>
<td>9.3 (6.8-11.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>RF positive, no. (%)</td>
<td>54 (75)</td>
<td>22 (71)</td>
<td>0.422</td>
</tr>
<tr>
<td>ACPA positive, no. (%)</td>
<td>58 (81)</td>
<td>18 (58)</td>
<td>0.018</td>
</tr>
</tbody>
</table>

- Cytokines
  - TNFα, lining, IOD/mm²: 30 (23-70) vs 30 (11-71), P = 0.933
  - CD55: 5345 (402-934) vs 569 (303-1066), P = 0.332

- Cellular Markers, Counts/mm²
  - CD3: 32 (37-49) vs 381 (220-678), P = 0.001
  - CD22: 0 (0-15) vs 88 (35-164), P = 0.001
  - CD68: 30 (0-144) vs 631 (253-1086), P = 0.001

- Table 2: Associations of clinical, serologic, and synovial parameters with lymphoid neogenesis

- Although previous work has demonstrated the sensitivity of immunohistochemistry, compared with PCR, in detecting CD21L+ cells, we performed PCR to detect CD21L messenger RNA (mRNA) in a subset of 12 patients. In 2 of the 12 samples, CD21L mRNA was detected. In both of these samples we also detected CD21L protein. One sample tested positive for CD21L protein but not for CD21L mRNA. (It should be noted that immunohistochemistry allows the detection of scarce, isolated cells.) Thus, these results suggest that the presence of FDCs was not underestimated in this study.

- In a previous study it was suggested that there is a distinction between highly organized, FDC-containing, "germinal center--like" lymphoid aggregates and a less organized form of lymphoid neogenesis in which T and B cells form aggregates but no FDCs or other germinal center--like features are present. Therefore, in a subanalysis, we compared patients with lymphoid neogenesis with FDC positivity (n = 8 [28%]) versus patients with lymphoid neogenesis without FDCs (n = 21 [72%]). The only significant differences were in disease activity, autoantibody status, or joint destruction. There was no significant difference in disease activity, autoantibody status, or joint destruction between these 2 groups (data not shown).

**LYMPHOID NEOGENESIS WITH FDC POSITIVITY IS NOT RELATED TO INCREASED INFLAMMATION.**
DISCUSSION

The current study was performed to investigate whether lymphoid neogenesis in RA synovium is related to specific clinical or immunologic features. Previous data suggesting that RA characterized by the presence of lymphocyte aggregates or ectopic germinal centers represents a specific subset were obtained in small cohorts of patients with end-stage, destructive disease. In contrast, the present study was performed in a large and well-characterized cohort of RA patients with active disease despite methotrexate treatment.

In comparison with previous data, we found lymphoid neogenesis in a relatively small number of patients (31%, versus 44–90% in previous studies). We also found lower frequencies of FDCs and T–B cell separation. The differences might be related to differences in antirheumatic drug treatments or selection of patients with end-stage, destructive RA in the earlier studies. In addition, differences may be explained in part by the specific definitions of lymphocyte aggregates used in the previous studies. We performed a detailed analysis of lymphocyte aggregates as originally proposed by Manzo et al., and subcategorized based on aggregate size.

We found that lymphoid neogenesis in rheumatoid synovial tissue coincides with features of inflammation such as increased numbers of infiltrating macrophages and expression of TNFα at the site of inflammation, and increased leukocyte and thrombocyte counts, ESRI, and CRP levels in peripheral blood. These findings do not explain whether the presence of lymphoid neogenesis in rheumatoid synovial tissue is a cause or a consequence of inflammation in RA. It has been suggested that synovial lymphoid neogenesis contributes to ectopic maturation of B cell responses, since clonal expansion, somatic hypermutation, and diversification of B cells have been described in RA synovial tissue. Otherwise, to date there has been no direct proof of functionality of lymphoid neogenesis. Since lymphoid neogenesis also occurs in non–autoantibody-associated forms of arthritis such as psoriatic arthritis and osteoarthritis, it could be a result of nonspecific inflammation.

We did not find evidence that synovial tissue containing FDC-positive aggregates represents a germinal center–like subset of lymphoid neogenesis, but we were able to confirm previous observations that lymphocyte aggregates are present in synovial tissue in a heterogeneous mix of different numbers and sizes. FDC-positive aggregates were detected adjacent to aggregates without FDCs or other germinal center–like features such as T–B cell separation. Additionally, we observed no increase in macrophage infiltration or cytokine expression in synovial tissue containing FDC positive aggregates. These data suggest that the presence of FDCs in the synovium may be secondary to inflammation, rather than a primary phenomenon.

Lymphoid neogenesis was related to elevated levels of biomarkers of inflammation but, in contrast, was not associated with clinical signs and symptoms. This suggests that synovial tissue lymphoid neogenesis is not related to systemic inflammation to an extent that translates into the clinical expression of the disease. Possible explanations for this are that the link between lymphoid neogenesis and local synovial inflammation is not pivotal, or that synovial inflammation and concurrent lymphoid neogenesis might vary between joints (although previous work indicated that there is little variation in synovial inflammation between different joints in the same patient). Clinical disease activity may also be influenced by factors other than synovial inflammation, including joint destruction. We found no evidence that lymphoid neogenesis is related to more erosive or nodular disease, as was suggested in an earlier study of a small patient cohort. This also indicates that either the link between lymphoid neogenesis and synovial inflammation is not dominant or the presence of lymphoid neogenesis and concurrent synovial inflammation may vary between joints and over time.

Previous work has indicated that the rheumatoid synovium is a potent autoantibody-producing organ, and the antibodies may form immune complexes in the joint, leading to complement fixation and macrophage activation. Synovial plasma cells may synthesize and secrete RF, ACPA, and other autoantibodies. In accordance with these findings, the levels of RF and ACPA are higher in synovial fluid than in peripheral blood. We have demonstrated in this study that the presence of circulating autoantibodies is not related to lymphoid neogenesis. We cannot completely exclude the possibility that lymphocyte aggregates were present but not detected in some autoantibody-positive RA patients, but we did minimize sampling error by analyzing 2 levels of a tissue block representing at least 6 synovial tissue samples. In fact, the frequency of detection of circulating ACPAs was lower in patients with lymphoid neogenesis. Thus, our data do not support the notion that lymphoid neogenesis in the synovium has a critical role in the production of RF and/or ACPA.

In conclusion, the findings presented here show that lymphoid neogenesis in the synovium does not define a clinically or immunologically distinct subtype of RA. The presence of lymphocyte aggregates and germinal center–like structures may be a secondary phenomenon due to chronic inflammation.

ACKNOWLEDGMENTS

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REFERENCES


THE RELATIONSHIP BETWEEN SYNOVIAL LYMPHOCYTE AGGREGATES AND THE CLINICAL RESPONSE TO INFLIXIMAB IN RHEUMATOID ARTHRITIS: A PROSPECTIVE STUDY
THE RELATIONSHIP BETWEEN SYNOVIAL LYMPHOCYTE AGGREGATES AND THE CLINICAL RESPONSE TO INFliximab IN RHEUMATOID ARTHRITIS: A PROSPECTIVE STUDY

Abstract

OBJECTIVE Some patients with rheumatoid arthritis (RA) exhibit lymphocyte aggregates in the synovium. This study was undertaken to address whether the presence of lymphocyte aggregates before treatment could serve as a biomarker for the clinical response to tumor necrosis factor (TNF) blockade, and to confirm whether the aggregation of synovial lymphocytes is reversible after anti-TNF treatment.

METHODS Synovial tissue biopsy samples were obtained from 97 patients with active RA before the initiation of infliximab treatment. Lymphocyte aggregates in the synovial tissue were counted and also graded for size. Logistic regression analysis was performed to identify whether the presence of lymphocyte aggregates could be a predictor of the clinical response at week 16. Furthermore, the effects of TNF blockade on lymphocyte aggregates were compared between patients with RA and patients with psoriatic arthritis (PsA).

RESULTS Fifty-seven percent of RA synovial tissue samples contained lymphocyte aggregates, and 32% of the patients had large aggregates. Aggregates were found in 67% of clinical responders compared with 38% of non-responders. The presence of aggregates at baseline was a highly significant predictor of the clinical response to anti-TNF treatment ($R^2 = 0.10$, $P = 0.008$). Positivity for lymphocyte aggregates increased the power to predict the clinical response ($R^2 = 0.29$), when analyzed in a prediction model that included baseline disease activity evaluated by the Disease Activity Score in 28 joints, anti–cyclic citrullinated peptide antibody positivity, and synovial TNFα expression. There was a reduction in lymphocyte aggregates after anti-TNF antibody therapy in both RA and PsA.

CONCLUSION RA patients with synovial lymphocyte aggregates have, on average, a better response to infliximab treatment than those with only diffuse leukocyte infiltration. Moreover, the aggregation of synovial lymphocytes is reversible after anti-TNF antibody treatment.

Introduction

Rheumatoid arthritis (RA) is an immune-mediated inflammatory disease of unknown etiology that affects the synovial tissue in multiple joints. Variability in both the cellular features $^{1,2}$ and molecular features $^{3,4}$ of the inflamed synovium, as well as the heterogeneous response to treatment $^{5,6}$, suggest that RA is a clinical syndrome comprising different pathogenetic subsets. Both the extent and the pattern of synovial lymphocyte infiltration are remarkably variable among different individuals with RA $^{1,2,6}$. In some tissues, a diffuse or scarce infiltration of T cells is present, while in others, B and T cells are organized in lymphocyte aggregates that may exhibit germinal center–like features $^{6,7}$.

It has been proposed that lymphocyte aggregates may be involved in the autoreactive humoral response observed in a subset of RA patients, comprising those who are positive for anti–cyclic citrullinated peptide antibodies (anti-CCP) and/or positive for rheumatoid factor (RF), resulting in amplification
and refinement of local autoantibody production. Conversely, other studies have suggested that the presence of lymphocyte aggregates is not directly related to a local germinal center–like humoral response, but rather could be attributed to a phenomenon secondary to the chronic inflammatory processes driving RA. Thus, the role of synovial lymphocyte aggregates in the pathogenesis of RA is still controversial. It is, at present, also unclear whether there is a differential response to treatment between RA patients with synovial lymphocyte aggregates and those without synovial lymphocyte aggregates.

We and other investigators in our group have recently shown that the clinical response to anti–tumor necrosis factor (anti-TNF) therapy in RA is related to the synovial tissue inflammation levels prior to treatment, providing proof of concept that synovial biomarkers may be used to predict the response to treatment. The objective of this study was to investigate the relationship between synovial lymphocyte aggregates and the response to anti-TNF therapy. Therefore, in a prospective study, we evaluated 97 patients with active RA starting infliximab treatment. Arthroscopic synovial biopsy samples were obtained before treatment, and the presence of synovial lymphocyte aggregates at baseline was assessed for any association with the clinical response at week 16. In addition, we obtained serial synovial biopsy samples before and after treatment in a subset of the RA patients, to confirm the previously reported effects of TNF blockade on synovial lymphocyte aggregates. To assess whether changes in synovial lymphocyte aggregates after TNF-blocking therapy are specific to rheumatoid synovial tissue, we also analyzed such changes after adalimumab treatment in a small cohort of patients with psoriatic arthritis (PsA).

## Patients and Methods

### Patients

To examine the relationship between the presence of synovial lymphocyte aggregates and the response to anti-TNF treatment, we obtained synovial tissue samples from 97 patients with RA before initiation of infliximab treatment. The baseline features of the larger cohort, including the presence of lymphocyte aggregates, have been described previously. Patients were selected for the present analysis based on the availability of evaluable synovial tissue at baseline, combined with standardized follow-up data on the response to infliximab treatment. The relationship between baseline synovial TNFα expression and clinical response in these patients has been reported previously.

All patients were being treated with stable dosages of methotrexate (5–30 mg/week) and had never taken biologic agents. In addition, all patients had active disease, defined by a Disease Activity Score in 28 joints (DAS28) of ≥ 3.2. Use of oral corticosteroids (<10 mg/day) and nonsteroidal antiinflammatory drugs was allowed if the dosage had not been changed within 1 month prior to baseline. Intraarticular steroid injections within the month prior to baseline were not allowed.

Disease characteristics and the presence of IgM-RF and anti-CCP (as measured by the second-generation anti-CCP enzyme-linked immunosorbent assay; Immunoscan RA [Mark 2], NO.RA-96RT from Eurodiagnostica, Arnhem, The Netherlands) were assessed at baseline. All patients were administered intravenous infusions of infliximab in a dose of 3 mg/kg at baseline and at weeks 2 and 6, and subsequently every 8 weeks. We determined the responder status by evaluating the reduction in the DAS28 after 16 weeks of therapy. RA patients with a reduction in the DAS28 of at least 1.2 (twice the measurement error of the DAS28 over time) were defined as responders, representing a clinically significant improvement. The clinical response was also determined according to the European League Against Rheumatism (EULAR) response criteria.

To investigate the effect of anti-TNF therapy on synovial lymphocyte aggregates, we analyzed serial synovial tissue samples from 15 patients who underwent arthroscopy in the same joint before treatment and 28 days after treatment. In 10 patients, we also performed arthroscopy 48 hours after the first infliximab treatment. To determine whether the synovial tissue response to anti-TNF antibody therapy is specific to RA or whether it is a more general phenomenon, we analyzed serial synovial biopsy samples from a second cohort, comprising 9 patients with active PsA who underwent arthroscopy and had never taken biologic agents. These patients were evaluated at baseline and at 28 days after the start of treatment with adalimumab (40 mg every other week). The clinical response in patients with PsA was defined by a decrease in the DAS28 score of at least 1.2 at 12 weeks.

All patients gave their written informed consent to participate. The study was approved by the Medical Ethics Committee of the Academic Medical Center at the University of Amsterdam.

### Synovial Biopsy, and Assessment of Lymphocyte Aggregates

All patients, under local anesthesia, underwent a miniarthroscopy of an actively inflamed knee, wrist, or ankle, as described previously in detail, and samples of the synovial tissue...
were studied. The following monoclonal antibodies were used to analyze the lymphocytic cell infiltrate: anti-CD3 (SK7; Becton Dickinson, San Jose, CA) to detect T cells, and anti-CD22 (CLB-B-ly/1, 6B11; Sanquin Research, Amsterdam, 3218, The Netherlands) to detect B cells. Anti-CD21–long isoform (anti-CD21L; a kind gift from Dr. Y. J. Liu, M. D. Anderson Cancer Center, Houston, TX) was used for detection of follicular dendritic cells (FDCs). Staining of cellular and cytokine markers was performed as described previously. The presence of lymphocyte aggregates was assessed on anti-CD3–stained sections. The presence of FDCs in lymphocyte aggregates was assessed at 3 different levels of the tissue. The size and number of lymphocyte aggregates and the presence of T or B cell aggregation were assessed at 2 different levels of the tissue, at least 50 µm apart, on sequential sections stained with CD3 and CD22. Thus, multiple sections representing different levels of a tissue block, consisting of at least 6 biopsy specimens, were examined to further minimize sampling error.

Aggregates were counted and graded on a 4-point scale (range 0–3) according to the number of cells in their diameter, as described previously. We calculated the total number of aggregates per section and the mean aggregate diameter per section. Grade 2 and grade 3 aggregates were termed large lymphocyte aggregates, while grade 1 aggregates were termed small lymphocyte aggregates. Germinal center–like structures were defined as lymphocyte aggregates containing FDCs.

STATISTICAL ANALYSIS. The primary analysis was focused on the comparison of the clinical response between patients with either small or large aggregates and patients without aggregates. Furthermore, we analyzed separately whether the presence of germinal center–like structures is related to the clinical response to anti-TNF antibody therapy. The chi-square test was used to compare patient characteristics between those patients with diffuse synovitis and those patients with lymphocyte aggregates. For comparison of continuous variables, we used the t-test or, if the data were skewed, the Mann-Whitney U test. To examine the relationship between clinical features and synovial parameters and the clinical response to anti-TNF treatment, we performed univariate Cox logistic regression, the Kruskal-Wallis test with the post hocGames-Howell test, and linear regression analysis, as appropriate.

To assess whether the presence of lymphocyte aggregates increased the predictive power to determine an association with clinical response to infliximab in a combined prediction model, we analyzed a multivariable prediction model that consisted of TNFα expression in the synovial lining at baseline, positivity for anti-CCP, and the DAS28 at baseline. We performed stepwise forward and backward multivariable logistic regression analyses to obtain estimates of the odds ratios, with outcome measures expressed as the natural log of the regression coefficient (eB). Collinearity diagnostics were performed to analyze the presence of multicollinearity. Hosmer and Lemeshow tests were performed to assess the goodness of-fit. Wilcoxon’s signed rank test was used for analysis of the lymphocyte aggregates in paired biopsy specimens. SPSS version 16.0 for Windows (SPSS, Chicago, IL) was used for all statistical analyses.

TABLE No.1

| Characteristics of the patients with rheumatoid arthritis |
|-----------------------------|-----------------|
| **N=97**                    |                 |
| **DEMOGRAPHICS**            |                 |
| Age (years)                 | 55 ± 13         |
| Female (%)                  | 67 (69%)        |
| **DISEASE STATUS**          |                 |
| Disease duration (months)   | 127 ± 116       |
| Erosive disease (%)         | 74 (76%)        |
| Rheumatoid Factor positive (%) | 72 (74%) |
| ACPA positive (%)           | 72 (74%)        |
| DAS28                        | 5.9 ± 1.1       |
| Patient global score (0-100 mm) | 60 ± 22 |
| ESR (mm/hr)                 | 34 ± 23         |
| C-reactive protein (mg/dl)  | 24 ± 29         |
| **DRUG TREATMENT**          |                 |
| Previous DMARDs             | 2.1 ± 1.5       |
| Methotrexate (mg/week)      | 18.2 ± 8.7      |
| Receiving corticosteroids (%) | 26 (27%) |
| Receiving NSAIDs (%)        | 50 (52%)        |

* Except where indicated otherwise, values are the mean ± SD.

**Anti-CCP** = anti–cyclic citrullinated peptide antibody; **DAS28** = Disease Activity Score in 28 joints; **ESR** = erythrocyte sedimentation rate; **DMARDs** = disease-modifying antirheumatic drugs; **NSAIDs** = nonsteroidal antiinflammatory drugs.

CHARACTERISTICS OF THE RA PATIENTS AT BASELINE.

Ninety-seven RA patients were analyzed. The demographic and clinical features of these patients are shown in Table 1. Low-dose oral corticosteroids were being taken by 27% of the patients. Patients had taken, and failed treatment with, a mean of 2.1 disease-modifying antirheumatic drugs (DMARDs) prior to inclusion in the study.

CLINICAL IMPROVEMENT AFTER INFILXIMAB TREATMENT.

Sixteen weeks after initiation of treatment with infliximab, the mean ± SD DAS28 decreased from 5.9 ± 1.1 to 4.2 ± 1.3 (P < 0.0001); the mean ± SD change in the DAS28 was 1.7 ± 1.3. Sixty-three of the 97 RA patients (65%) experienced a decrease in the DAS28 of ≥1.2. Twenty-two patients (23%) had a good response to infliximab treatment according to the EULAR response criteria, 51 patients (53%) had a moderate response according to the EULAR response criteria, and 24 patients (25%) did not fulfill the EULAR response criteria. Association of the presence of synovial lymphocyte aggregates with the
The acute-phase response. Of the 97 patients with RA, 42 (43%) had diffuse synovial inflammation, 24 (25%) had small lymphocyte aggregates, and 31 (32%) had large lymphocyte aggregates. The synovial tissue of patients with large aggregates contained significantly more small aggregates compared with those with only small aggregates or those with only diffuse synovitis (P = 0.019). Disease duration and use of corticosteroids were not related to the presence of synovial lymphocyte aggregates.

Of the 97 synovial biopsy samples, 89 could be evaluated by staining for the presence of FDCs. Seven samples (8%) showed CD21L-positive staining, which was observed within large aggregates in all 7 samples. Separate clusters of T cells and B cells were found in 7 of the 31 patients with large aggregates, but separate clusters were not detected in small aggregates. Patients with aggregates had a higher erythrocyte sedimentation rate and higher C-reactive protein level compared with those without aggregates (P = 0.005 and P < 0.05, respectively). As reported previously, the presence of circulating autoantibodies was not related to the presence of synovial lymphocyte aggregates.

**FIGURE No.1a**

![Graph of presence of lymphocyte aggregates in synovial tissue](image)

**FIGURE No.1b**

![Graph of decrease in DAS28](image)

**PREDICTIVE POWER OF THE PRESENCE OF SYNOVIAL LYMPHOCYTE AGGREGATES AT BASELINE IN RELATION TO THE CLINICAL RESPONSE TO INFILIXIMAB TREATMENT IN RA.**

Aggregates were present in 42 (67%) of 63 clinical responders (defined as a decrease in the DAS28 of ≥1.2) and in 13 (38%) of 34 nonresponders (P = 0.007 between groups). Univariate Cox logistic regression analysis confirmed that the presence of lymphocyte aggregates at baseline was related to the clinical response (R² = 0.10, P = 0.008). The positive predictive value was 76% and the negative predictive value was 50%.

Subsequently, we analyzed the patients for clinical response to treatment according to the EULAR response criteria. Aggregates were present in 16 (73%) of the 22 EULAR good responders, in 30 (59%) of the 51 EULAR moderate responders, and in 9 (38%) of the 24 patients who did not respond to infliximab treatment according to the EULAR response criteria (Figure 1A). Kruskal-Wallis analysis and a post hoc test (Games-Howell test) showed that lymphocyte aggregates were significantly more often present in good responders compared with nonresponders (95% confidence interval [95% CI] -0.69, -0.01; P = 0.041) (Figure 1A).

Furthermore, patients were analyzed for treatment response according to
the absolute decrease in the DAS28. Univariate linear regression analysis showed that the presence of lymphocyte aggregates was predictive of the absolute decrease in the DAS28 (R² = 0.041, P = 0.026) (Figure 1B).

Separate analyses of only those synovial tissue samples with large aggregates suggested a relationship with clinical response, but the relationship with aggregate size did not reach statistical significance when the clinical response was analyzed as categories of improvement according to the EULAR criteria (P = 0.52) (Figure 1C) or dichotomously as a decrease in the DAS28 of ≥ 1.2 (R² = 0.025, P = 0.19). However, the presence of large lymphocyte aggregates was a significant predictor of the clinical response when the response was analyzed as the absolute decrease in the DAS28 (R² = 0.033, P = 0.041) (Figure 1D).

The presence of FDCs in large aggregates, as determined by the expression of CD21L, was not predictive of the clinical response; these were present in only 7 of 97 biopsy samples. Taken together, our findings indicate that the presence of lymphocyte aggregates (defined as one group with either small or large aggregates) is a significant predictor of the response to infliximab treatment in RA.

**FIGURE No.2a**

**FIGURE No.2b**

CONTRIBUTION OF LYMPHOCYTE AGGREGATES TO A COMBINED MODEL FOR THE PREDICTION OF CLINICAL RESPONSE TO INFliximab.

In a recent study in which the patients from the current study were included, we identified synovial TNFα expression, the baseline DAS28, and the presence of anti-CCP antibodies as predictors of the clinical response to infliximab. The dichotomy of the DAS28, when applied as a measure of clinical response using a decrease of ≥ 1.2 to define improvement, was chosen because it is used in daily clinical practice and is required for prolongation of reimbursement for TNF blocking therapy by insurance companies in The Netherlands. Analysis of this combined prediction model in our previous study showed that the model was modestly predictive of the clinical response to infliximab at week 16 (R² = 0.19) (with inclusion of baseline TNFα expression, P = 0.009, eB 1.1, 95% CI 1.1, 1.9; with inclusion of anti-CCP positivity, P = 0.044, eB 2.8, 95% CI 1.0, 9.2; and with inclusion of baseline DAS28, P = 0.040, eB 1.6, 95% CI 1.0, 2.6).

The results from our current study showed that the addition of lymphocyte aggregates as a variable into this model improved the prediction of the clinical response to infliximab (R² = 0.29, by forward stepwise method). Collinearity diagnostics showed that
all variables had a tolerance of > 0.95 and a variance inflation factor close to 1, indicating that no significant multicollinearity had occurred. The variables were included in the following order in the prediction model: inclusion of baseline TNFα expression ($P = 0.016, e^r = 1.2, 95\% CI 1.0, 1.4$), lymphocyte aggregates ($P = 0.023, e^r = 3.3, 95\% CI 1.2, 9.4$), anti-CCP positivity ($P = 0.019, e^r = 4.0, 95\% CI 1.36, 12.8$), and, finally, baseline DAS28 ($P = 0.048, e^r = 1.6, 95\% CI 1.0, 2.7$). The positive predictive value of the model was 85% and the negative predictive value was 53%. A backward stepwise method yielded the same results.

DECREASE IN SYNOVIAL LYMPHOCYTE AGGREGATES AFTER TNF BLOCKADE IN BOTH RA AND PSA.

Serial synovial tissue samples were obtained from 15 RA patients before the initiation of infliximab treatment and on day 28 after treatment. In 9 of the 15 patients, lymphocyte aggregates were present at baseline. After 28 days, the number of aggregates decreased in 6 of these 9 aggregate-positive patients (Figure 2A) and the size of the aggregates decreased in 7 patients, whereas we observed an increase in the number of aggregates in 3 patients (Figure 2A) and an increase in the size of the aggregates in 2 patients. These reductions in the number and size of the aggregates from baseline to day 28 did not reach statistical significance, possibly due to the relatively small number of patients as well as the variability in response. We also obtained synovial biopsy tissue from 9 of these patients at 48 hours after the first administration of infliximab, 6 of whom had lymphocyte aggregates at baseline. In 5 of the 6 aggregate-positive patients, there was a trend toward a decrease in the size and number of lymphocyte aggregates within 48 hours after the first infusion with infliximab (Figure 2B).

To determine whether the decrease in the number and size of the lymphocyte aggregates after treatment was specific to RA or was, perhaps, a more general phenomenon, we studied the synovial tissue response to adalimumab treatment in 9 patients with PsA. This patient cohort was compared with patients with PsA ($n = 9$) who received placebo. In 7 of the 9 patients treated with adalimumab, lymphocyte aggregates were present at the time of the baseline biopsy. In 6 of 7 patients, there was a decrease in the size and number of lymphocyte aggregates 28 days after the initiation of adalimumab treatment ($P = 0.028$ and $P = 0.043$, respectively, versus baseline) (Figure 2C). Because lymphocyte aggregates are found in both RA and PsA, and because they appear to decrease after anti-TNF therapy in both inflammatory forms of arthritis in a similar way, we subsequently pooled the data from all patients with inflammatory arthritis whose synovial tissue was positive for lymphocyte aggregates and compared the values before and after anti-TNF treatment, using the same time points, to get more statistical power. In these 16 patients, there was a significant decrease in the size and number of synovial lymphocyte aggregates 28 days after the start of anti-TNF therapy ($P = 0.028$ and $P = 0.044$, respectively, versus baseline) (results not shown and Figure 2D), consistent with the observations described in previous reports.20

DISCUSSION

Since the clinical response to anti-TNF therapy is heterogeneous, there is a clear need for biomarkers that can identify different pathogenic subsets that are associated with the response to or lack of response to TNF-antagonist therapy.20-22 We have recently provided proof of concept to confirm that synovial biomarkers predictive of the response to anti-TNF therapy might be identified.20 In the present study, we investigated the relationship between the pretreatment presence of synovial lymphocyte aggregates and the primary clinical response to infliximab treatment, in a prospective study of a large cohort of well-characterized patients with RA. The results revealed a highly significant relationship between the presence of synovial lymphocyte aggregates at baseline and the primary clinical response defined at 16 weeks. When the presence of synovial lymphocyte aggregates was added into a combined prediction model with synovial TNFα expression, the DAS28 at baseline, and the presence of anti-CCP antibodies, the presence of lymphocyte aggregates increased the prediction of response from 19% to 29%. Of interest, the aggregation of synovial lymphocytes was also shown to be reversible after anti-TNF antibody treatment, both in patients with RA and in patients with PsA.

Our findings appear, at first sight, to be in clear contrast with those from a previous study in which synovial lymphocyte aggregates were not identified as a predictor of the response to anti-TNF therapy.20 There are several differences between the 2 studies that may help to resolve the apparent discrepancy. First, in the other study, lymphocyte aggregate-positive patients were defined by the presence of large aggregates rather than the presence of either small and/or large aggregates. In our study, we did not find a statistically significant relationship between the presence of aggregates and the response to infliximab therapy (defined according to the EULAR criteria or defined dichotomously as a decrease in the DAS28 of ≥1.2) when only large aggregates were taken into account, although there was a trend toward significance.

Second, in contrast to our study, the patient cohort in the other study was more heterogeneous, in that patients with early, untreated RA, along with DMARD inadequate responders and anti-TNF inadequate responders, were included.22 Those patients were also treated with a different DMARD background medication. During followup, patients were sequentially treated with gold salts, methotrexate, leflunomide, or different TNF blockers depending on the DAS28. It is likely that the markedly large number of variables in that study may have made it difficult to detect the relationship between the presence of synovial lymphocyte aggregates and the response to anti-TNF therapy.

Third, in the other study, the patients with synovial lymphocyte aggregates had a significantly longer disease duration compared with those without lymphocyte aggregates.20 Of importance, a high proportion of them had failed previous treatment with other TNF antagonists, and thus represented a therapy-resistant subgroup whose data were followed up in a subsequent study.22 Therefore, enrichment of the cohort of patients with synovial lymphocyte aggregates with the subset of patients who had previously failed TNF blockade may have been an important confounding factor. In our study, we only included
patients who had failed treatment with at least 2 conventional DMARDs, including methotrexate; previous use of TNF antagonists was an exclusion criterion. Fourth, duration of followup after the initiation of anti-TNF therapy was variable in the other study, with a mean followup of 43 months. In contrast, we chose to select a fixed end point at 16 weeks to ascertain the primary response to infliximab treatment, since the secondary response defined at later time points could have been influenced by totally unrelated mechanisms, including the development of human antichimeric antibodies against infliximab or human anti-human antibodies against adalimumab.

The use of a very stringent study design allowed us to identify synovial lymphocyte aggregates as a highly significant predictor of the response to infliximab therapy. The relationship observed between synovial lymphocyte aggregates and the response to anti-TNF therapy is consistent with previous circumstantial evidence that indicated 1) a correlation between synovial lymphocyte aggregates and synovial inflammation, and 2) a relationship between synovial inflammation and the response to anti-TNF therapy. When the presence of lymphocyte aggregates was added into a combined prediction model for the prediction of the clinical response to infliximab, it increased the explained variance of response to 29%. Consistent with the clinical experience, in which it has been observed that the response to TNF blockade is not a dichotomous phenomenon, there was no distinct threshold value for scores for lymphocyte aggregates in the synovium of patients with RA. Therefore, the predictive value of the presence of synovial lymphocyte aggregates alone or in combination with other tested predictors of response is statistically significant and of great scientific interest, but cannot be translated into a predictive test in individual patients.

The results presented herein also confirm that aggregation of synovial lymphocytes represents a reversible phenomenon after resolution of inflammation, which is consistent with previous observations in patients with RA and patients with PsA, and supports the notion that lymphocyte aggregates are formed secondary to the inflammatory process. Previous studies have shown that TNF blockade results in decreased expression of cytokines, chemokines, and adhesion molecules, which are all required for secondary lymphoid organ formation. The decrease in lymphocyte aggregates after anti-TNF treatment is consistent with the protective effect of TNF blockade on joint destruction, since previous work has shown that the presence of small lymphocyte aggregates is related to the development of bone erosions.

Taken together, our findings reveal a highly significant relationship between the presence of synovial lymphocyte aggregates at baseline and the primary clinical response to anti-TNF antibody treatment, but the clinical response cannot be predicted completely, thus indicating the involvement of other, as yet unknown mechanisms. Future work should expand the search for other biomarkers and molecular networks (for instance, with the use of microarray analysis) as well as combinations of clinical variables, to achieve an effective approach that would increase the percentage of patients exhibiting a robust response to TNF blockade.

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tissue.


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EARLY EFFECTS OF RITUXIMAB ON THE SYNOVIAL CELL INFILTRATE IN PATIENTS WITH RHEUMATOID ARTHRITIS
EARLY EFFECTS OF RITUXIMAB ON THE SYNOVIAL CELL INFILTRATE IN PATIENTS WITH RHEUMATOID ARTHRITIS

Abstract

OBJECTIVE: To study the specific effects of rituximab treatment on the synovium in patients with rheumatoid arthritis (RA) early after initiation of treatment.

METHODS: Seventeen RA patients underwent an arthroscopic synovial biopsy procedure directly before and 1 month after receiving 2 infusions of the chimeric anti-CD20 monoclonal antibody rituximab (1,000 mg on days 1 and 15; both without methylprednisolone premedication). Immunohistochemical analysis was performed to characterize the cell infiltrate. Stained tissue sections were analyzed by digital image analysis. Statistical analysis was performed using Wilcoxon’s signed rank test.

RESULTS: No significant change in the Disease Activity Score 28-joint assessment was found at 4 weeks after the first rituximab infusion. At 2 and 4 weeks after infusion, B cells in peripheral blood were almost completely depleted.

Most B cells in the synovium were found in large lymphocyte aggregates. Interestingly, a significant reduction in B cell numbers at sites of inflammation was observed 4 weeks after treatment (median 26 cells/mm² [interquartile range 4–150] before treatment and 11 cells/mm² [interquartile range 0–29] after treatment; P < 0.02). B cells disappeared completely in 3 patients, whereas there was partial depletion in 11 patients. In the other 3 patients, no B cells were present in biopsy tissues obtained either pretreatment or posttreatment. No reductions in other synovial cell populations were observed at 4 weeks.

CONCLUSION: Rituximab treatment leads to a rapid and significant decrease in synovial B cell numbers, but not in all patients. Whether the variable tissue response is related to the clinical response over time remains to be clarified.

Introduction

Rituximab, a chimeric monoclonal antibody directed against the CD20 antigen expressed by B cells, is a promising new biologic agent for the treatment of rheumatoid arthritis (RA). A single course of 2 infusions of rituximab was reported to significantly improve disease symptoms for up to 48 weeks in RA patients with high levels of disease activity despite methotrexate treatment. The safety and efficacy of the drug were recently confirmed in 2 phase IIb studies. Although rituximab is efficacious, its exact mechanism of action has yet to be elucidated. In the peripheral blood, rituximab causes a selective, transient depletion of pre-B cells as well as naive, mature, and memory B cells, leaving stem cells and plasma cells unaffected. This can be explained by the binding of rituximab to CD20, which may lead to cell-mediated cytotoxicity, complement-mediated lysis, and apoptosis. There is at present no information available concerning the effects of rituximab on the primary target of the disease, the rheumatoid synovium. Animal studies have suggested that the kinetics of rituximab-induced B cell depletion vary among different tissues. Depletion of B cells from lymph nodes may occur within days, whereas in the peritoneal cavity, it can take weeks to complete. In addition, marginal-zone B cells of the spleen and B cells located in germinal centers in lymphoid tissues appear to be partly resistant to depletion by anti-CD20 anti-
bodies in a human CD20–transgene mouse model. The persistent presence of B cells in germinal centers after treatment with rituximab has also been described in a patient with Graves’ disease. These observations suggest that the susceptibility of B cell subsets to rituximab treatment may be dependent on the specific microenvironment. Factors that may influence resistance to B cell depletion, such as CD55 (decay-accelerating factor [DAF]), a complement inhibitory protein, and B lymphocyte stimulator (BLYS), a major B cell survival factor, could affect the specific effects of rituximab treatment in the inflamed synovium. Another factor that could influence the susceptibility of B cells to cell death is the use of corticosteroids as premedication. Therefore, we examined the specific effects of rituximab treatment on the inflamed synovium in patients with RA. This is the first study to examine B cells in the inflamed RA synovium early after initiation of treatment with rituximab.

**PATIENTS AND METHODS**

**PATIENTS AND TREATMENT PROTOCOL.** Patients with RA diagnosed according to the 1987 criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) were studied. Inclusion criteria were the presence of active disease, defined as ≥ 4 tender and ≥ 4 swollen joints (of 28 joints assessed), at study enrollment; an erythrocyte sedimentation rate (ESR) of ≥ 28 mm/hour or a serum C-reactive protein (CRP) level of ≥ 15 mg/liter; or morning stiffness for ≥ 45 minutes. Patients who were negative for both rheumatoid factor (RF) and anti–cyclic citrullinated peptide (anti-CCP) antibodies were excluded.

All study patients had to be receiving stable doses of methotrexate (5–30 mg/week) for at least 28 days prior to enrollment. Stable prednisone therapy (≤10 mg/day) and nonsteroidal antiinflammatory drug treatment were allowed. All other disease-modifying antirheumatic drugs (DMARDs) and biologic agents were withdrawn at least 4 weeks prior to study inclusion, with a washout period for infliximab and adalimumab treatment of >8 weeks prior to randomization. The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center/University of Amsterdam. All patients signed informed consent forms before inclusion in the study.

The patients were treated with 2 infusions (1,000 mg each) of rituximab (Roche, Woerden, The Netherlands) on days 1 and 15 after premedication with clemastine fumarate (2 mg intravenously) and acetaminophen (1 gm orally). Premedication with methylprednisolone was not allowed, since this could have influenced the synovial cell infiltrate.

Patients were assessed for disease activity using the Disease Activity Score in 28 joints (DAS28) at the time of synovial biopsy. Biopsies were obtained before and 4 weeks after the first infusion of rituximab.

**BLOOD LYMPHOCYTE POPULATIONS.** B cells (CD19+) and T cell subsets (CD3+, CD4+, CD8+) (all from Becton Dickinson, San Jose, CA) were detected in peripheral blood samples by real-time fluorescence-activated cell sorting using a FACSCalibur Flow Cytometer (Becton Dickinson) at baseline and at 2 weeks and 4 weeks after the first rituximab infusion. CD19 was chosen as a marker because of its similar expression to CD20 on B cell subsets, without interference with the circulating rituximab antibody. The lower limit of detection of CD19+ B cells was set at 0.01 x 10^9 CD19+ cells/liter.

**SYNOVIAL BIOPSY.** An arthroscopy of the same actively inflamed joint (knee, ankle, or wrist) under local anesthesia was performed on all patients before and 4 weeks after the first infusion of rituximab, as previously described in detail. To minimize sampling error (variance < 20%), at least 6 biopsy samples were obtained during each procedure. Specimens were directly embedded en bloc in TissueTek OCT (Miles Diagnostics, Elkhart, IN) in a mold and subsequently snap-frozen.

**IMMUNOHISTOCHEMISTRY.** From each tissue block, consisting of the 6 different biopsy samples, serial sections (5 μm) were cut with a cryostat and stained with the following mouse monoclonal antibodies: anti-CD3 (SK7; Becton Dickinson),...
Seventeen patients were included in the trial (14 women and 3 men) (Table 1). The median age of the patients was 55 years (range 22–75 years), and the median duration of disease was 13 years (range 10 months to 50 years). Erosions were present in all patients. Seven patients had nodular disease, 13 patients were seropositive for IgM-RF (median 55 [interquartile range (IQR) 24–138]), and all patients were anti-CCP positive (median 208 [IQR 53–1,031]). The median DAS28 score at baseline was 6.7 (IQR 6.10–7.65). All patients were treated with stable dosages of methotrexate (median 15 mg/week [IQR 10–23]) and 10 patients were taking prednisone at a fixed dosage (median 5 mg/day [IQR 0–10]). On average, the patients had failed treatment with a median of 3 DMARDs (IQR 2–4.5) and a median of 2 biologic agents (IQR 1–3) before study entry.

The DAS28 scores did not change significantly at 4 weeks after initiation of rituximab treatment in these patients.

FIGURE No.1

Change in the number of CD22+ B cells in serial synovial tissue samples obtained before and 4 weeks after initiation of rituximab treatment. Shown are the results in individual rheumatoid arthritis patients.
**Baseline characteristics of the study patients***

<table>
<thead>
<tr>
<th>DEMOGRAPHICS</th>
<th>BEFORE RITUXIMAB</th>
<th>AFTER RITUXIMAB</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range) years</td>
<td>55 (22 - 75)</td>
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<td></td>
</tr>
<tr>
<td>Female, no. (%)</td>
<td>14 (82)</td>
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<td></td>
</tr>
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</table>

**DISEASE STATUS**

<table>
<thead>
<tr>
<th>Disease duration, median (range) years</th>
<th>Before</th>
<th>After</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erodisive disease, no. (%)</td>
<td>13 (0.9 - 50)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nodular disease, no. (%)</td>
<td>17 (100)</td>
<td></td>
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</tr>
</tbody>
</table>

* IgM-RF = IgM rheumatoid factor; IQR = interquartile range; anti-CCP = anti-cyclic citrullinated peptide; DAS28 = Disease Activity Score 28-joint assessment; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs.
study patients who had not received premedication with methylprednisolone (median 6.7 [IQR 6.10–7.65] before treatment and 6.6 [IQR 6.28–7.26] after treatment; P = 0.253).

In addition, the serum levels of anti-CCP antibodies and IgM-RF were unaltered after 4 weeks (P = 0.124 and P = 0.514 versus pretreatment values, respectively).

DEPLETION OF CIRCULATING B CELLS

At 2 and 4 weeks after rituximab infusion, CD19+ B cells in peripheral blood were undetectable (median 0.13 x 10^9/liter [IQR 0.10–0.39] at baseline; <0.01 x 10^9/liter [IQR 0.00–0.00] at both 2 and 4 weeks after treatment) (Table 2). The total T cell numbers and T cell subset numbers did not change significantly.

SPECIFIC REDUCTION OF SYNOVIAL B CELLS EARLY AFTER INITIATION OF RITUXIMAB TREATMENT, BUT NOT IN ALL PATIENTS.

In 13 patients, lymphocyte aggregates were found in the pretreatment biopsy sample (76%). Ten of these patients had grade 3 aggregates (58.8%), 3 patients had grade 2 (17.6%), and 4 patients had only grade 1 or no aggregates at all (23.5%). B cells were observed almost exclusively in grade 2 and 3

FIGURE No.2

![Change in the number of CD22+ B cells in representative serial synovial tissue samples obtained from 2 different rheumatoid arthritis patients before (A and C) and 4 weeks after (B and D) initiation of rituximab treatment. Different patterns of depletion were identified. In some patients, there was complete B cell depletion (compare A and B), while in other patients, few B cells were depleted (compare C and D). (Original magnification x 20.)](image)

TABLE No.3

Lymphocyte aggregate counts, by grade, total numbers, and mean diameter, in rheumatoid arthritis patients before and 1 month after rituximab treatment*

<table>
<thead>
<tr>
<th></th>
<th>LYMPHOCYTE AGGREGATES BEFORE RITUXIMAB</th>
<th>LIYMPHOCYTE AGGREGATES AFTER RITUXIMAB</th>
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<tbody>
<tr>
<td></td>
<td>GRADE 1</td>
<td>GRADE 2</td>
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</tr>
<tr>
<td>1</td>
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<tr>
<td>mean</td>
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<td>6</td>
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</tbody>
</table>

* The number of lymphocyte aggregates per section was counted, and the aggregates were graded on a scale of 1–3, where 1 = 1–5 cells in maximal radius, 2 = 5–10 cells in maximal radius, and 3 > 10 cells in maximal radius. For calculation of the mean aggregate diameter per section, an average aggregate radius of 3 was taken for grade 1 aggregates, 7 for grade 2, and 13 for grade 3.
aggregates, with the majority being present in grade 3 aggregates. At baseline, there was a positive correlation between the numbers of CD22⁺ B cells and the numbers of CD4⁺ T cells (P < 0.001), CD4⁺ and CD8⁺ T cells (P < 0.001 and P = 0.026, respectively), CD38⁺ plasma cells (P < 0.001), grade 2 and 3 aggregates (P < 0.001 and P = 0.003, respectively), and the ESR and CRP levels (P = 0.008 and P = 0.014, respectively).

There was clear heterogeneity of B cell infiltration between patients. Importantly, using statistical analysis for paired data, we detected a significant decrease in the number of CD22⁺ B cells in the synovium 4 weeks after initiation of rituximab treatment (median 26 cells/mm² [IQR 4–150] before treatment and 17 cells/mm² [IQR 0–29] after treatment; P = 0.016) (Table 2 and Figure 1). In 3 patients, B cells disappeared completely, whereas in 11 patients, there was only a partial decrease in B cell numbers (Figure 2). In 3 patients, no B cells were present in either the pre- or posttreatment biopsy specimens. No correlation between the change in the number of B cells and the change in DAS28 scores at 4 weeks was found. At this time point, there was no statistically significant decrease in the total aggregate number (P = 0.245), the total number of grade 3 aggregates (P = 0.139), or the mean aggregate diameter (P = 0.432) (Table 3), which could be explained by the fact that T cells were unaffected.

As shown in Table 2, the reduction in B cells was specific, since there was no decrease in the number of fibroblast-like synoviocytes, T cell populations, or plasma cells at that time point. In contrast, there was a trend toward an increase in the number of sublining macrophages.

DISCUSSION

The results presented here demonstrate the early effects of rituximab on rheumatoid synovial tissue. The effects are specific, since premedication with methylprednisolone, which could have influenced the cell infiltrate, was omitted.

Consistent with previous studies, significant numbers of B cells were present in the rheumatoid synovium in a subset of the patients. B cells were mostly found in moderate (grade 2) and large (grade 3) lymphocyte aggregates, and they colocalized with large numbers of CD4⁺ T cells. In contrast with the rapid and profound depletion of peripheral blood B cells in all patients, there was a variable response in the synovial tissue. This might be explained by such factors as BLyS and complement-inhibiting factors that are expressed in the synovium. In addition, the interaction between B cells and other cells, including T cells, follicular dendritic cells, and synovial fibroblasts, could protect B cells against undergoing cell death.

These observations confirm and extend data obtained in animal models. In mouse models, the kinetics of B cell depletion and the sensitivity of B cells to rituximab varied among different tissues. In a human CD20–transgene mouse model, B cells in the peripheral blood and lymphoid tissues were highly sensitive to cell death in response to rituximab treatment, while B cells located in germinal centers in lymphoid tissues were far less sensitive to depletion. This could be explained by the protective effect of B cell–activating factors such as BLyS and the requirement for B cells to access the circulation for efficient depletion. Consistent with these findings, B cells in germinal centers in the thyroid of a patient with Graves’ disease were found to be present despite rituximab 7 months after treatment. In contrast to these studies, a disruption of germinal centers in human RA synovial tissue engrafted into SCID mice was seen 3 days after treatment with anti-CD20 antibodies. This difference might be explained by the different treatment regimens used and the drug kinetics in this particular model.

We found an increase in the number of sublining macrophages 4 weeks after initiation of treatment, at a time when no clinical improvement had yet occurred. This might be explained by the withdrawal of biologic agents and DMARDs other than methotrexate prior to study inclusion, consistent with our previous studies showing an increase in sublining macrophages in placebo-treated patients. Thus, it is tempting to speculate that a reduction in macrophage numbers might follow after a longer follow-up period.

The observations presented herein clearly show specific depletion of B cells in the inflamed synovium early after the initiation of rituximab treatment. It should be noted, however, that depletion is not complete and does not occur in all patients. Persistent synovial tissue B cells might still drive the local inflammatory process in these patients and could be a source of the low numbers of circulating B cells (CD19⁺, IgD⁺, CD27⁺) that have been described after treatment with rituximab in patients with RA and systemic lupus erythematosus. The persistence of these B cells could also be responsible for an early relapse of RA activity. Future studies will need to address whether a more profound depletion can be achieved over time and whether evaluation of synovial B cells could assist in predicting the clinical response and deciding when to give the patient a second course of treatment. Examination of synovial biopsy samples obtained at later time points is also needed to provide insight into the secondary effects on other cell populations in the inflamed joint.
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CD22 IS NOT EXPRESSED MERELY ON B CELLS: 
COMMENT ON THE ARTICLE BY VOS ET AL
CD22 IS NOT EXPRESSED MERELY ON B CELLS: COMMENT ON THE ARTICLE BY VOS ET AL.

To the Editor

We read with interest the recent report by Vos and colleagues on the early depleting effects of rituximab in the synovial tissue of patients with rheumatoid arthritis (RA), showing that the depletion of CD22+ cells was incomplete 1. Recently, we reported the depleting effects of rituximab in paired samples of peripheral blood, bone marrow, and synovium 2. Our study revealed a complete depletion of the CD20+ subset of B cells in synovium, as shown by staining the cytoplasmic tail of the CD20 membrane protein. Our results and those of Vos et al are seemingly contradictory, and the existence of a CD22+ CD20- B cell subset may be relevant to the pathogenic mechanisms of RA. Therefore, we investigated the specificity and sensitivity of CD22, CD19, and CD20 as markers of B cells.

Briefly, peripheral blood mononuclear cells from 5 healthy volunteers were obtained through isolation over a Ficoll and freshly stained with the following markers: fluorescein isothiocyanate (FITC)–conjugated anti-CD20 (clone 2H7); phycoerythrin-conjugated anti-CD19 (clone HIB5); allophycocyanin (APC)–conjugated anti-CD22 (clone S-HCL-1); and FITC-conjugated anti-CD3 (clone 5K7) (all from BD Biosciences, San Jose, CA). After incubation for 30 minutes in the dark, cells were washed and read on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with the FlowJo software program (Tree Star, Ashland, OR). We observed that 97.6% of CD19+ cells and 96.7% of CD20+ cells were CD22 positive. However, only 78.1% and 75.2% of CD22+ cells were positive for CD19 and CD20, respectively (P = 0.04, by Mann-Whitney U test) (Table 1).

In conclusion, these data indicated that CD22 is not a specific marker for B cells, raising the possibility that in the study by Vos et al the residual CD22 positivity after rituximab treatment can be explained by the presence of cell types not belonging to the B cell lineage. In this context, Han et al previously reported that in healthy persons basophils can be isolated with a purity of 99.4% by sorting CD22+,CD19- lymphocytes 3. Mast cells (tissue-infiltrating basophils) do not seem to express CD22 on their membrane but do show intracellular messenger RNA expression of CD22 4. Therefore, we believe further research into the residual CD22+ expressing cells is warranted, and that the results of CD22 single-staining should be interpreted with caution when used in the context of B cell depletion with rituximab.

<table>
<thead>
<tr>
<th>GATED POPULATION</th>
<th>CD22</th>
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<tr>
<td>CD22+ cells</td>
<td>100</td>
<td>78.1</td>
<td>75.2</td>
</tr>
<tr>
<td>CD19+ cells</td>
<td>97.6</td>
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REFERENCES

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REPLY

To the Editor

We appreciate the interest of Dr. Teng and his colleagues in our report. Based on their analysis of peripheral blood mononuclear cells from 5 healthy donors, they suggest that the residual CD20+ positivity in rheumatoid synovial tissue observed in a subset of the patients in our study after rituximab treatment does not necessarily represent persistent B cell infiltration but could be explained by expression of CD20+ by basophils or perhaps mast cells in the synovium. We need to reject this hypothesis for the following reasons. Previous work has shown that basophil granulocytes are absent in the synovium of patients with rheumatoid arthritis (RA) and mast cells do not express CD20 at the protein level. We have confirmed our previous results by demonstrating CD19+ cells in the synovium after rituximab treatment, with results similar to those observed with CD22 staining (Figure 1).

Recently, we also showed a variable tissue response 16 weeks after rituximab treatment. Three other groups of investigators have confirmed our results using CD19 and CD20 as markers (8 weeks and 24 weeks after the initiation of treatment). Taken together, the evidence is clear that B cells may persist in the synovium in some patients, although on average there is a marked reduction in the number of such cells, as shown in our study.

How could we explain the complete depletion of CD20+ B cells in the synovium in the study by Teng and colleagues, when 4 other groups of investigators observed a variable tissue response? First, we need to consider false-negative results due to technical reasons. Teng et al used an antibody directed against the cytoplasmic tail of CD20 to detect synovial B cells after treatment with rituximab. When rituximab binds to CD20, this may induce redistribution of the CD20 molecule into lipid rafts, which induces proximity with molecules involved in signal transduction.

In conclusion, ample evidence suggests that B cell infiltration in the synovium may be persistent in a subset of patients with RA after rituximab treatment. It is tempting to speculate that the recently described persistence of synovial plasma cells in nonresponders to rituximab treatment is related to the persistence of these synovial B cells, which are thought to serve as their precursors.

In addition, oligomers with other CD20 molecules may be formed. Because redistribution of CD20 requires the cytoplasmic tail, it is conceivable that this process may interfere with the sensitivity of the immunohistologic method. Thus, the results reported by Teng et al need to be confirmed by staining for CD19 or CD22. Second, we need to take into account the difference in sensitivity of the method used to quantify the stained tissue sections. Whereas Teng et al evaluated the sections by semiquantitative analysis using a 5-point scale, we quantified the results by fully quantitative digital image analysis, which is a more sensitive method. An alternative explanation for the discrepancy between the results described by Teng et al and those observed by other groups of investigators is the use of high-dose corticosteroids, which may have biased the results: the patients received 100 mg of methylprednisolone twice, and they were allowed to receive up to 20 mg of oral prednisolone daily and also 80 mg of intraarticular prednisolone after the first arthroscopy (which could have an effect on the contralateral joints as well). Thus, complete depletion of CD20+ B cells in the tissue might be explained in part by use of high-dose corticosteroids rather than by the effect of rituximab alone. As we have shown previously, prednisolone treatment has marked effects on synovial B cells. Consequently, the changes in the synovium and bone marrow shown by Teng and colleagues are not necessarily specific for rituximab treatment but may be related to combination therapy with corticosteroids.

In conclusion, ample evidence suggests that B cell infiltration in the synovium may be persistent in a subset of patients with RA after rituximab treatment. It is tempting to speculate that the recently described persistence of synovial plasma cells in nonresponders to rituximab treatment is related to the persistence of these synovial B cells, which are thought to serve as their precursors.


FIGURE No.1

FIGURE 1. Change in the number of CD19+ B cells in synovial tissue 4 weeks after rituximab treatment. Data are presented as box plots, where the boxes represent the 25th to 75th percentiles, the lines within the boxes represent the median, and the lines outside the boxes represent the 10th and 90th percentiles. Circles indicate outliers. * P<0.05
SYNOVIAL
TISSUE RESPONSE
TO RITUXIMAB:
MECHANISM OF
ACTION AND
IDENTIFICATION
OF BIOMARKERS
OF RESPONSE
SYNOVIAL TISSUE RESPONSE TO RITUXIMAB: MECHANISM OF ACTION AND IDENTIFICATION OF BIOMarkers OF RESPONSE

OBJECTIVE: To investigate the synovial tissue in patients with rheumatoid arthritis (RA) treated with rituximab and to identify possible predictors of clinical response.

METHODS: A total of 24 patients with RA underwent synovial biopsy before, 4 and 16 weeks after initiation of rituximab treatment (without peri-infusional corticosteroids to prevent bias). Immunohistochemical analysis was performed and stained sections were analysed by digital image analysis. Linear regression analysis was used to identify predictors of clinical response.

RESULTS: The 28-joint Disease Activity Score (DAS28) was unaltered at 4 weeks, but significantly reduced at 16 and 24 weeks. Serum levels of IgM-rheumatoid factor (RF) decreased significantly at 24 weeks and anticitrullinated peptide antibody (ACPA) levels at 36 weeks. Peripheral blood B cells were depleted at 4 weeks and started to return at 24 weeks. Synovial B cells were significantly decreased at 4 weeks, but were not completely depleted in all patients; there was a further reduction at 16 weeks in some patients. We found a significant decrease in macrophages at 4 weeks, which was more pronounced at 16 weeks. At that timepoint, T cells were also significantly decreased. The reduction of plasma cells predicted clinical improvement at 24 weeks.

CONCLUSIONS: The results support the view that B cells orchestrate local cellular infiltration. The kinetics of the serological as well as the tissue response in clinical responders are consistent with the notion that rituximab exerts its effects in part by an indirect effect on plasma cells associated with autoantibody production, which could help explain the delayed response after rituximab treatment.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder affecting synovial tissue in multiple joints. Early treatment with disease-modifying antirheumatic drugs (DMARDs) has become the cornerstone of therapy. Recently, new biological therapies, including rituximab, have become available. Rituximab is a chimaeric monoclonal antibody directed against the CD20 antigen expressed by B cells, which significantly improves disease symptoms in patients with high levels of disease activity despite treatment with methotrexate (MTX) or tumour necrosis factor (TNF) blockers 1–3. This clinical effect strongly supports the notion that B cells play a critical role in the pathogenesis of RA, although the exact mechanism of rituximab treatment in RA remains to be elucidated.

We have previously shown that rituximab treatment causes a rapid and specific decrease in numbers of B cells at the primary site of inflammation, the rheumatoid synovium 4, which was recently confirmed in another study 5. The early synovial tissue response varies between patients, which is in contrast with the marked B cell depletion observed in the peripheral blood of nearly all patients with RA. Interestingly, in the earlier, smaller studies
there was no significant decrease in numbers of inflammatory cells other than synovial B cells 4–8 weeks after initiation of treatment.

Currently, no data are available on the synovial tissue response to rituximab treatment after more prolonged follow-up and its predictive value related to clinical improvement. The current study was performed to investigate the kinetics of this response in detail and to identify possible predictors of clinical response in patients with RA.

**PATIENTS AND METHODS**

**PATIENTS AND TREATMENT PROTOCOL.** A total of 24 patients were included in this study analysing synovial biopsies at three timepoints: before treatment, at 4 weeks and 16 weeks after initiation of rituximab treatment; 17 of these patients participated in a previously reported study on the synovial tissue response to rituximab at 4 weeks only. The patients had active RA; active disease was defined as having >4 tender joints and >4 swollen joints of 28 joints assessed, and at least one of the following: erythrocyte sedimentation rate (ESR) >28 mm/h, serum C-reactive protein (CRP) levels >15 mg/litre, or morning stiffness >45 min. In addition, patients needed to be positive for IgM-RF and/or anti-citrullinated peptide antibodies (ACPA) and have active arthritis (defined by the presence of pain and swelling) of a wrist, knee or ankle joint, amenable for arthroscopy.

All study patients were on stable doses of MTX (5–30 mg/week) for at least 28 days prior to enrolment. Stable prednisone therapy (10 mg/ day) and non-steroidal anti-inflammatory drug (NSAID) treatments were allowed. All other DMARDs and biological agents were withdrawn at least 4 weeks prior to study inclusion, with a washout period for leflunomide, infliximab, adalimumab and etanercept of 8 weeks prior to randomisation. The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center/University of Amsterdam, and all patients gave written informed consent before participation in the study.

Treatment consisted of two infusions of 1000 mg of rituximab (Roche, Woerden, The Netherlands) on days 1 and 15 after premedication with 2 mg clemastine fumarate intravenously and 1000 mg acetaminophen orally. Peri-infusional treatment with corticosteroids and weeks 4, 16, and 24 weeks after the first infusion of rituximab. To minimise sampling error at least six biopsy samples were obtained from different sites in the joint during each procedure. Specimens were directly embedded in bloc in TissueTek OCT (Miles Diagnostics, Elkhart, Indiana, USA) and subsequently snap-frozen in liquid nitrogen.

**IMMUNOHISTOCHEMISTRY.** From each frozen tissue block serial sections (5 mm) were cut and stained with the following mouse monoclonal antibodies: anti-CD19 (SK; Becton Dickinson), anti-CD4 (SK3; Becton Dickinson) and anti-CD8 (DK25; Dako, Glostrup, Denmark) to detect T cells, anti-CD22 (CLB-B-ly; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) for CD21 expression, and anti-CD23 (clone MHM6; Dako) to detect macrophages, anti-CD25 (clone 6F; Serotec, Oxford, UK) to detect fibroblast-like synoviocytes, anti-CD38 (clone B-84; Immunotech, Marseille, France) to detect plasma cells and anti-CD23 (clone MHM6; Dako) as well as anti-CD21L (a kind gift from Dr Y Liu, Schering-Plough, Dardilly, France) to detect follicular dendritic cells (FDCs), as described previously. Sections with non-assessable tissue, defined by the absence of an intimal lining layer, were not analysed. For control sections, the primary antibodies were omitted or irrelevant antibodies were applied.

To determine the distribution of B cells and FDCs in lymphocyte aggregates, we evaluated two separate tissue sections (each section representing one level of the six biopsy samples that were embedded en bloc), located 50 mm apart, which were stained with the anti-CD22, anti-CD23 and anti-CD21L antibodies. CD22 rather than CD19 was used to detect B cells in tissue, as it results in more reliable staining on the tissue level. The presence of FDCs was assessed by CD23 staining and subsequently confirmed by CD21L staining.

**DIGITAL IMAGE ANALYSIS.** All sections were coded and randomly analysed by computer-assisted image analysis using a Leica DM-RXA light microscope and a computer programme (Leica Microsystems QWin software (both from Leica, Cambridge, UK) and an independent observer (Marjolein Vinkenoog, Division of Clinical Immunology and Rheumatology of the Academic Medical Centre/University of Amsterdam, The Netherlands) who was unaware of the clinical data performed the acquisition and image analysis. For all markers, 18 high-power fields were analysed, as previously described and validated. CD68 expression was analysed separately in the intimal lining layer and the synovial sublining. CD23 expression was analysed on two levels as described above, and the mean number of B cells was used for statistical analysis. Data are expressed as the number of cells per mm², representing cell density. Lymphocyte aggregates were counted and classified as previously described.

**BLOOD LYMPHOCYTE POPULATION.** Flow cytometry on fresh peripheral blood samples was performed at baseline and at 4, 16 and 24 weeks after the first rituximab infusion. B cells and T cell subsets were detected by real-time flow cytometry using FACS (FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, California, USA) with antibodies against CD19, CD23, CD4 and CD8 (all from Becton Dickinson). CD19 was chosen because of its similar expression to CD20 on B cell subsets, without interference with the circulating rituximab antibody. The lower limit of detection of CD19 + B cells was set at 0.016 × 10⁹ cells/ litre.
gates with a radius of 2–5 cells were defined as grade 1, those with a radius of 5–10 cells were graded as 2, and those with a radius of >10 cells were graded as 3. The total number of grade 1, 2 and 3 was added up per section. The resulting figure was termed the total number of aggregates.

**Statistical Analysis.** Changes in the DAS28 were compared using the Student paired t test. Changes in peripheral blood lymphocytes, synovial cell populations, as well as serum levels of total IgM, total IgG, IgM-RF and ACPA were compared using Wilcoxon signed rank test for paired data. A mixed linear model was used as a repeated measurements method to confirm the results of the separate paired t test for analysis of changes in synovial parameters.

Correlations between changes in synovial cell populations, serum IgM-RF and ACPA levels and the DAS28 between baseline, 4 weeks and 16 weeks after treatment were determined by Spearman correlation coefficient. Differences in baseline synovial cell populations and changes in cell populations between responders and non-responders to treatment were determined by the Mann–Whitney U test for unpaired data.

For analysis of possible predictive biomarkers for clinical response, clinical response was determined as a decrease in DAS28 of ≥1.2 at 24 weeks *, according to the European League Against Rheumatism (EULAR) response criteria * and the decrease in DAS28 between baseline and week 24. First, Mann–Whitney U test for unpaired data (for dichotomous analysis of response) and Spearman correlation coefficient (for continuous analysis of response) were used to identify variables related to the clinical response. Significantly related parameters were subsequently analysed by univariate linear regression analysis to assess the predictive value. Finally, these parameters were analysed together with multiple linear regression analysis in a backward model.

**Results.**

Baseline characteristics of the study patients.

| TABLE No.1 |
| Baseline characteristics of the study patients. |

**DEMOGRAPHICS**

| Median age, years (range) | 55 (22-75) |
| Female, no (%) | 18 (75) |

**DISEASE STATUS**

| Median disease duration, years (range) | 12 (0.9-50) |
| Erosive disease, no (%) | 24 (100) |
| Nodular disease, no (%) | 9 (38) |
| Median IgM-RF, kU/liter (IQR) | 75 (46-159) |
| Median ACPA, kU/liter (IQR) | 240 (95-1031) |
| Median DAS28 (IQR) | 6.5 (1.1) |
| Median ESR, mm/hr (IQR) | 37 (19-55) |
| Median CRP, mg/dl (IQR) | 24 (10-76) |

**MEDICATIONS**

| Median no. of previous DMARDs (IQR) | 4 (2-3) |
| Median no. of previous biologic agents (IQR) | 2 (1-3) |
| Median methotrexate dosage, mg/week (IQR) | 15 (10-25) |
| Corticosteroids, no. (%) | 16 (67) |
| Median prednisone dosage, mg/day (IQR) | 5 (0-10) |

* ACPA, anti-cyclic citrullinated peptide; CRP, C-reactive protein; DAS28, 28-joint Disease Activity Score; DMARDs, disease-modifying antirheumatic drugs; ESR, erythrocyte sedimentation rate; IQR, interquartile range; RF, rheumatoid factor.

**Clinical and demographic details are shown in table 1.**

**Clinical response to treatment with rituximab.**

One patient withdrew from the study after 20 weeks because of insufficient clinical response. For analysis related to response at week 24 the observations from her week 20 visit were carried forward. The DAS28 was unaltered at 4 weeks (peri-infusional corticosteroids were not allowed), but there was a significant decrease in DAS28 at 16 and 24 weeks (compared to baseline: mean (SD) decrease of 1.6 (1.1) and 1.6 (1.6), respectively; both P < 0.001). At 24 weeks 16 of the 24 patients (67%) had a decrease in DAS28 of at least 1.2. In all, 3 patients (13%) had a good response according to the EULAR response criteria, 14 patients (58%) a moderate response and 7 patients (29%) did not fulfil the EULAR response criteria.
DECREASED IGM-RF AND ACPA LEVELS AFTER RITUXIMAB TREATMENT.

The IgM-RF and ACPA levels were not available for the week 24 visit and week 36 for the patient who withdrew from the study after 20 weeks because of insufficient clinical response. For another patient the visit at week 36 was not performed because of personal circumstances. Thus, serial IgM-RF and ACPA levels were available for 22 patients.

There was a highly significant decrease in serum levels of IgM-RF at 16 (P = 0.006) and 24 weeks (P < 0.001) after treatment (fig 1). There was a trend towards lower ACPA levels at 24 weeks with a statistically significant decrease after 36 weeks (P = 0.0155) (fig 1). At 24 weeks the serum levels of IgM-RF and ACPA decreased significantly more than those of their respective antibody classes (P = 0.001 for IgM-RF compared with total serum IgM; P = 0.026 for ACPA compared with total serum IgG levels).

DEPLETION OF B CELLS IN PERIPHERAL BLOOD.

CD19+ B cells in PB were undetectable 4 and 16 weeks after rituximab treatment (table 2). Low numbers of B cells could be measured in 2/24 patients at week 4 and 4/24 patients at week 16 (0.01 x10^9/litre). At 24 weeks B cells started to return in 13 of 23 analysed patients (median 0.01 x10^9/litre (IQR 0.00–0.02). The total numbers of T cells and T cell subsets did not change significantly after treatment (table 2).

THE EFFECTS OF RITUXIMAB ARE NOT LIMITED TO SYNOVIAL B CELLS: CHANGES IN PLASMA CELLS, T CELLS, FDCS AND MACROPHAGES.

Out of the total of 24 patients analysed, baseline biopsies of 2 patients did not pass synovial tissue quality control; of these patients only samples taken after 4 and 16 weeks were included in the analysis. In two other patients the biopsy samples taken after 16 weeks and in one patient the biopsy taken after 4 weeks was of insufficient quality to be included in the analysis.

Extension of the study population in the present study confirmed our previous observations showing a highly significant reduction of synovial B cells at 4 weeks, but not in all patients (fig 2, table 2). Similar results were obtained when we used anti-CD19 antibodies to detect synovial B cells by immunohistochemistry (data not shown). There was no statistically significant additional reduction of synovial B cells at 16 weeks on the group level, but there was a clear trend towards more pronounced B cell depletion in 7 of the 15 patients who had persistent B cells at week 4 (median 39 cells/mm² (IQR 12–89) and 4 (0–22), at respectively 4 and 16 weeks after rituximab infusion). In five patients there was clear persistence of B cells (median 52 (IQR 9–551) and 101 (63–313) at baseline and at 16 weeks, respectively).

Since B cells are precursors of plasma cells, and B cell depletion could indirectly result in a decrease in short-lived plasma cells associated with autoantibody production we examined the consequences of rituximab treatment for plasma cell numbers in the synovium. There was a marked reduction of plasma cells in a subset of the patients (fig 2, table 2). On the group level this change did not reach statistical significance due to the variability of the response.

The number of T cells was unaltered at 4 weeks, but there was a significant decrease in T cell numbers at 16 weeks (fig 2, table 2). Consistent with the reduction of synovial B cells and T cells (the major cell populations in the lymphocyte aggregates), we observed a trend towards reduced numbers of lymphocyte aggregates at 4 weeks with a significant decrease of aggregates of all sizes at 16 weeks (fig 2, table 2). FDCs were found in four patients at baseline, but were undetectable in all patients 16 weeks after treatment. Hence, rituximab treatment had a clear effect on lymphocyte aggregates and germinal centres (defined by the presence of FDCs).

Of interest, we also found a significant decrease in intimal macrophages at 4 weeks, which was even more pronounced at 16 weeks (fig 2, table 2). In addition, there was...
a trend towards lower numbers of sublining macrophages at 4 weeks with a statistically significant reduction at 16 weeks (fig 2, table 2). Using repeated measurement methods for analysis of changes in synovial cell populations between baseline and 4 and 16 weeks after treatment yielded the same results as separate paired t test analysis.

Persistence of B cells after 16 weeks was correlated with persistence of plasma cells ($r = 0.70; P < 0.001$), T cells ($r = 0.69; P < 0.001$), and lymphocyte aggregates ($r = 0.72; P < 0.001$) at the same timepoint.

**CHANGES IN SYNOVIAL PLASMA CELLS PREDICT CLINICAL RESPONSE.**

Since the clinical response to rituximab treatment may be variable, we studied whether changes in synovial cell populations were related to clinical improvement.

Of importance, there were no baseline characteristics of the synovium that could significantly predict clinical response to treatment ($P = 0.005$), but not so in nonresponders. Similarly, there was a significant difference in reduction of intimal macrophages between responders and non-responders ($P = 0.002$) (fig 4B and 5). Within the clinical responder group there was a significant decrease in plasma cells after treatment ($P = 0.005$), but not so in nonresponders. Similarly, there was a significant reduction in intimal macrophages between responders and non-responders ($P = 0.008$; fig 4D), with a similar trend for sublining macrophages (fig 4F).

Linear regression analysis was performed to establish the predictive value of the changes in synovial cell populations for the size of the clinical response after 24 weeks. Consistent with the analyses described above, we found a positive correlation between the change in intimal macrophages ($r = 0.51; P = 0.04$) as well as plasma cells ($r = 0.46, P = 0.003$) between 4 and 16 weeks on the one hand and the decrease in DAS28 after 24 weeks on the other. Accordingly, linear regression analysis revealed

**TABLE No.2**

Cells in peripheral blood and synovial tissue obtained from patients with rheumatoid arthritis before, 4 and 16 weeks after rituximab treatment.

<table>
<thead>
<tr>
<th>CD19</th>
<th>P *</th>
<th>CD3</th>
<th>P *</th>
<th>CD4</th>
<th>P *</th>
<th>CD8</th>
<th>P *</th>
<th>CD138</th>
<th>P *</th>
<th>CD68L</th>
<th>P *</th>
<th>Aggregates</th>
<th>P *</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.13 (0.09-0.19)</td>
<td>&lt;0.01 (0.00-0.00)</td>
<td>&lt;0.001</td>
<td>&lt;0.01 (0.00-0.00)</td>
<td>&lt;0.001</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1.32 (1.01-1.68)</td>
<td>1.27 (1.00-1.90)</td>
<td>0.78</td>
<td>1.26 (1.00-1.87)</td>
<td>0.49</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.93 (0.52-1.14)</td>
<td>0.78 (0.69-1.53)</td>
<td>0.64</td>
<td>0.85 (0.69-1.22)</td>
<td>0.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.29 (0.19-0.48)</td>
<td>0.34 (0.20-0.52)</td>
<td>0.51</td>
<td>0.34 (0.22-0.49)</td>
<td>0.38</td>
<td></td>
<td></td>
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<td></td>
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</tr>
</tbody>
</table>

CD68+ macrophages were analysed separately in the intimal lining layer (L) and synovial sublining (SL).

Values for peripheral blood lymphocytes are median (interquartile range (IQR)) $10^9$/litre, for cellular markers in synovium median (IQR) cells/mm$^2$. The total no. of lymphocyte aggregates (aggregates) was counted per section.* Wilcoxon signed rank test for paired data between baseline and week 4. ** Wilcoxon signed rank test for paired data between baseline and week 16.
that the decrease in plasma cells between 4 and 16 weeks could predict the decrease in DAS28 at 24 weeks after treatment ($R^2 = 0.26$, $P = 0.002$).

The reduction of macrophages also showed a trend for predicting clinical improvement at week 24 in the univariate analysis ($P = 0.051$), but when analysed together with the decrease in plasma cells in a multiple linear regression model, it was not an independent predictor of the decrease in DAS28 ($P = 0.216$, partial correlation 0.399). When linear regression analysis was performed on the combination of changes in plasma cells between 4 and 16 weeks and the baseline DAS28 using multiple linear regression analysis, the decrease in plasma cells continued to predict the decrease in DAS28 at week 24 ($P = 0.034$), whereas the baseline DAS28 alone could not predict the clinical response ($P = 0.623$). Interestingly, the change in plasma cell numbers was also correlated with a decrease in the ACPA levels at week 16 ($r = 0.52$, $P = 0.03$).

When we used the change in synovial cells between baseline and 16 weeks, similar trends were observed. As noted above and shown in Table 2 and Fig 2, the major changes in synovial cell populations other than B cells were observed between 4 and 16 weeks after treatment, secondary to the changes in synovial B cells.

Collectively, the results suggest that the clinical response can be predicted by changes in cell types other than B cells, especially the number of synovial plasma cells that are derived from B cells. This change is also correlated to the reduction in serum ACPA levels.
FIGURE No.3

Differences in synovial B cells at baseline (A), respectively changes in B cells after rituximab treatment (B and C), in clinical responders versus non-responders. There was no statistically significant difference in B cell numbers at baseline or in the reduction in synovial B cells between responders and non-responders. The circles represent outliers (values of more than 1.5 box lengths from the upper or lower edge of the box; the box length is the interquartile range). * P <0.05, ** P <0.01.

FIGURE No.4a

Differences in synovial plasma cells at baseline (A), respectively changes in plasma cells after rituximab treatment (B and C), in clinical responders versus non-responders. There was no statistically significant difference in plasma cell numbers at baseline or in the reduction in synovial plasma cells between responders and non-responders. The circles represent outliers (values of more than 1.5 box lengths from the upper or lower edge of the box; the box length is the interquartile range). * P <0.05, ** P <0.01.
FIGURE No.4b

FIGURE No.5

FIGURE 4. Differences in synovial plasma cells (A), intimal macrophages (C), sublining macrophages (E) and T cells (G) at baseline respectively changes in these cells (B, D, F, H) after rituximab treatment in clinical responders versus nonresponders. There was no statistically significant difference in these cells at baseline between responders versus nonresponders. In light of the kinetics of the changes after treatment (fig 2), we compared the decrease in synovial cell populations other than B cells between 4 weeks and 16 weeks in relationship to clinical response. There was a highly significant difference in reduction of intimal macrophages (P = 0.008), respectively plasma cells (P = 0.002), between responders compared to nonresponders with a similar trend for sublining macrophages. The circles represent outliers (values of more than 1.5 box lengths from the upper or lower edge of the box; the box length is the interquartile range). *P < 0.05, **P < 0.01.

FIGURE 5. Change in the number of CD138+ plasma cells in representative serial synovial tissue samples obtained at 4 (A and C) and 16 (B and D) weeks after initiation of rituximab treatment. Different patterns of response were identified. In patients who responded to treatment we observed a reduction in plasma cells between 4 and 16 weeks after treatment (compare A and B), while in patients who did not fulfil the response criteria, plasma cells persisted (compare C and D) (Original magnification x20). Linear regression analysis revealed a significant relationship between the decrease in plasma cell numbers and the decrease in 28-Joint Disease Activity Score (DAS28) at week 24.
DISCUSSION

The results presented here confirm the previously reported variable tissue response of B cells after rituximab treatment, in contrast to the nearly complete depletion of B cells in the peripheral blood. Moreover, this study shows for the first time the secondary effects on cell populations other than synovial B cells, supporting the concept that B cells orchestrate synovial inflammation. In particular the change in synovial plasma cells (derived from B cells) between 4 and 16 weeks after initiation of treatment is related to the clinical response over time. These findings are consistent with the kinetics of the gradual clinical response and the slow but sure decrease in levels of circulating antibodies observed after rituximab treatment.

B cells may drive the inflammatory processes involved in RA by different mechanisms. First, B cells may drive synovial inflammation by production of autoantibodies; they are the precursors of short-lived plasma cells associated with production of autoantibodies, such as IgM-RF and ACPA. Second, B cells are effective antigen-presenting cells and activators of T cells. Third, B cells may promote synovial inflammation by producing pro-inflammatory cytokines and chemokines. Thus, depletion of B cells could interfere with different mechanisms involved in the pathogenesis. The results from the present study show that rituximab treatment may indeed deplete B cells at the primary site of inflammation, the synovium, although there is persistence of synovial B cells in a subset of patients. The discrepancy with the complete B cell depletion observed in peripheral blood in nearly all patients might be explained by the expression of protective factors in the tissue, such as BLYS and CD40L, as well as the requirement for B cells to access the circulation for efficient depletion. This difference underscores the importance of analysis of different compartments to understand the effects of treatment, as has also been shown after, for example, Campath-1H treatment. The variable response in the synovial tissue with regard to B cell depletion suggests that the standard therapeutic regimen is perhaps not optimal in all patients. Of note, persistence of synovial B cells was related to persistence of plasma cells. Future studies need to address the question whether it is possible to induce clinical improvement in non-responders with persistent B cells and plasma cells in the synovium. Conceivably, a subset of patients would benefit from a more intense dosing schedule. It is also possible that persistence of plasma cells in non-responders is related to the presence of long-lived plasma cells in the synovium of a subset of patients. Plasma cells with different longevity could be induced by mechanisms such as epitope spreading. For these patients alternative approaches may be considered, interfering with, for example, APRIL (a proliferation-inducing ligand) and B lymphocyte stimulator (BLyS). The present study strengthens the rationale for evaluating changes in biomarkers after targeted therapies interfering with B cells and plasma cells to further optimise the clinical response in the context of personalised medicine.

The relationship between the change in plasma cells and clinical improvement suggests that rituximab exerts its effects at least in part by an indirect effect on short-lived autoreactive plasma cells that are associated with the production of autoantibodies. Consistent with these results, there was a reduction in RF and ACPA levels after treatment; the reduction in plasma cells was directly related to the decrease in ACPA levels at week 16. These data are also in agreement with a previous study showing a trend towards lower synovial immunoglobulin synthesis 2 months after rituximab treatment, although that study was not powered to detect statistically significant changes. A role for autoantibodies in RA has been suggested since the discovery of RF in RA and regained interest in the late nineties. The previously reported decrease in RF and ACPA levels after rituximab treatment relative to minor changes in total immunoglobulins suggests a role for short-lived plasma cells in their production. This notion is supported by observations in a mouse model; rheumatoid factor transgenic mice were crossed with mice of the autoimmune-prone MRL/lpr strain, after which a spontaneous rheumatoid factor response developed. This response was mediated by continuous generation of short-lived plasmablasts. In addition, it has been suggested that small immune complexes containing autoantibodies may drive synovial inflammation by triggering Fcγ receptor IIIA, which is expressed by intimal macrophages. The results presented here would support the concept that autoantibody production by B cells and plasma cells is critically involved in promoting synovial inflammation.

Depletion of B cells did not only indirectly result in a decrease in synovial plasma cells, but there was also an effect on other major cell populations, such as T cells and macrophages. This indicates that B cells have an important role in sustaining the inflammatory cell infiltrate in the rheumatoid synovium. The decrease in synovial T cells and the disruption of lymphocyte aggregates and germinal centres, as shown by the disappearance of FDCs, supports the hypothesis that B cells influence T cell activity and organisation in the synovial tissue. Lymphocyte aggregates could be disrupted by the absence of B cell derived factors such as lymphotixin beta. However, it is quite likely that the explanation is more complex, since previous work has shown that, in contrast to large follicles, relatively small lymphocyte aggregates usually contain very few B cells. Interestingly, B cell depletion also diminished macrophage infiltration, which is in agreement with the concept that there is a consistent relationship between clinical improvement and changes in synovial macrophages, independent of the primary mechanism of action of the treatment. Together, the change in T cells and macrophages could be explained by an indirect effect of B cell depletion on the expression of proinflammatory cytokines and chemokines involved in cell migration and retention, although this remains to be shown.

In conclusion, rituximab treatment results in a variable response on synovial B cells with secondary changes in numbers of other inflammatory cells, leading to diminished synovial inflammation. There is a direct relationship between the decrease in synovial plasma cells and clinical improvement over time.

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B Cells and B Cell directed therapies in Rheumatiod Arthritis

response


primary efficacy and safety at twenty-four weeks. (3) controlled, phase III trial evaluating results of a multicenter, random (7)ized, double-blind, placebo-con (6)rolled, dose-ranging trial. (1)ARTHRITIS RHEUM 007:5:79–80.


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Chapter 7

THE RELATIONSHIP BETWEEN
THE TYPE I
INTERFERON
SIGNATURE AND
THE RESPONSE
TO RITUXIMAB
IN RHEUMATOID
ARTHRITIS
THE RELATIONSHIP BETWEEN THE TYPE I INTERFERON SIGNATURE AND THE RESPONSE TO RITUXIMAB IN RHEUMATOID ARTHRITIS

Abstract

OBJECTIVE: To analyze the relationship between the type I interferon (IFN) signature and clinical response to rituximab in rheumatoid arthritis (RA) patients.

METHODS: Twenty RA patients were treated with rituximab (cohort 1). Clinical response was defined as decrease in disease activity score evaluated in 28 joints (DAS28) and according to EULAR response criteria at week 12 and 24. In peripheral blood mononuclear cells the presence of an IFN signature was analyzed measuring the expression levels of three interferon response genes by quantitative PCR. After comparison with healthy controls, patients were qualified as IFN high or IFN low. The data were confirmed in a second independent cohort (n = 31). Serum IFNα bioactivity was analyzed using a reporter assay.

RESULTS: In cohort 1, there was a better clinical response to rituximab in the IFN low group. Consistent with this finding, IFN low patients had a significantly stronger reduction in DAS28 and more often achieved an EULAR response at week 12 and 24 compared to IFN high patients in cohort 2. The pooled data showed a significantly greater reduction in DAS28 in IFN low patients at week 12 and 24 compared to the IFN high group and more often an EULAR response at week 12. Accordingly, serum IFNα bioactivity at baseline was inversely associated with the clinical response, although this result did not reach statistical significance.

CONCLUSION: The type I IFN signature negatively predicts the clinical response to rituximab treatment in RA, supporting the notion that IFN signalling plays a role in RA immunopathology.

Introduction

Type I interferons (IFNs) are cytokines that regulate antiviral immune responses. Surprisingly, among a range of auto-antibody associated autoimmune conditions a proportion of patients display a dominant type I interferon (IFN) signature in their peripheral blood mononuclear cells (PBMCs). These diseases include rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), dermatomyositis, Sjögren’s syndrome, systemic sclerosis and multiple sclerosis. In several small cohorts of patients with these conditions the presence of a type I IFN signature was associated with the presence and the titer of auto-antibodies. It has therefore been hypothesized that patients with a type I IFN signature suffer from a pathogenetic subset in which type I IFN stimulating auto-antigens enhance the humoral immune response.

Rituximab has shown clinical efficacy in many of these conditions. Most of the evidence has been gathered in RA. However, rituximab does not induce a clinical response in all patients and does not decrease the level of all autoantibodies. Type I interferons have been associated with enhancement of B cell survival, since they stimulate B cell survival directly and through the production of BlyS and APRIL. Therefore, we hypothesized that patients with a type I interferon signature respond less well to rituximab treatment.
Patients and methods

Patients and treatment protocol. Patients were included from 2 cohorts of RA patients that were reported previously. Patients had active RA (Disease Activity Score evaluated in 28 joints (DAS28) ≥ 3.2) despite methotrexate treatment. The study protocol was approved by the Ethics Committee of the participating centers (cohort 1: UMCU, cohort 2: AMC); all patients gave written informed consent. Patients were treated with 2 infusions of 1000 mg rituximab (day 1 and 15). Pre-medication with methylprednisolone was omitted in the AMC cohort as previously described. In both cohorts the DAS28-ESR was used to evaluate disease activity. The clinical response was defined by the decrease in DAS28 at week 24 compared to baseline. Paired serum and PBMC samples were collected at baseline.

Blood sampling for RNA isolation. Blood was drawn in heparin tubes. PBMCs were isolated using a Ficoll gradient and subsequently stored at liquid nitrogen until RNA isolation. RNA isolation was performed using the RNeasy mini kit #74106 and QIAcube #9001293 (Qiagen, Venlo, the Netherlands). A DNAse digestion step was included in the protocol to remove genomic DNA. RNA concentrations were determined using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, MA).

Real-time PCR. RNA (0.5 μg) was reverse transcribed into cDNA using a Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturers’ instructions. Quantitative real-time PCR (qPCR) was performed using an ABI Prism 7900HT Sequence detection system (Applied Biosystems, Foster City, CA) using SybrGreen (Applied Biosystems). Relative expression of OAS1, ISG15 and Mx2 was normalized to 18S RNA expression. To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies a standard curve was constructed. An IFN score was calculated by adding the relative expression values for the three IFN-inducible genes. We defined which patients showed an increased IFN response gene expression, by calculating the 95% limits of the healthy controls; normal values were defined as the mean expression of the 3 IFN-response genes, plus 1.96 times the standard deviation.

Reporter cell assay for type I IFN. The reporter cell assay for type I IFN has been described in detail elsewhere. In this assay, reporter cells were used to measure the ability of patient sera to induce IFN-induced gene expression. The reporter cells (WISH cells, no. CCL-25; American Type Culture Collection) were cultured with 50% patient sera for 6 h and then lysed. cDNA was made from total cellular mRNA and gene expression levels were then quantified using qPCR. Forward and reverse primers for the genes MX-1 (myxovirus resistance 1), PKR (dsRNA-activated protein kinase), and IFIT-1 (IFN-induced protein with tetratricopeptide 1), which are known to be highly and specifically induced by IFNα, were used in the reaction and relative expression determined in comparison to a housekeeping gene control (HPRT1). The type I IFN activity was calculated by determining the mean and standard deviation of the relative expression of each of the three genes in a population of healthy individuals, determining the number of standard deviations above the mean of healthy donors for each of the three genes for each patient sample, and determining the sum of those three standard deviation values.

Serum ELISAs. Serum levels of IgM-rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPA; anti-CCP2 ELISA, Immunoscan RA, Mark 2, Euro Diagnostica, Arnhem, the Netherlands) were determined at baseline and weeks 4, 16, 24 and 36 after treatment. Baseline serum BAFF and APRIL levels were measured by ELISA (R&D and Bender).

Statistical analysis. The Student’s t test and Mann-Whitney U test were used where appropriate for comparison of baseline data between the two cohorts and IFN high and IFN low patients. Linear and logistic regression analysis was used where appropriate for prediction analyses. The Wilcoxon signed rank test for paired data was used to analyze the decrease in auto-antibodies.

tested this hypothesis in 2 independent cohorts of RA patients commencing rituximab treatment.
The Type I IFN Signature in PBMCS Predicts the Clinical Response to Rituximab Treatment.

As a primary endpoint for analysis, we used the clinical response at week 24 as measured by the decrease in DAS28 and EULAR response criteria. To gain more understanding about the dynamics of the clinical response we also analysed the clinical response at week 12.

First, we performed an exploratory analysis in the test cohort, cohort 1. Six of the 20 (30%) patients had an IFN high signature. The decrease in DAS28 after 12 and 24 weeks was markedly lower in the IFN high group compared to the IFN low group, although the difference did not reach statistical significance, possibly due to the relatively small number of patients (mean ± SD: -0.52 ± 0.75 compared to -1.49 ± 1.04; P = 0.056, R² = 0.19 at week 12 and -0.67 ± 0.9 compared to -1.70 ± 1.60; P = 0.15, R² = 0.11 at week 24, using linear regression analysis [Figure, panel A]). When analyzing the EULAR response, less IFN high patients achieved a EULAR response compared to IFN low patients (7 out of 18 versus 10 out of 13; P = 0.043, R² = 0.18 at week 12 and 9 out of 18 versus 11 out of 13; P = 0.059, R² = 0.17 at week 24).

Subsequently, because we observed a comparable pattern in both cohorts, we pooled the data for univariate regression analysis. Using this approach in the patients, the IFN signature was a significant predictor of the decrease in DAS28 after rituximab treatment (0.71 ± 0.8 for IFN high versus 1.6 ± 0.97 for IFN low; R² = 0.20; P = 0.001 at week 12 and 0.90 ± 1.48 for IFN high versus 1.96 ± 1.43 for IFN low R² = 0.12; P = 0.012 at week 24 [Figure, panel C]). Fewer IFN high patients achieved a EULAR response at week 12 compared to IFN low patients (2 out of 6 versus 10 out of 14; P = 0.13, R² = 0.16 using logistic regression analysis) but differences were less clear cut at week 24 (3 out of 6 versus 8 out of 14; P = 0.77, R² = 0.01). Next, we tested the relationship between IFN signature and clinical response to rituximab in a larger, independent cohort, which served as validation cohort (cohort 2). Eighteen of 31 patients (58%) had a type I IFN high signature at baseline. Consistent with the results in cohort 1, the decrease in DAS28 12 and 24 weeks after initiation of rituximab treatment was lower in IFN high patients compared to the IFN low group (-0.77 ± 0.83 compared to -1.67 ± 0.92; P = 0.008, R² = 0.22 at week 12 and -0.98 ± 1.60 compared to -2.30 ± 1.30; P = 0.16; P = 0.027 at week 24 [Figure, panel B]). When analysing the EULAR response, less IFN high patients achieved a EULAR response compared to IFN low patients (7 out of 18 versus 10 out of 13; P = 0.043, R² = 0.18 at week 12 and 9 out of 18 versus 11 out of 13; P = 0.059, R² = 0.17 at week 24).

Consistent with these findings, serum IFN bioactivity negatively predicted the decrease in DAS28, which did not reach statistical significance (P = 0.06, R² = 0.12 at week 12; P = 0.11, R² = 0.09 at week 24) and the achievement of a EULAR response at week 12 (P = 0.055, R² = 0.20 at week 12 and P = 0.24, R² = 0.07 at week 24).

Since type I interferons have been associated with enhancement of B cell survival in the tissues, we explored whether the IFN high signature might be associated with persistence of circulating autoantibodies. The levels of IgM-RF and ACPA decreased significantly in both IFN low and IFN high patients, but the reduction of both IgM-RF and ACPA appeared indeed more pronounced in the IFN low group. In IFN high patients the IgM-RF titer (n = 19) decreased from (median [IQR]) 639 (274 - 1197) to 270 (63 – 396) kU/ liter (P = 0.055, R² = 0.16, R² = 0.19 at week 24) and the achievement of a EULAR response at week 12 (P = 0.055, R² = 0.20 at week 12 and P = 0.24, R² = 0.07 at week 24).

Analysis of the relationship between the IFN signature and the relative decrease in DAS28 after rituximab yielded similar results (Figure, panel D-F). Using multivariate regression analysis in a forward model, we found that the IFN signature remained a predictor of the decrease in DAS28 to rituximab treatment, even when correcting for SJC at baseline (R² = 0.16, P = 0.003 and R² = 0.09, P = 0.029 at week 12 and 24, respectively). Consistent with these findings, serum IFN bioactivity negatively predicted the decrease in DAS28, which did not reach statistical significance (P = 0.06, R² = 0.12 at week 12; P = 0.11, R² = 0.09 at week 24).
Table No.1

Comparison of patient characteristics between the two cohorts.

<table>
<thead>
<tr>
<th>DEMOGRAPHICS (N=51)</th>
<th>AMC (N=31)</th>
<th>UMC (N=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, no. (%)</td>
<td>24 (77)</td>
<td>12 (60)</td>
<td>0.187</td>
</tr>
<tr>
<td>Age, median (range) years</td>
<td>54 (28-72)</td>
<td>59 (32-70)</td>
<td>0.354</td>
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</table>

<table>
<thead>
<tr>
<th>BASELINE DISEASE STATUS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM-RF positive, no. (%)</td>
<td>26 (84)</td>
</tr>
<tr>
<td>ACPA positive, no. (%)</td>
<td>28 (90)</td>
</tr>
<tr>
<td>DAS28, mean ± SD</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td>ESR, median (range) mm/hour</td>
<td>37 (4-86)</td>
</tr>
<tr>
<td>CRP, median (range) mg/dl</td>
<td>20 (2-112)</td>
</tr>
<tr>
<td>Disease duration, median (range) years</td>
<td>12 (1-30)</td>
</tr>
<tr>
<td>Symptomatic secondary Sjögren’s syndrome (%)</td>
<td>3 (10)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MEDICATION</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Concomitant methotrexate, no. (%)</td>
<td>31 (100)</td>
</tr>
<tr>
<td>Concomitant methotrexate dosage, median (range)</td>
<td>15 (5-30)</td>
</tr>
<tr>
<td>Concomitant oral prednisone, no. (%)</td>
<td>20 (65)</td>
</tr>
<tr>
<td>Concomitant oral prednisone dosage, median (range)</td>
<td>5 (5-10)</td>
</tr>
</tbody>
</table>

* IgM-RF = IgM rheumatoid factor; SD = standard deviation; ACPA = anti-citrullinated peptide antibodies; DAS28 = Disease Activity Score 28-joint assessment; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein.

Table No.2

Comparison of clinical and biological characteristics between IFN high and IFN low patients.

<table>
<thead>
<tr>
<th></th>
<th>IFN high (n=24)</th>
<th>IFN low (n=27)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon serum bioactivity, median IQR [n=28]</td>
<td>0.49 (0.0 – 0.99)</td>
<td>0.0 (0.0 – 0.48)</td>
<td>0.012</td>
</tr>
<tr>
<td>DAS28, mean ± SD [n=51]</td>
<td>6.2 ± 1.0</td>
<td>6.6 ± (1.0)</td>
<td>0.16</td>
</tr>
<tr>
<td>Swollen joint count, median ± SD [n=51]</td>
<td>9 ±5</td>
<td>14 ± 6</td>
<td>0.013</td>
</tr>
<tr>
<td>Tender joint count, median ± SD [n=51]</td>
<td>14 ± 7</td>
<td>15 ± 6</td>
<td>0.43</td>
</tr>
<tr>
<td>VAS disease activity, median ± SD [n=51]</td>
<td>44 ± 2</td>
<td>46 ± 16</td>
<td>0.64</td>
</tr>
<tr>
<td>ESR, median ± SD [n=51] mm/hour</td>
<td>38 (19-55)</td>
<td>45 (21-61)</td>
<td>0.50</td>
</tr>
<tr>
<td>CRP, median ± SD [n=51] mg/dl</td>
<td>19 (5-112)</td>
<td>269 ± 117</td>
<td>0.38</td>
</tr>
<tr>
<td>IgM-RF positive, no. (%) [n=51]</td>
<td>21 (88)</td>
<td>22 (81)</td>
<td>0.56</td>
</tr>
<tr>
<td>ACPA positive, no. (%) [n=51]</td>
<td>20 (86)</td>
<td>23 (83)</td>
<td>0.56</td>
</tr>
<tr>
<td>IgM-RF titer, median ± SD [n=51] IU/ml</td>
<td>81 (2-128)</td>
<td>62 (2-128)</td>
<td>0.57</td>
</tr>
<tr>
<td>ACPA titer, median ± SD [n=51] IU/ml</td>
<td>352 (0-4958)</td>
<td>240 (0-1366)</td>
<td>0.31</td>
</tr>
<tr>
<td>Serum BLyS level [n=28]</td>
<td>1265 (1039-1518)</td>
<td>1246 (964-1536)</td>
<td>0.76</td>
</tr>
<tr>
<td>Serum APRIL [n=28]</td>
<td>11 (6-24)</td>
<td>24 (14-50)</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* IFN = interferon; IQR = interquartile range; IgM-RF = IgM rheumatoid factor; SD = standard deviation; ACPA = anti-citrullinated protein antibodies; DAS28 = Disease Activity Score 28-joint assessment; VAS = visual analogue scale; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; BLyS = B Lymphocyte Stimulator; APRIL = A Proliferation Induced Ligand.
In this study we found that patients with a type I IFN signature in PBMCs respond less well to rituximab treatment than patients without an IFN signature. A possible confounder in this study could have been the lower baseline disease activity, as measured by the swollen joint count, in IFN high patients. However, we found that the IFN signature also predicted the decrease in DAS28 when we controlled for the swollen joint count in a multivariate regression model. We found a more pronounced difference in the clinical response between IFN low and IFN high patients at week 12 compared to week 24 after rituximab treatment. This may be caused by the relatively small size of the patient cohorts. Alternatively, IFN high patients may respond more slowly to rituximab than IFN low patients.

The data presented here suggest that the type I IFN signature is not just an epiphenomenon in RA, but might be involved in its pathogenesis. RA is traditionally viewed as a TNFα driven disease. This concept is amongst others based on the effectiveness of TNFα blocking agents in RA and results from genetic studies in which an association was found between TNFα related genes and ACPA positive RA versus type I IFN related genes and ACPA negative RA\cite{17-19}. According to this model ACPA positive RA is predominantly driven by TNFα, based on in vitro observations that TNFα downmodulates the effects of type I IFNs and vice versa. However, the results from the current study, together with other observations, contradict this model.

First, in a recent genome wide association study both single nucleotide polymorphisms in TNF related genes and IFN related genes were associated with autoantibody positive RA\cite{20}. Furthermore, TNFα, IFNα and IFNβ are simultaneously expressed in RA synovial tissue. In line with these data we found that plasma TNFα levels were at least equal in IFN high compared to IFN low patients (data not shown). Taken together, these data suggest that the presence of TNFα and type I IFNs is not mutually exclusive. Instead of the single-cytokine based paradigm the data support a concept of multiple immune mechanisms that act simultaneously in RA.

In contrast to our hypothesis, we could not find a direct relationship between the response to rituximab, the IFN signature, increased levels of APRIL and BlyS and persistence of autoantibodies. This is supported by available data in the literature, which suggest a complex contribution of type I IFNs to RA pathogenesis and response to therapy. The production of type I IFN is not only stimulated by viruses, but also by apoptotic/necrotic material, RNA binding proteins, osteopontin and autoantigen-containing immune complexes\cite{17,21-26}. Furthermore, gene polymorphisms may contribute to excessive IFN signalling to subphysiologic activating stimuli\cite{27}. Subsequently, type I IFNs may not only stimulate the production of APRIL and BlyS and directly enhance B cell survival but also stimulate T cells and dendritic cells\cite{17,26}. At the same time, IFNβ is able to reduce the secretion of pro-inflammatory cytokines like IL-6, matrix metalloproteinases (MMPs), and prostaglandin E2 by fibroblast-like synoviocytes. In addition, IFNβ has anti-angiogenic properties and may inhibit osteoclastogenesis\cite{28,29}.

**FIGURE No.1**

DIFFERENCE IN DECREASE IN DAS28 AFTER TREATMENT BETWEEN IFN HIGH AND IFN LOW PATIENTS. THE DIFFERENCE IN ABSOLUTE AND RELATIVE DECREASE IN DAS28 AFTER TREATMENT BETWEEN IFN HIGH AND IFN LOW PATIENTS WAS COMPARRED USING LINEAR REGRESSION ANALYSIS. BOTH IN COHORT 1 (A,D [N=20]) AND COHORT 2 (B,E [N=31]) PATIENTS WITH AN IFN HIGH SIGNATURE SHOWED A SMALLER DECREASE IN DAS28 AT WEEK 12 AND 24 COMPARED TO PATIENTS WITH AN IFN LOW SIGNATURE. ANALYSIS OF POOLED DATA YIELDED SIMILAR RESULTS (C,F). MEAN VALUES AND STANDARD DEVIATION ARE SHOWN. NS = NOT SIGNIFICANT, * P <0.05, ** P < 0.01.
The relationship between the type I IFN signature and the humoral autoimmune response in RA was analyzed in a number of previous studies. In a first study the type I IFN signature was found in a subset of RA patients and correlated with autoantibody-associated diseases, similar to findings in other autoantibody-associated diseases. Also, the type I IFN signature was identified in a subset of autoantibody-positive arthralgia patients who later developed RA. A direct relationship between the IFN signature and autoantibody responses in RA could not be confirmed in a subsequent investigation in a larger cohort, in whom the IFN signature was found to occur equally often in seropositive and seronegative RA patients. However, the presence of an IFN signature was associated with persistence of ACPA after TNF blockade. Taken together with the association between polymorphisms in IFN-related genes and both seropositive and seronegative RA, the current and previous data suggest that IFN-related autoimmunity does not directly determine humoral autoimmunity in RA, but may promote and sustain humoral autoimmunity responses in seropositive patients.

In RA the level of type I IFN bioactivity has been associated with the clinical response to TNF blockade, although these results need to be replicated since they have been variable for different cohorts. Pilot data from an RA patient cohort treated with TNF antagonists suggested that relatively high plasma levels of type I IFN activity prior to initiation of therapy were associated with better clinical response than those agents that lower plasma type I IFN activity. Patients with high type I IFN activity may respond better to TNF blockade because of the anti-inflammatory effects of their disease-associated high IFNβ levels. Alternatively, patients with an IFN signature may have an overall higher inflammatory activity than IFN low patients and may respond better to TNF blockade because of higher TNFα activity. This is in line with the earlier finding that patients with higher levels of synovial inflammation and synovial TNFα expression respond better to TNF blockade. The concept of a lower inflammatory activity in IFN low patients is supported by the fact that IFN low patients do not show an alternative gene activation signature in their PBMCs, but have a gene signature comparable to healthy controls.

How can we explain the finding that patients with an IFN high signature respond less well to rituximab? First, IFN high patients could have a disease that is less B cell-dependent. This appears unlikely, since all patients in our study were RF and/or ACPA positive. Furthermore, current evidence suggests non-response to rituximab is associated with persistence of B cells. Conceivably, higher inflammatory activity in IFN high patients may relatively protect B cells against the depleting effects of rituximab. Although we found similar systemic levels of BlyS and APRIL in IFN high and IFN low patients, type I IFNs may promote survival of pathogenic B cells in lymphoid tissue or bone marrow. This might involve contact-dependent interactions with T cells, dendritic cells and stromal cells and membrane-bound survival factors like CXCL12, VCAM, membrane-bound BlyS or heparan-sulphate bound APRIL. With regard to the clinical response to rituximab, although BlyS and APRIL are thought to rescue B cells from B cell depletion, systemic levels of BlyS and APRIL do not predict clinical response to rituximab. Nonetheless, the reason for the decreased clinical response to rituximab in IFN high patients remains speculative until more data become available.

The decreased clinical response to rituximab in IFN high RA patients seemingly contradicts the clinical responsiveness of IFN-associated diseases to rituximab. However, the presence of high type I IFN activity does not necessarily imply that these patients will respond better to rituximab treatment. In contrast, recent randomized controlled trials failed to show clinical efficacy of rituximab in patients with non-nephritis lupus, although the explanation for the negative findings is still a subject of controversy. Our data suggest that rituximab might be less effective in SLE because of the higher systemic type I IFN levels in SLE patients.

RA is a heterogeneous condition with a heterogeneous response to therapy. It would be important to identify biomarkers that may be used to predict the response to targeted treatment. The presence of solely the IFN signature would obviously not allow treatment decisions with regard to initiation of rituximab therapy. In general, the combination of multiple (clinical and biological) markers bears the most promise for meeting the needs of useful predictors of response to treatment. Our data support the evaluation of the IFN signature in future studies assessing the validity of combinations of markers in relationship to the response to rituximab treatment.

In conclusion, this study is the first to show a relationship between the IFN high signature and the clinical response to rituximab through an as yet undefined mechanism.
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CHAPTER 8

CLINICAL RESPONSE, PHARMACOKINETICS, DEVELOPMENT OF HUMAN ANTI-CHIMERIC ANTIBODIES, AND SYNOVIAL TISSUE RESPONSE TO RITUXIMAB TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS
CLINICAL RESPONSE, PHARMACOKINETICS, DEVELOPMENT OF HUMAN ANTI-CHIMERIC ANTIBODIES, AND SYNOVIAL TISSUE RESPONSE TO RITUXIMAB TREATMENT IN PATIENTS WITH RHEUMATOID ARTHRITIS

Abstract

OBJECTIVES: To analyze whether persistence of synovial B lineage cells and lack of clinical response to rituximab treatment in rheumatoid arthritis (RA) patients are associated with low rituximab serum levels and anti-rituximab antibody (ARA) formation.

METHODS: Fifty-eight RA patients were treated with rituximab. The clinical response was determined 24 weeks after each treatment course using the disease activity score evaluated in 28 joints (DAS28) and EULAR response criteria. Rituximab serum levels, ARAs and synovial B lineage cell numbers were determined before and after treatment.

RESULTS: Four weeks after treatment rituximab serum levels were highly variable. Low rituximab levels were associated with ARA formation (in 5 patients [8.6%]) and high baseline erythrocyte sedimentation rate. Interestingly, serum rituximab levels were not related to persistence of synovial B lineage cells or clinical response. Furthermore, response to treatment and re-treatment was similar in ARA positive compared to ARA negative patients.

CONCLUSION: There is clear variability in serum levels after rituximab treatment, but rituximab levels are not lower in patients with persistence of synovial B lineage cells or lack of clinical response. The current treatment schedule suffices to induce and maintain a clinical response, even when ARAs are formed.

Introduction

Rituximab is an effective therapy for rheumatoid arthritis (RA). Recent studies have shown that rituximab induces an incomplete B cell depletion in the synovial tissue of a subset of RA patients and that persistence of synovial B lineage cells and (small numbers of) B cell subsets in the peripheral blood is associated with lack of clinical response. This might theoretically be explained by suboptimal rituximab levels in these patients due to a high initial B cell load, early formation of anti-rituximab antibodies (ARA) or other factors influencing pharmacokinetics. Therefore, we analyzed the relationship between these parameters in a cohort of RA patients starting rituximab treatment. The data were confirmed in an independent cohort.
PATIENTS AND METHODS

PATIENTS. Patients were included from 2 studies on the synovial tissue response to rituximab in RA that were reported previously [6]. Patients had active RA (Disease Activity Score evaluated in 28 joints (DAS28) > 3.2) despite methotrexate treatment. The study protocol was approved by the Ethics Committee of the participating centers; all patients gave written informed consent.

TREATMENT REGIMEN. Patients were treated with 2 infusions of 1000 mg rituximab (day 1 and 15). Pre-medication with methylprednisolone was omitted in the AMC cohort. In both cohorts the DAS28 was obtained at baseline and after 24 weeks. A clinically significant decrease in disease activity was defined according to the EULAR response criteria [6]. Patients were retreated after at least 24 weeks [6].

MEASUREMENT OF RITUXIMAB LEVELS AND ARAS. Rituximab levels and ARAs were measured after 4, 12 and 24 weeks (LUMC) or 4, 16 and 24 weeks (AMC).

IMMUNOHISTOCHEMISTRY. Synovial biopsies were collected by arthroscopy in 17 patients of the LUMC cohort and 24 patients of the AMC cohort as described previously [6]. In the AMC cohort frozen sections were stained with anti-CD20 (Becton Dickinson, San Jose, CA) and anti-CD22 (CLB-B-ly; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) to detect plasma cells. In the LUMC cohort paraffin-embedded sections were stained with anti-cytoplasmic CD20 (clone L26) to detect B cells, anti-human CD138 (clone B-B4; Immunotech, Marseille, France) to detect plasma cells. In the LUMC cohort paraffin-embedded sections were stained with anti-human CD138 (clone B-B4; Serotec, Oxford, UK) to detect plasma cells. The immunohistochemical stainings were quantified using digital image analysis (AMC) or semiquantitative evaluation, respectively (LUMC [6]). The relationship between CD20+ B cells and rituximab levels was only analyzed for baseline samples, since rituximab bound to CD20 might interfere with the detection of B cells using anti-CD20 [6].

STATISTICAL ANALYSIS. Student’s paired t-tests were used to evaluate the change in DAS28 after treatment. Univariate linear and univariate logistic regression analyses were calculated, where appropriate, to first assess the relationship between baseline patient characteristics, ARAs and rituximab levels; second between rituximab levels and persistence of synovial B lineage cells; and third between rituximab serum levels, ARAs, and clinical response determined by the decrease in DAS28 and the EULAR response (moderate/good versus none).

FIGURE No.1

RESULTS

PATIENT CHARACTERISTICS.

Clinical response to the first and second treatment course was available for in total 58 and 47 patients, respectively. Clinical characteristics and clinical response are shown in Table 1.

VARIABILITY IN SERUM LEVELS OF RITUXIMAB AND PREDICTORS OF VARIABILITY.

Rituximab levels measured 4 weeks after the first infusion were remarkably variable with a range of 0.3 – 362 (median 110) μg/ml (Figure 1A). ARAs were detectable in 2 patients who had received methylprednisolone and in 3 who did not receive this pre-medication. Since the incidence of ARA formation was low, the two cohorts were combined, when possible, for further analyses involving ARAs. Rituximab levels in ARA positive patients were lower compared to ARA negative patients, when possible, for further analyses involving ARAs. Rituximab levels measured 4 weeks after treatment (right). Data are represented by geometric means and 95% confidence intervals; *p < 0.05. While rituximab clearance was highly variable, neither ARA formation nor rituximab serum levels were related to the clinical response at week 24.

Baseline ESR negatively predicted rituximab levels at week 4 in both patient cohorts (AMC cohort: r = -0.17, P = 0.018; LUMC cohort: r = -0.23, P = 0.007); in the AMC cohort.
TABLE No.1

Patient characteristics and clinical response.

<table>
<thead>
<tr>
<th>DEMOGRAPHICS (N=58)</th>
<th>AMC (N=30)</th>
<th>LUMC (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female, no. (%)</td>
<td>24 (80)</td>
<td>20 (71)</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>BASELINE DISEASE STATUS</th>
<th>AMC (N=30)</th>
<th>LUMC (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgM-RF positive, no. (%)</td>
<td>25 (83)</td>
<td>24 (86)</td>
</tr>
<tr>
<td>ACPA positive, no. (%)</td>
<td>27 (90)</td>
<td>23 (82)</td>
</tr>
<tr>
<td>DAS28, mean (± SD)</td>
<td>6.5 ± 1.1</td>
<td>6.0 ± 1.2</td>
</tr>
<tr>
<td>ESR, median (range) mm/hour</td>
<td>37 (4-86)</td>
<td>46 (5-139)</td>
</tr>
<tr>
<td>CRP, median (range) mg/dl</td>
<td>29 (1.9-112)</td>
<td>25 (2.0-114)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MEDICATION</th>
<th>AMC (N=30)</th>
<th>LUMC (N=28)</th>
</tr>
</thead>
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<tr>
<td>Concomitant methotrexate, no. (%)</td>
<td>30 (100)</td>
<td>21 (75)</td>
</tr>
<tr>
<td>Concomitant leflunomide, no (%)</td>
<td>0 (0)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Corticosteroids, no. (%)</td>
<td>21 (70)</td>
<td>11 (39)</td>
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</table>

<table>
<thead>
<tr>
<th>CLINICAL RESPONSE 24 WEEKS AFTER COURSE 1 (N=58)</th>
<th>AMC (N=30)</th>
<th>LUMC (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28, mean (± SD)</td>
<td>5.0 ± 1.9</td>
<td>4.5 ± 1.2</td>
</tr>
<tr>
<td>EULAR good (%)</td>
<td>4 (13)</td>
<td>5 (18)</td>
</tr>
<tr>
<td>EULAR moderate (%)</td>
<td>15 (50)</td>
<td>17 (61)</td>
</tr>
<tr>
<td>EULAR none (%)</td>
<td>11 (37)</td>
<td>4 (14)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CLINICAL RESPONSE 24 WEEKS AFTER COURSE 2 (N=47)</th>
<th>AMC (N=22)</th>
<th>LUMC (N=25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DAS28, mean (± SD)</td>
<td>4.5 ± 1.7</td>
<td>3.9 ± 1.1</td>
</tr>
<tr>
<td>EULAR good (%)</td>
<td>5 (23)</td>
<td>9 (36)</td>
</tr>
<tr>
<td>EULAR moderate (%)</td>
<td>10 (46)</td>
<td>14 (56)</td>
</tr>
<tr>
<td>EULAR none (%)</td>
<td>7 (32)</td>
<td>2 (8)</td>
</tr>
</tbody>
</table>

* ACPA, anti-citrullinated peptide antibodies; AMC, Academic Medical Centre/University of Amsterdam; CRP, C-reactive protein; DAS28, Disease Activity Score 28-joint assessment; ESR, erythrocyte sedimentation rate; EULAR, European League Against Rheumatism; IgM-RF, IgM rheumatoid factor; LUMC, Leiden University Medical Centre.

TABLE No.2

Prediction of decrease in synovial B lineage cells by rituximab (RTX) levels at week 4.

<table>
<thead>
<tr>
<th>RTX LEVELS WK 4</th>
<th>AMC (N=30)</th>
<th>LUMC (N=28)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persistence of CD22+ B cells at wk 4</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Persistence of CD19+ B cells at wk 4</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>Change CD22+ B cells wk 4-16</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>Change CD19+ B cells wk 4-16</td>
<td>0.62</td>
<td></td>
</tr>
<tr>
<td>Change CD138+ plasma cells wk 4-16</td>
<td>0.13</td>
<td></td>
</tr>
<tr>
<td>CD138+ plasma cells wk 0-12</td>
<td>0.71</td>
<td></td>
</tr>
</tbody>
</table>

ARAS

| Change CD22+ B cells wk 0-16 | 0.24       |             |
| Change CD19+ B cells wk 0-16 | 1.00       |             |
| Change CD138+ plasma cells wk 0-16 | 0.76   |             |
| Change in CD79a+ B/plasma cells wk 0-12 | 0.10   |             |
| CD138+ plasma cells wk 0-12 | 0.37       |             |

Logistic regression analysis was used to calculate the relationship between rituximab levels and persistence of synovial B cells at week 4; linear regression analysis was used to calculate the relationship between rituximab levels at week 4 and the subsequent change in synovial B lineage cells. A Academic Medical Centre/University of Amsterdam; L Leiden University Medical Centre.

Similar trends were also found for baseline CRP and DAS28 (for CRP: $r^2 = -0.23, P = 0.006$; for DAS28: $r^2 = -0.13, P = 0.032$).

However, no relationship was found between rituximab levels after 4 weeks and body surface area, gender, use of oral prednisolone, dosage of methotrexate, or use of methylprednisolone pre-medication.

Synovial B cells persist despite detectable rituximab levels in peripheral blood.

In the AMC cohort the change in synovial CD19+ and CD22+ B cells was analyzed 4 and 16 weeks after initiation of treatment. A marked decrease in synovial B cells was found 4 weeks after the first infusion. While in some patients a further decrease in B cells...
Synovial B cells persisted in a subset of patients (in 47% and 35% of patients after 4 and 16 weeks, respectively). We compared serum rituximab levels in patients with persistence of synovial B cells at week 4 to those in patients without detectable synovial B cells at that time point (i.e. 2 weeks after the second infusion when therapeutically active levels of rituximab are expected). Of interest, serum rituximab levels did not differ between these groups (Table 2; Figure 2A). Similarly, the rituximab levels at week 4 did not predict whether synovial B cells persisted or decreased further after 16 weeks (Figure 2B). Also, rituximab levels at week 4 did not predict the persistence of plasma cells at week 16.

These data were confirmed in the LUMC cohort. Rituximab levels at week 4 or 12 did not correlate with persistence of synovial CD79+ B cells or CD138+ plasma cells (Table 2).

**VARIABILITY IN RITUXIMAB LEVELS AND ARA FORMATION ARE NOT RELATED TO THE CLINICAL RESPONSE TO RITUXIMAB.**

Consistent with the results presented above clinical non-responders did not have lower rituximab levels compared to responders ([AMC] $P = 0.81$, $P = 0.33$ for week 4 and 16; [LUMC] $P = 0.58$, $P = 0.11$ for week 4 and 12). ARA positive patients experienced a similar decrease in DAS28 and EU-LAR response 24 weeks after the first and second treatment course compared to ARA negative patients ($P = 0.87$ and $P = 0.32$, for the response to course 1 and 2, respectively; Figure 2C,D).
DISCUSSION

We examined whether persistence of synovial B lineage cells and lack of clinical response are related to low rituximab serum levels. We show that ARAs formation and differences in baseline disease activity are partly responsible for a marked variability in serum rituximab levels after therapy. Nevertheless, patients with ARAs or relatively low rituximab levels experience on average similar depletion of synovial B lineage cells and a similar clinical response compared to those without ARAs or higher serum levels of rituximab.

The relationship between rituximab levels, ARAs and systemic inflammation is in line with earlier observations in patients treated with infliximab 11. Conceivably, patients with high systemic inflammation have a higher B cell load, although we found no direct correlation with synovial or circulating B cell numbers. Alternatively, (therapeutic) antibodies might be cleared more rapidly in these patients.

The data suggest that persistence of B cells after rituximab may be explained by expression of local survival factors rather than suboptimal rituximab levels. Furthermore, the current rituximab treatment regimen results in drug levels that remain in the therapeutic range (defined by response in terms of clinical signs and symptoms) even when patients form ARAs. These findings are in line with 2 dose-ranging studies that showed no statistically significant difference in ACR20, ACR50, or ACR70 response between patients treated with 2500 mg compared to those treated with 2x1000 mg rituximab 14-15. It should be noted that the group of ARA positive patients was relatively small and that higher serum levels could perhaps result in a clinical response of longer duration. The present study was not designed to address this possibility, since all patients were re-treated after 24 weeks if the DAS28 ≥ 3.2 5. Other limitations include the lack of data on rituximab levels at earlier time points and data on drug levels in the synovium. Although the data suggest that perhaps lower doses of rituximab might be used in some patients, it is obviously too early to recommend this for clinical practice until more data on the effects of both clinical signs and symptoms and structural outcomes will become available. Moreover, there is a clear need for the identification of biomarkers that may help to further optimise rituximab treatment in individual patients.

ACKNOWLEDGMENTS

We would like to thank the pharmacologist Marleen Kemper and the research technicians ELS De Groote, Ann Van Houten and Hein de Wijke.

REFERENCES


11. van Gestel AM, Haagasma CI, van Riel PL. Validation of rheumatoid arthritis improvement criteria that include simplified joint counts. Arthritis Rheum 1999; 41:1845-1856.


CHAPTER 9

DISEASE ACTIVITY-GUIDED RITUXIMAB THERAPY IN RHEUMATOID ARTHRITIS: THE EFFECTS OF RETREATMENT IN INITIAL NON-RESPONDERS VERSUS INITIAL RESPONDERS
DISEASE ACTIVITY-GUIDED RITUXIMAB THERAPY IN RHEUMATOID ARTHRITIS: THE EFFECTS OF RETREATMENT IN INITIAL NON-RESPONDERS VS INITIAL RESPONDERS

Abstract

OBJECTIVE. To explore the efficacy of re-treatment with rituximab in patients with rheumatoid arthritis (RA) who were initial nonresponders to treatment, and to evaluate the effects of rituximab in RA when retreatment is given in a standardized way based on the Disease Activity Score in 28 joints (DAS28), according to the international consensus statement.

METHODS. Patients with RA who had a DAS28 of >3.2 received up to 3 courses of rituximab at intervals of at least 6 months, regardless of whether the patient had responded to the first course of treatment with rituximab.

RESULTS. Of the 30 patients with RA who were included in the study, 26 could be evaluated for the efficacy of treatment after 6 months. Eighteen patients qualified for re-treatment at 6 months, 6 patients were re-treated at a later time point because of a disease relapse, and 2 other patients were not re-treated because they had low disease activity. Seven of the 24 patients who qualified for re-treatment had not exhibited clinical improvement after the first treatment course. These patients typically did not respond to subsequent courses of rituximab. Of interest, in the 17 patients who had exhibited a clinical response to the first course of rituximab, the second and third treatment courses resulted in European League Against Rheumatism responses similar to those observed after the first course, and no major relapses occurred before re-treatment.

CONCLUSION. Rituximab re-treatment is not effective in patients who do not exhibit clinical improvement after the first treatment course, which is consistent with the notion that such patients represent a different pathogenetic subset of RA. Patients who initially responded to rituximab treatment experienced sustained benefit from DAS28-based systematic re-treatment according to the international consensus statement.

Introduction

Rituximab, a chimeric monoclonal antibody targeting CD20 expressed on B cells, is an effective and safe treatment of rheumatoid arthritis (RA) 1–3. Currently, a course of rituximab treatment consists of 2 infusions administered during a 2-week period. According to a recent consensus statement, rituximab treatment should be repeated if patients experience a clinical response to the first treatment course and significant disease activity remains or recurs 4. Currently, there are no data on re-treatment of patients with RA who do not exhibit clinical improvement after the first course of rituximab.

In patients who experience a clinical response to rituximab, the number of synovial B cells 5,6 and especially B cell–derived plasma cells 7 decreases after rituximab treatment, which is consistent with the original hypothesis that rituximab treatment may break a self-perpetuating course of proliferation of self-reactive pathogenic B cell clones causing RA 8,9. Apparently, rituximab is not able to break this course of inflammation in patients whose RA is unresponsive to therapy. The reasons for the variable response are unknown, but suboptimal depletion of B cells and B cell–derived plasma cells may be associated with autoimmunity in the tissue of patients who do not experience a clinical response to the first course of rituximab 7,10. If this hypothesis were true, such patients theoretically might benefit from re-treatment, resulting in
more pronounced B cell depletion. Alternatively, patients without a clinical response to the first course of rituximab may represent a different pathogenetic subset of the clinical syndrome termed RA. In this case, a clinical response to retreatment obviously cannot be expected in patients who did not respond to treatment initially.

The recently published international consensus statement on the use of B cell–targeted treatment with rituximab in patients with RA recommends repeating treatment if patients experienced a clinical response to a first treatment course, at least 6 months have passed, and significant disease activity persists or a disease flare occurs. This consensus statement is based on the experience in open-label extension studies of registration trials. In these studies, physicians were allowed to re-treat patients with active disease after 4 months or 6 months, at their own discretion; this approach introduced some variability. In these studies, clinical responses were maintained and perhaps slightly improved after each course, and major disease flares were prevented. It is important to study whether use of the currently advised standardized re-treatment schedule is able to maintain a clinical response and prevent major disease flares, for the following reasons. First, recurrent disease flares are invalidating for the patient. Second, disease flares exert a disproportionately large effect on radiographic progression. Third, the persistence of B cells, and in particular B cell–derived plasma cells, in the synovial tissue of some patients after rituximab treatment suggests that retreatment before disease flares may be required to improve the clinical response.

To determine whether an initial nonresponse to rituximab treatment predicts a lack of response to subsequent courses of treatment, and to evaluate the clinical effects of systematic, disease activity–guided re-treatment with rituximab in accordance with the international consensus statement, we retreated patients with RA according to a standardized re-treatment schedule based on the Disease Activity Score in 28 joints (DAS28), independent of the clinical response to the first course. Patients whose DAS28 was ≥3.2 after at least 6 months were re-treated.

### PATIENTS AND METHODS

**Patients.** The study group comprised patients with a diagnosis of RA according to the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for the classification of RA and in whom disease remained active despite methotrexate (MTX) treatment. Active disease was defined by the presence of ≥4 tender joints and ≥4 swollen joints (of 28 joints assessed), as well as at least 1 of the following criteria: erythrocyte sedimentation rate ≥ 28 mm/hour, serum C-reactive protein level ≥ 15 mg/liter, or morning stiffness lasting ≥45 minutes. Patients negative for both IgM rheumatoid factor (IgM-RF) and anti–citrullinated protein antibodies (ACPA) were excluded from the study.

All study patients were receiving treatment with MTX (5–30 mg/week) for at least 3 months, with stable dosages for 4 weeks prior to inclusion. Stable low-dose prednisone therapy (≤10 mg/day) and nonsteroidal antiinflammatory drug (NSAID) treatment were allowed. Treatment with all other disease-modifying antirheumatic drugs (DMARDs) and biologic agents was withdrawn at least 4 weeks prior to study inclusion, with the exception of leflunomide, etanercept, adalimumab, and infliximab treatment of ≥8 weeks prior to inclusion. No alteration of DMARD therapy was allowed during the study period. The study protocol was approved by the Medical Ethics Committee of the Academic Medical Center/University of Amsterdam, and all patients gave written informed consent.

**TREATMENT REGIMEN AND CLINICAL EVALUATION.** Patients were treated with 2 infusions of rituximab (1,000 mg on days 1 and 15) (Roche, Woerden, The Netherlands). Pretreatment included clemastine (2 mg intravenously) and acetaminophen (1 gm orally). In contrast to routine clinical practice, premedication with methylprednisolone was not given during the first course, since the patients had also participated in a study on the effects of rituximab on biomarkers, and this could have introduced bias. After an interval of at least 6 months after the start of the first rituximab treatment course, patients whose DAS28 was ≥3.2 received re-treatment with a second course of rituximab. We allowed a maximum delay of 1–2 months between the decision to re-treat and the administration of rituximab, for logistic reasons. The DAS28 was determined at baseline and every month after treatment and subsequent re-treatment. A clinically significant decrease in disease activity was defined as a moderate or good response according to the European League Against Rheumatism (EULAR) criteria, as measured during at least 2 consecutive study visits. A relapse of disease activity was defined as an increase of ≥0.6 in the DAS28 from the lowest achieved value. Patients were followed up for up to 2 years.

**STATISTICAL ANALYSIS.** In addition to descriptive statistics, we used Student’s paired t-tests to evaluate the change in the DAS28 after treatment, because these data were normally distributed. Changes in serum immunoglobulin titers were evaluated using the nonparametric Wilcoxon signed rank test for paired data.
The clinical and demographic characteristics of the 30 patients who were included in the study are shown in Table 1. All patients had active disease despite MTX treatment, with a mean DAS28 of 6.5. All patients were positive for IgM-RF and/or ACPAs. Twenty-five patients had previously been treated with ≥ 1 tumor necrosis factor (TNF) blocker. The reasons for withdrawal differed between patients and for each compound: in 5 patients, 1 or more TNF blockers were withdrawn because of side effects; in 17 patients, 1 or more TNF blockers were withdrawn because of inefficacy (primary inefficacy in 10 patients and secondary inefficacy in 7 patients); in 3 patients, the first TNF blocker was withdrawn because of side effects, and a second and/or third agent was withdrawn because of inefficacy. All patients were treated with MTX (range 5–30 mg), with the dosage remaining stable during followup. Twentyone patients received concomitant oral low-dose prednisone (range 5–10 mg/day). One patient was treated with oral prednisone at a dosage of 20 mg/day, because of persistent high disease activity 1–2 months after the second treatment course; 6 months after the start of treatment, the dosage was decreased.

### TABLE No.1

**Characteristics of the 30 patients***

<table>
<thead>
<tr>
<th>Demographics</th>
<th>(n=30)</th>
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<tbody>
<tr>
<td>Age, median (range) years</td>
<td>56 (22-75)</td>
</tr>
<tr>
<td>Female, no. (%)</td>
<td>24 (80)</td>
</tr>
<tr>
<td><strong>Disease status</strong></td>
<td></td>
</tr>
<tr>
<td>Disease duration, median (range) years</td>
<td>12 (1-50)</td>
</tr>
<tr>
<td>Erosive disease, no. (%)</td>
<td>30 (100)</td>
</tr>
<tr>
<td>Nodular disease, no. (%)</td>
<td>11 (37)</td>
</tr>
<tr>
<td>IgM-RF, median (IQR) kU/l</td>
<td>62 (35-141)</td>
</tr>
<tr>
<td>ACPA, median (IQR) kU/l</td>
<td>352 (126-1268)</td>
</tr>
<tr>
<td>DAS28, mean (± SD)</td>
<td>6.5 (1.1)</td>
</tr>
<tr>
<td>ESR, median (IQR) mm/hour</td>
<td>37 (22-52)</td>
</tr>
<tr>
<td>CRP, median (IQR) mg/l</td>
<td>29 (12-64)</td>
</tr>
<tr>
<td><strong>Medication</strong></td>
<td></td>
</tr>
<tr>
<td>No. of previous DMARDs, median (range)</td>
<td>4 (2-9)</td>
</tr>
<tr>
<td>No. of previous biologic agents, median (range)</td>
<td>2 (0-4)</td>
</tr>
<tr>
<td>Methotrexate dosage, median (range) mg/week</td>
<td>15 (5-30)</td>
</tr>
<tr>
<td>Corticosteroids, no. (%)</td>
<td>21 (70)</td>
</tr>
<tr>
<td>Prednisone dosage, median (IQR) mg/day</td>
<td>7.5 (5-10)</td>
</tr>
</tbody>
</table>

* IgM-RF = IgM rheumatoid factor; IQR = interquartile range; ACPA = anti–citrullinated protein antibody; DAS28 = Disease Activity Score in 28 joints; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DMARDs = disease-modifying antirheumatic drugs.

### FIGURE No.1

**Treatment flow chart.**

EULAR = European League Against Rheumatism.
to 12.5 mg/day. This patient was excluded from the efficacy analysis.

**TABLE No.2**

<table>
<thead>
<tr>
<th>Total no. of AEs† (n = 30)</th>
<th>359</th>
</tr>
</thead>
<tbody>
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<td>Grade 1 Δ</td>
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<tr>
<td>Grade 2</td>
<td>20</td>
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<tr>
<td>Grade 3</td>
<td>20</td>
</tr>
<tr>
<td>Grade 4</td>
<td>0</td>
</tr>
<tr>
<td>SUSARS ‡</td>
<td>5</td>
</tr>
<tr>
<td>- arterial embolism</td>
<td>1</td>
</tr>
<tr>
<td>- pulmonary embolism</td>
<td>1</td>
</tr>
<tr>
<td>- severe infusion reaction</td>
<td>2</td>
</tr>
<tr>
<td>- toxic hepatitis</td>
<td>1</td>
</tr>
<tr>
<td>No. of infections B</td>
<td>48</td>
</tr>
<tr>
<td>Infections per patient-year</td>
<td>0.9</td>
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<tr>
<td>No. of serious infections §</td>
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<td>Urinary tract infections</td>
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<td>- cystitis</td>
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<tr>
<td>- urosepsis</td>
<td>1</td>
</tr>
<tr>
<td>Respiratory infections</td>
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<tr>
<td>- sinusitis</td>
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</tr>
<tr>
<td>- infectious bronchitis</td>
<td>8 ~</td>
</tr>
<tr>
<td>- pneumonia</td>
<td>3</td>
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<tr>
<td>Skin infections</td>
<td>0</td>
</tr>
<tr>
<td>- erysipelas</td>
<td>2</td>
</tr>
<tr>
<td>- cellulitis</td>
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<tr>
<td>- infection burn wound</td>
<td>2</td>
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<tr>
<td>Fever of unknown origin</td>
<td>2</td>
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<tr>
<td>Fungal infections</td>
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<tr>
<td>- cutaneous mycosis</td>
<td>2</td>
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<td>- oral candidiasis</td>
<td>1</td>
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<tr>
<td>Viral infections</td>
<td>1</td>
</tr>
<tr>
<td>- herpes zoster</td>
<td>1</td>
</tr>
<tr>
<td>- recurrence herpes labialis</td>
<td>2</td>
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</tbody>
</table>

**FIGURE No.2**

- **A.** Disease Activity Score in 28 joints (DAS28) over time in 16 patients whose rheumatoid arthritis initially responded to rituximab therapy and who then received systemic disease activity-guided treatment. Values are the mean ± SD, where the circles represent the mean. B. Number of months since the preceding course of rituximab in patients who initially had a response to treatment. n = re-treatment was not given because the DAS28 was < 3.2.

**RESULTS OF SYSTEMATIC RE-TREATMENT IN INITIAL RESPONDERS.**

Seventeen patients who had responded to the first course of rituximab treatment (14 with a moderate EULAR response and 3 with a good EULAR response) received a second course of treatment (Figure 1). Eleven of the 17 initial responders...
ers qualified for a second course 6 months after the first course, because of a DAS28 ≥ 3.2, and (due to logistic reasons) were re-treated at 7–8 months. Six patients experienced a relapse at a later time point (between 338 and 500 days), after which they received a second course (Figure 2). The DAS28 did not return to baseline levels in any of the patients who experienced a relapse (Figure 3). In the 17 initial responders, the second treatment course resulted, on average, in clinical improvement: 4 patients experienced a good EULAR response, 10 had a moderate EULAR response, and only 2 did not fulfill the EULAR response criteria. One patient experienced an infusion-related reaction at the time of re-treatment, after which rituximab was discontinued.

The 4 patients who fulfilled the EULAR criteria for a good response after the second course of treatment did not receive a third course, because of a long-lasting decrease in disease activity (DAS28 ≥ 3.2) after the second treatment course. The remaining 12 patients received a third treatment course, which also resulted in sustained clinical responses: 2 patients experienced a good EULAR response, 10 had a moderate EULAR response, and 1 patient had a poor EULAR response. One patient experienced an infection (Table 2). One patient was admitted to the hospital for intravenous antibiotic treatment of urosepsis. Two patients experienced recurrence of herpes labialis, and 1 patient had herpes zoster infection. There were no serious opportunistic infections or infections with Mycobacterium tuberculosis. In 4 patients, IgM levels were below the lower limit of normal after 6 months (n = 1), 1 year (n = 1), and 2 years (n = 2). In these patients, we observed oral candidiasis (n = 2), cutaneous mycosis (n = 1), and pneumonia (n = 1). Recurrent cystitis occurred in a patient after curettage and in a patient with a previous history of recurrent cystitis. In none of the patients did the IgG or IgA level decrease to below the lower limit of normal.

FIGURE No.3

For the assessment of safety, all 30 patients participating in the study were analyzed (Table 2). The rate of infections requiring antibiotics was 0.9/patient-year during 2 years of followup. Infections consisted of urinary tract infections, respiratory tract infections, skin infections, and fungal and viral infections (Table 2). One patient was admitted to the hospital for intravenous antibiotic treatment of urosepsis. Two patients experienced recurrence of herpes labialis, and 1 patient had herpes zoster infection. There were no serious opportunistic infections or infections with Mycobacterium tuberculosis. In 4 patients, IgM levels were below the lower limit of normal after 6 months (n = 1), 1 year (n = 1), and 2 years (n = 2). In these patients, we observed oral candidiasis (n = 2), cutaneous mycosis (n = 1), and pneumonia (n = 1). Recurrent cystitis occurred in a patient after curettage and in a patient with a previous history of recurrent cystitis. In none of the patients did the IgG or IgA level decrease to below the lower limit of normal.

FIGURE No.3

SAFETY OF THE DAS28-GUIDED RE-TREATMENT SCHEDULE.

For the assessment of safety, all 30 patients participating in the study were analyzed (Table 2). The rate of infections requiring antibiotics was 0.9/patient-year during 2 years of followup. Infections consisted of urinary tract infections, respiratory tract infections, skin infections, and fungal and viral infections (Table 2). One patient was admitted to the hospital for intravenous antibiotic treatment of urosepsis. Two patients experienced recurrence of herpes labialis, and 1 patient had herpes zoster infection. There were no serious opportunistic infections or infections with Mycobacterium tuberculosis. In 4 patients, IgM levels were below the lower limit of normal after 6 months (n = 1), 1 year (n = 1), and 2 years (n = 2). In these patients, we observed oral candidiasis (n = 2), cutaneous mycosis (n = 1), and pneumonia (n = 1). Recurrent cystitis occurred in a patient after curettage and in a patient with a previous history of recurrent cystitis. In none of the patients did the IgG or IgA level decrease to below the lower limit of normal.

FIGURE No.3

SAFETY OF THE DAS28-GUIDED RE-TREATMENT SCHEDULE.

For the assessment of safety, all 30 patients participating in the study were analyzed (Table 2). The rate of infections requiring antibiotics was 0.9/patient-year during 2 years of followup. Infections consisted of urinary tract infections, respiratory tract infections, skin infections, and fungal and viral infections (Table 2). One patient was admitted to the hospital for intravenous antibiotic treatment of urosepsis. Two patients experienced recurrence of herpes labialis, and 1 patient had herpes zoster infection. There were no serious opportunistic infections or infections with Mycobacterium tuberculosis. In 4 patients, IgM levels were below the lower limit of normal after 6 months (n = 1), 1 year (n = 1), and 2 years (n = 2). In these patients, we observed oral candidiasis (n = 2), cutaneous mycosis (n = 1), and pneumonia (n = 1). Recurrent cystitis occurred in a patient after curettage and in a patient with a previous history of recurrent cystitis. In none of the patients did the IgG or IgA level decrease to below the lower limit of normal.
This study is the first to show the clinical response to repeated rituximab treatment in patients with RA that was unresponsive to the first treatment course. In addition, we evaluated re-treatment of patients whose RA responded to a first course of rituximab treatment according to the international consensus statement, using a systematic disease activity–guided re-treatment schedule. Patients with active disease (defined as a DAS28 of ≥ 3.2) received re-treatment at least 6 months after the previous course. We observed that patients who did not respond to the first course of rituximab therapy generally did not respond to subsequent courses. Furthermore, we showed that treatment according to the disease activity–guided strategy is able to sustain the clinical response in initial responders to rituximab treatment and prevent major disease flares.

Systematic re-treatment was generally well tolerated, and there was no clear-cut safety signal compared with previous trials. The relatively high number of adverse events can be explained by the high frequency of comorbidities in our study population, consisting mainly of therapy-refractory RA in patients with high disease activity. We did not observe any opportunistic infections or tuberculosis, although 1 patient experienced a recurrence of herpes zoster shortly after commencing rituximab treatment.

Of importance, the lack of a response to rituximab treatment appears to be a constant feature in patients whose RA does not respond to the first treatment course. This observation is consistent with the notion that perhaps RA is not a single pathogenetic entity, but comprises different subsets leading to similar common final pathways. Disease mechanisms independent of B cells might be driving synovial inflammation in patients not responding to rituximab treatment. It is also conceivable that B cell proliferation and plasma cell formation may continue to occur despite treatment with anti-CD20 antibodies. Differential inflammatory expression of lymphocyte survival factors, such as B lymphocyte stimulator, APRIL, or interleukin-6, might be involved in the persistence of these cells. It has also been suggested that Fc-γ receptor polymorphisms could explain the lack of efficacy in nonresponders to rituximab treatment, but recent data on patients with malignant lymphoma do not support this hypothesis. An alternative explanation may be an effect of complement inhibitory proteins, such as CD55, which could render patients insensitive to rituximab treatment.

Disease activity–guided re-treatment with rituximab according to the international consensus statement, at intervals of at least 6 months, was able to maintain the clinical response and prevent major disease relapses, although it should be noted that the majority of the patients experienced some increase in disease activity before re-treatment. Still, the baseline DAS28 was not reached in any of the patients. We cannot exclude the possibility that the minor increase in disease activity observed after 6 months could have been prevented by re-treatment within the 6-month interval. Ideally, in a treatment-to-target strategy, repeat treatment should be given at the time point at which a clinical response is expected and the target DAS28 is not achieved (i.e., after 4 months), but data on the safety of this approach are as yet unavailable.

Among patients whose RA initially responded to rituximab treatment, the clinical response tended to be more pronounced after the second course of treatment. This observation is consistent with the data obtained from the open-label extension studies of the registration trials. It fits the hypothesis that fixed re-treatment before disease flare may induce a further decrease in synovial inflammation by extending the period during which the level of B cell renewal is reduced and the level of proinflammatory survival signals is low. Similarly, in patients with chronic lymphocytic leukemia or indolent non-Hodgkin’s lymphoma, maintenance with (low-dose) rituximab after induction therapy is effective in enhancing the response rate and prolonging therapy-free survival.

In conclusion, RA patients whose disease initially fails to respond to rituximab are unlikely to exhibit a response to subsequent treatment courses. In patients who have an initial response to rituximab, disease activity–guided re-treatment with rituximab according to the current international consensus statement is effective in sustaining clinical response and preventing major disease flares.

ACKNOWLEDGMENTS

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RE-TREATMENT OF RHEUMATOID ARTHRITIS PATIENTS WHO WERE INITIAL NONRESPONDERS TO RITUXIMAB: COMMENT ON THE ARTICLE BY THURLINGS ET AL.
RE-TREATMENT OF RHEUMATOID ARTHRITIS PATIENTS WHO WERE INITIAL NONRESPONDERS TO RITUXIMAB: COMMENT ON THE ARTICLE BY THURLINGS ET AL.

To the Editor

In a recent article, Thurlings et al reported the efficacy of subsequent cycles of rituximab in rheumatoid arthritis (RA) patients whose disease had failed to respond to the first course of treatment. The authors have previously demonstrated that clinical nonresponse to rituximab is associated with the persistence of B-lineage cells in the synovium. This is consistent with the findings of our own studies and the studies of other investigators on synovium. It is also consistent with the results of our studies of peripheral blood. In fact, we have never observed nonresponse in patients with fast and complete B cell depletion, as measured by highly sensitive flow cytometry. These findings suggest that patients whose RA fails to respond to 1 cycle of rituximab may have a form of disease that potentially would be treatable via B cell depletion, if depletion were enhanced. Administering a second cycle of rituximab after a relatively short interval might be one potential strategy to enhance depletion. However, Thurlings et al concluded that a lack of response after 2 cycles of rituximab may indicate a B cell–independent pathogenic mechanism. This conclusion requires the demonstration that depletion becomes complete following the second cycle of rituximab. In some patients, depletion may be even less efficient after subsequent cycles of rituximab, due to the development of human antichimeric antibodies. No data describing peripheral blood or synovial depletion were presented in the report by Thurlings and colleagues. Interpreting clinical responses in patients with highly resistant RA can be difficult. In the study by Thurlings et al, a small number of patients with a persistent lack of response were selected from a population with highly resistant RA. A comparison of baseline clinical characteristics between responders and nonresponders may have been helpful. Data on the components of the Disease Activity Score in 28 joints (DAS28) and absolute DAS values in the group of nonresponders before and after each cycle would have been interesting to compare with the data that were presented for responders. The question of the mechanism of nonresponse to rituximab is crucial to our understanding of the role of B cells in RA. While an assessment of the clinical efficacy of subsequent cycles of rituximab in nonresponders is valuable, it may be premature, with only limited clinical data and with no data regarding blood or synovial depletion, to draw conclusions about the pathogenesis of disease in this group.

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could be detected in 5 of 22 patients (4 responders and 1 nonresponder). After the second treatment course, small numbers of B cells (0.01 × 10^9/liter) could be detected in 5 of 22 patients (4 responders and 1 nonresponder). We also analyzed antirituximab antibody formation (human antichimeric antibodies [HACAs]), using a previously described assay. HACAs were observed in serum (obtained when rituximab was at trough levels before the second course) in 1 of 30 patients. Two of these patients experienced a moderate response (according to the EULAR criteria) to the first treatment course, and 1 patient did not respond. During the infusion of the second rituximab course, 1 patient (an initial responder) experienced a serious infusion-related reaction, and rituximab treatment was discontinued. The other 2 HACA-positive patients experienced the same clinical response after the second treatment course (1 moderate responder and 1 nonresponder). Hence, the extent of peripheral blood CD19+ B cell depletion and HACA formation were not related to the clinical response. Taken together, our data suggest that re-treatment of initial nonresponders is not likely to induce a robust clinical response after a second course of rituximab. The mechanism of nonresponse needs further study.

**FIGURE No.2**

**FIGURE 1.** Disease Activity Score in 28 joints (DAS28) after 2 treatment courses (crse) in individual rheumatoid arthritis patients who were initial nonresponders to rituximab treatment. No significant changes in the DAS28 were observed after re-treatment.

**REFERENCE**


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ATACICEPT IN PATIENTS WITH RHEUMATOID ARTHRITIS. RESULTS OF A MULTICENTER, PHASE IB, DOUBLE-BLIND, PLACEBO-CONTROLLED, DOSE-ESCALATING, SINGLE- AND REPEATED-DOSE STUDY
ATACICEPT IN PATIENTS WITH RHEUMATOID ARTHRITIS: RESULTS OF A MULTICENTER, PHASE IB, DOUBLE-BLIND, PLACEBO-CONTROLLED, DOSE-ESCALATING, SINGLE- AND REPEATED-DOSE STUDY

Abstract

OBJECTIVE: Atacicept is a recombinant fusion protein that binds and neutralizes B lymphocyte stimulator and a proliferation-inducing ligand. The purpose of this study was to investigate the tolerability, pharmacokinetics, and pharmacodynamics of atacicept treatment in patients with rheumatoid arthritis (RA) and to collect exploratory data on clinical outcomes.

METHODS: In this multicenter, phase IB, randomized, placebo-controlled, dose-escalating trial, 73 patients were enrolled into 6 escalating-dose cohorts. Patients received atacicept or placebo as single doses (70, 210, or 630 mg) or as repeated doses given at 2-week intervals (3 doses of 70 mg, 3 doses of 210 mg, or 7 doses of 420 mg), followed by 10 weeks of trial assessments, with a follow-up assessment at 3 months after the final dose.

RESULTS: Atacicept was well tolerated, with few differences between treatment groups and no obvious safety concerns. The pharmacokinetics profile was nonlinear, but was consistent and predictable across all doses and regimens. Treatment-related decreases in immunoglobulin (particularly IgM) and rheumatoid factor levels were evident, and a clear decrease in anti–citrullinated protein antibodies was observed in the cohort that received 7 doses of 420 mg. The B cell response was biphasic, with an initial transient increase (dominated by memory B cells) followed by a dose-related decrease (dominated by mature B cells). Clinical assessments showed trends toward improvement with the 3-month treatment. Little effect on the erythrocyte sedimentation rate or C-reactive protein levels was seen.

CONCLUSION: Atacicept was well tolerated both systemically and locally. The results demonstrated that the biologic activity of atacicept was consistent with its mechanism of action.

Introduction

Rheumatoid arthritis (RA) is a chronic syndrome characterized by nonspecific, usually symmetric, inflammation of the peripheral joints. Although the introduction of earlier aggressive treatment with disease modifying antirheumatic drugs (DMARDs) has played a major role in improving many patient outcomes, RA is still associated with long-term morbidity and early mortality.

The role of T cells in the pathogenesis of RA is well established, while that of B cells is not as well understood. B cells could potentially act as antigen-presenting cells, secreting proinflammatory cytokines, producing autoantibodies, and activating T cells, which then infiltrate synovial tissue. The production of rheumatoid factors (RFs; antibodies that bind immunoglobulin) and anti–citrullinated protein antibodies (ACPsAs) is among the characteristic...
features of RA. These autoantibodies are generally thought to be associated with disease severity and prognosis 4–7, although the association with ACPAs is a more controversial subject. Other autoantibodies detectable in the serum or synovial fluid of patients with RA include antinuclear factors, antineutrophil cytoplasmic antibodies, and antibodies to native type II collagen, citrullinated peptides, and gp130-RAPS, the RA antigenic peptide–bearing soluble form of gp130 8. The relevance of autoantibody-producing autoreactive B cells in RA has been highlighted by the success of therapeutic B cell depletion 9–11. Although the precise consequences of the decrease in the production of RFs and other autoantibodies are unknown, it has been proposed that small immune complexes containing RFs may activate macrophages in the synovial membrane via the Fcγ receptor 9. These findings suggest that B cells are an appropriate therapeutic target.

Atacicept is a recombinant fusion protein that binds and neutralizes the activity of B lymphocyte stimulator (BLyS; trademark of Human Genome Sciences, Rockville, MD), a cytokine shown to be a key regulator of B cell maturation, proliferation, and survival, and APRIL, a proliferation-inducing ligand, which is another tumor necrosis factor (TNF) family member that is closely related to BLyS 8. BLyS and APRIL are produced by a wide variety of cell types, and act on B cells to enhance survival, proliferation, antigen presentation, and class-switch recombination at various stages of B cell development 12. Three TNF receptor–related receptors have been identified that have unique binding affinities for BLyS and APRIL: TACI, BCMA, and BAFF receptor (BAFFR). TACI and BCMA bind both BLyS and APRIL, while BAFF-R appears to bind only BLyS with high affinity 13,14. APRIL also binds, with as yet unknown consequences, to heparan sulfate proteoglycans, such as syndecan 1/CD138 15.

Atacicept contains the BLyS/APRIL-binding extracellular portion of the TACI molecule fused to the Fc portion of human IgG1 and can be used to neutralize BLyS and APRIL and prevent them from binding to their receptors on B lymphocytes. Decreasing the levels of BLyS and APRIL in vivo is expected to result in a decrease in circulating mature B cells and Ig-secreting cells, and a consequent drop in Ig levels.

An important role of BLyS in the pathogenesis of autoimmune disorders is supported by the observation that transgenic mice expressing high levels of BLyS exhibit immune cell disorders and display symptoms similar to those in patients with systemic lupus erythematosus (SLE) and Sjögren’s syndrome 16. In addition, serum levels of BLyS and APRIL are, on average, elevated in patients with RA and SLE 17–20, although there is considerable overlap in the observed ranges of the serum concentrations of BLyS and APRIL in, for example, patients with RA as compared with healthy controls. Of interest, the levels of both BLyS and APRIL are higher in RA synovial fluid than in blood, particularly in the presence of significant joint inflammation, which suggests that these ligands may play an important role in the inflamed synovial compartment 19–21.

In addition to elevated levels of BLyS and APRIL homotrimers, circulating heterotrimeric complexes of BLyS and APRIL have also been shown to be elevated in serum from patients with systemic immune–based rheumatic diseases (including SLE, RA, Reiter’s syndrome, psoriatic arthritis, polymyositis, and ankylosing spondylitis), and have been shown to induce B cell proliferation in vitro 22. Among Ig fusion proteins for TACI, BCMA, and BAFF-R, only atacicept can block the biologic activity of all of these complexes 22 and may have therapeutic utility in limiting the extent of tissue damage in RA.

Therefore, we conducted a phase Ib, randomized, double-blind, placebo-controlled, escalating-dose study at 7 clinical centers in Australia, The Netherlands, Russia, the former state of Serbia and Montenegro, and the UK to evaluate single and multiple doses of atacicept administered over 1 month and 3 months to patients with RA.
PATIENTS AND METHODS

The study (Merck Serono study code 25072) was conducted in compliance with the Declaration of Helsinki (2000 version) and with the International Conference on Harmonisation Harmonised Tripartite Guideline for Good Clinical Practice. Approval was obtained from the Local Ethics Committees before study initiation, and written informed consent was obtained from all patients before performing any study procedures.

STUDY OBJECTIVES. The primary objective was to assess the systemic and local tolerability of single and repeated subcutaneous doses of atacicept in patients with RA. Secondary objectives were to assess the pharmacokinetics and pharmacodynamics of atacicept in patients with RA following single and repeated subcutaneous doses, to characterize biomarkers specific to the mechanism of action of atacicept and to document markers of disease activity and progression.

STUDY DESIGN. A total of 73 RF-positive patients with RA were included in the study and were grouped into 6 escalating-dose cohorts. Within each cohort, patients were randomized 3:1 to receive subcutaneous atacicept or placebo as single doses (70, 210, or 630 mg) or as multiple doses administered at 2-week intervals (3 doses of 70 mg, 3 doses of 210 mg, or 7 doses of 420 mg) (Figure 1). Dose escalation was authorized by a Safety Review Board upon review of the safety data. Predetermined dosing intervals for multiple doses were confirmed by a Pharmacokinetics/Pharmacodynamics Review Board, based on the results of pharmacokinetics and pharmacodynamics analyses in earlier cohorts.

Trial assessments were performed at baseline and continued for 10 weeks following administration of study drug, with a followup assessment at 3 months after the final dose.

PATIENT POPULATION. Adults of either sex who had active moderate-to-severe RA, which was defined as ≥6 swollen joints, ≥6 tender joints, and a C-reactive protein (CRP) concentration ≥25 mg/liter or an erythrocyte sedimentation rate (ESR) ≥28 mm/hour, were recruited. The main inclusion criteria were a disease duration of at least 6 months, failure of treatment with ≥5 DMARDs, RF positivity, and willingness to avoid pregnancy during the study and for 3 months after the last administration of study drug.

The primary exclusion criteria included any previous treatment with rituximab (anti-CD20 antibody) or anti-BLyS antibody, use of other biologic agents within 3 months before study day 1; prednisone dosage >10 mg/day or methotrexate dosage >17.5 mg/week; use of DMARDs other than methotrexate within 28 days before study day 1; history of, or prophylactic treatment for, tuberculosis; and significant concomitant illness or organ dysfunction.

PROCEDURES. Following randomization and baseline assessments, the first dose of study drug was administered subcutaneously into the anterior abdominal wall of each patient on study day 1. To prevent binding, the medication was administered by a nurse who was otherwise uninvolved in the study. Postdose blood and urine samples were collected for assessment of the pharmacokinetics, pharmacodynamics, safety, and disease activity. Samples were obtained at predefined intervals up to 14 weeks postdose (single-dose cohorts), 18 weeks postdose (repeated-dose cohorts 2 and 4), and 26 weeks postdose (repeated-dose cohort 6), with a followup assessment—3 months after the final dose (Figure 1).

OUTCOME MEASURES. Safety and tolerability assessments included physical examination, vital signs, electrocardiograms (EKGs), laboratory analyses (hematology, coagulation, clinical chemistry, and urinalysis), adverse events, and injection-site reactions. Assays for binding antibodies to atacicept were performed at baseline and the final followup assessment. Assays for neutralizing antibodies were performed if binding antibodies were detected. Antibody titers to tetanus toxoid and diptheria toxoid were compared between baseline and the final followup assessment to determine vaccine immunization status.

The following pharmacokinetics variables were measured using enzyme-linked immunosorbent assays (ELISAs): free atacicept, atacicept–BLyS complex, and composite atacicept (free atacicept plus atacicept–BLyS complex) and anti-atacicept antibodies specific for atacicept (ZymoGenetics, Seattle, WA), goat polyclonal antibodies specific for biotin-conjugated BLyS (R&D Systems, Wiesbaden, Germany), or goat polyclonal antibodies to biotin-conjugated TACI-ly (R&D Systems) were incubated with 1:10 dilutions of patient samples, standard, or control samples for 1 hour in streptavidin precoated microplates. After washing, biotinylated anti-BLyS mouse monoclonal antibody (ZymoGenetics) was incubated at room temperature for 1 hour. After washing, HRP-conjugated biotinylated anti-BLyS mouse monoclonal antibody (ZymoGenetics) was washed in a 3:1 molar ratio, and 50 ng/ml of serum for the composite antibodies. The mean spiking recoveries performed to test the accuracy for low, medium, and high analyte concentrations in RA patient samples corresponded to 82.5–97.0%, 93.9%, and 102.0–125.8% recovery rates, respectively, in the 3 assays.

The analyte concentration in patient samples was recalculated using a standard curve, applying a polynomial second-order-fitting algorithm. All samples were measured in triplicate. As assay performance criteria, a precision of <15% for the coefficient of variation (CV) in the standard samples and <<20% in the patient samples were accepted. The lower limits of quantification of the assays were 3.2 ng/ml of serum for free atacicept, 10 units/ml of serum for atacicept–BLyS complex (1 unit/ml corresponding to 1.82 ng/ml of atacicept to 0.44 ng/ml of BLyS in a 3:1 molar ratio), and 50 ng/ml of serum for the composite antibodies. The lower limit of quantification of the assays was 31.2 ng/ml of BLyS in the serum. The mean spiking recoveries for low, medium, and high
concentrations of the analytes in RA patient samples corresponded to 101–113% recovery rates.

IgG (and IgG1–4 subclasses), IgM, IgA, ACPAs, and RFs (IgA, IgM, and IgG) were assessed in blood samples as markers of biologic activity, using conventional laboratory tests. A panel of cell types (B and T cell subsets, natural killer [NK] cells, and monocytes) was assessed in antibody-stained peripheral blood samples by 4-color flow cytometry. A contract research organization (Esoterix, Groningen, The Netherlands) performed blood sample processing, antibody staining, and acquisition, analysis, and quality control of the data. Serum CRP levels, ESRs, and urinary hydroxypyridinoline (HP): lysylpyridinoline (LP) ratios were also measured as disease activity markers.

Disease assessments included the Disease Activity Score 28-joint assessment (DAS28) (22), tender and swollen joint counts (in 28 joints), patient’s assessment of pain (using a 0–100-mm visual analog scale [VAS]), physician’s global assessment of disease activity (using a Likert scale), patient’s global assessment of disease activity (using a 0–100-mm VAS), patient’s assessment of physical function (using the Health Assessment Questionnaire [HAQ]), and the duration of morning stiffness. Achievement of the American College of Rheumatology 20% improvement criteria (ACR20) was assessed based on these data.

STATISTICAL ANALYSIS. The required sample size was determined so that the total numbers of patients would allow for the initial assessment of systemic and local tolerability as well as the pharmacokinetic and pharmacodynamic properties of atacicept. The safety analysis set was defined as all patients who received at least 1 injection of investigational treatment (analyzed as treated patients). The intent-to-treat analysis set was defined as all patients who were randomized. The 2 populations ended up being identical. Given the study’s safety focus and the small number of patients per cohort, only descriptive statistics and graphic representations were used. Continuous efficacy parameters were summarized over time, using actual values and percentages of change from baseline. Categorical parameters were summarized over time, using only frequencies and percentages. Imputation of missing values was used only for disease progression outcomes, and only in cases of study withdrawal due to disease progression. For binary variables (withdrawal due to disease progression), missing values were set at “no response” after withdrawal. For continuous variables, missing values were left missing. Treatment-emergent adverse events and features of the medical history were coded using the Medical Dictionary for Regulatory Activities (MedDRA; version 8.0). Past and concomitant medications were coded using the WHO Drug Dictionary (Quarter 1, 2004). Pharmacokinetics and pharmacodynamics were assessed through noncompartmental analysis, using WinNonlin Professional software, version 5.0.1 (Pharsight, Mountain View, CA).

CHARACTERISTICS OF THE STUDY POPULATION.

Patients were enrolled from the former state of Serbia and Montenegro (n = 35), Russia (n = 29), The Netherlands (n = 7), Australia (n = 1), and the UK (n = 1). Overall, 73 patients were randomized and received treatment: 18 patients received placebo (combined placebo group), 6 patients each in the 3 single-dose cohorts received atacicept (cohorts 1, 3, and 5; 1 dose of 70 mg, 1 dose of 210 mg, and 1 dose of 630 mg, respectively), 9 patients each in cohorts 2 and 4 received atacicept (3 doses of 70 mg and 3 doses of 210 mg, respectively), and 19 patients in cohort 6 received atacicept (7 doses of 420 mg) (Figure 1).

Baseline characteristics were reasonably wellbalanced across treatment groups, considering the small numbers of patients. Overall, the mean ± SD age at study inclusion was 56 ± 8 years, and the mean ± SD disease duration at study inclusion was 7.9 ± 6 years. The majority of patients were female (81%). The mean ± SD body weight was 75 ± 14 kg. A history of smoking was reported by 34% of patients. All patients were RF+, as required by the protocol. The overall mean ± SD scores for the disease
Summary of safety data, by treatment group*

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<tr>
<td>MedDRA ‘skin and subcutaneous tissue disorders’ – patients, n (%)</td>
<td>0</td>
<td>0</td>
<td>2 (22%)</td>
<td>1 (17%)</td>
<td>0</td>
<td>0</td>
<td>4 (21%)</td>
<td>7 (10%)</td>
<td></td>
</tr>
<tr>
<td>Local reactions – patients, n (%):</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Any (itching, swelling, erythema, bruising, and/or ‘other’‡)</td>
<td>2 (11%)</td>
<td>0</td>
<td>3 (33%)</td>
<td>4 (66%)</td>
<td>3 (33%)</td>
<td>1 (17%)</td>
<td>11 (58%)</td>
<td>24 (33%)</td>
<td></td>
</tr>
<tr>
<td>Erythema</td>
<td>0</td>
<td></td>
<td>3 (33%)</td>
<td>3 (50%)</td>
<td>1 (11%)</td>
<td>1 (17%)</td>
<td>7 (37%)</td>
<td>15 (21%)</td>
<td></td>
</tr>
<tr>
<td>Pain (VAS &gt;0)</td>
<td>2 (11%)</td>
<td>0</td>
<td>1 (11%)</td>
<td>3 (50%)</td>
<td>2 (22%)</td>
<td>1 (17%)</td>
<td>8 (42%)</td>
<td>17 (23%)</td>
<td></td>
</tr>
</tbody>
</table>

* See Patients and Methods for a description of the individual cohorts. MedDRA Medical Dictionary for Regulatory Activities; VAS = visual analog scale (range 0–100 mm). † Two further serious adverse events (an electrocardiogram suggestive of myocardial ischemia and a fractured arm) were reported in patients who withdrew before receiving any treatment. One serious adverse event (death from lung cancer) was reported 8 months after completion of treatment in a patient in cohort 4. ‡ Reported in >2 patients overall. § Other reactions consisted of stinging and pain (1 patient each).
All treated patients underwent final followup evaluations.

**TOLERABILITY OF ATACICEPT.**

Overall, 32 patients (44%) reported 80 treatment-emergent adverse events (Table 1). Only 3 of these events were considered severe (arthralgia in cohort 4 atacicept group, and a rheumatoid nodule and an RA exacerbation in cohort 6 atacicept group), and half were considered unrelated or unlikely to be related to the study medication. There was no notable difference in the frequency of infection-related adverse events between patients who received atacicept and those who received placebo or between the treatment groups (Table 1). No infection-related events were considered serious or severe. Events reported by >2 patients were fatigue, nasopharyngitis, chronic pyelonephritis, anemia, and headache. Two of the 3 patients with chronic pyelonephritis had a history of the condition at baseline. One patient in cohort 3 experienced worsening of chronic pyelonephritis and bronchopneumonia (resolving following treatment with oral antibiotics), with onset of symptoms at 6 weeks and 8 weeks, respectively, after a single dose of atacicept. Both events were considered to be of moderate severity and possibly related to the study medication, despite the interval between symptom onset and study treatment. Notably, the patient’s white blood cell counts and immunoglobulin levels were within normal ranges throughout the trial.

Skin and subcutaneous tissue disorders (according to the MedDRA system) were more frequent in patients receiving atacicept. However, the small study population and the small number of reported events do not permit us to draw any conclusions about a causal relationship. Two patients (cohort 2 and cohort 6, both receiving atacicept) experienced urticaria 4–6 hours after a dose of study medication; 1 of them had a history of drug allergies. A third patient (cohort 6 receiving atacicept) experienced macular erythema and pruritus, and a fourth patient (cohort 6 receiving atacicept) had a rash. Only 1 of these 4 patients discontinued treatment; the remaining 3 did not experience recurrence of symptoms with subsequent doses. One serious adverse event was reported during the study: pneumothorax occurred in this patient (cohort 3 receiving atacicept), which was related to a bronchoscopy for investigation of an abnormality present at baseline.

One death was reported poststudy in a 60-year-old man (cohort 4 receiving atacicept). This patient had a 40-year history of smoking and died of lung cancer ~8 months after completing study treatment. Local injection site symptoms were reported in 24 of the 73 patients. The most frequent local reaction was mild-to-moderate erythema, which was reported in 15 patients (36 mild erythema events, 3 moderate erythema events). Mild itching, swelling, bruising, and other
symptoms (stinging and pain) were each reported 8 times overall. VAS pain scores >0 were reported by 17 patients, but the scores were generally low (median of 15 on the 0–100-mm scale).

Assessment of data from the hematology, biochemistry, urine, coagulation, vital signs, and EKG studies showed no trends over time and few notable differences between treatment groups. The results of these evaluations did not suggest any potential safety concerns. No binding antibodies to atacicept were detected. There were no appreciable changes in the titers of antibodies to tetanus toxoid or diphtheria toxoid following atacicept treatment.

PHARMACOKINETICS OF ATACICEPT.

Atacicept displayed nonlinear pharmacokinetics, characterized by a greater than dose-proportional increase in free atacicept, along with a less than dose-proportional, saturated increase in exposure to atacicept–BLyS complex (Figure 2). The evidence for nonlinearity was weaker for exposure to composite atacicept (free atacicept plus atacicept–BLyS complex). This may be because the nonlinearities of the individual pharmacokinetics of free atacicept and atacicept–BLyS complex offset each other within the composite atacicept measurement.

The concentration–time profiles of free and composite atacicept displayed multiphasic pharmacokinetics, with fairly rapid absorption for this class of molecules (time to maximum concentration ~24 hours after the first dose), rapid distribution phases that were complete by 7–14 days after administration, and a prolonged terminal phase (terminal half-life 600–1,500 hours [25–53 days]).

Very little, if any, accumulation of free atacicept was observed with multiple doses. The accumulation of composite atacicept was moderate, while the accumulation of atacicept–BLyS complex continued throughout the entire dosing period (up to 7 doses every 2 weeks in cohort 6). There were indications that a pseudo–steady state would be achieved shortly after the seventh dose in this cohort.

Despite their nonlinearity, the pharmacokinetics profiles of atacicept were very consistent and predictable across all doses and between single and multiple doses for all 3 variables (free atacicept, atacicept–BLyS complex, and composite atacicept).

Although the small number of patients who underwent synovial fluid sampling (n = 4) limits the conclusions that can be drawn, there was evidence that atacicept was detectable in inflamed joints. The levels of free atacicept and atacicept–BLyS complex in synovial fluid were approximately one-third and one-half the levels in serum, respectively. In these patients, the concentrations of BLyS in synovial fluid before atacicept administration were ~4 times higher than those measured in serum, as might have been predicted from the values reported in published studies (20,21).

PHARMACODYNAMICS OF ATACICEPT.

Immunoglobulins. Immunoglobulin values showed prompt decreases following the first dose of atacicept, which continued with repeated dosing. In atacicept-treated patients in cohort 6, maximum reductions were seen on day 85. IgG values were reduced by a median of 21%, IgA by 37%, and IgM by 54%, compared with baseline (Figure 3A). The median IgA values showed a dose-dependent decline; values on day 85 were below baseline in all cohorts. Most obviously in the 3-month cohort 6, IgA values returned toward baseline after treatment cessation; however, they had not yet reached prestudy levels by the final assessment (12 weeks after the final dose). For IgG, the median values were more variable, especially in the placebo and cohort 1 atacicept groups.

Three months after a single or final dose, IgG values were generally at or near baseline levels. IgG subclasses (IgG1–4) showed decreases with treatment that were roughly parallel to those observed for the total IgG values. Treatment-related decreases were most evident for IgM. In the combined placebo group, median IgM values did not vary substantially from baseline levels during the observation period. In contrast, all atacicept groups showed a rapid decrease in IgM following the first dose, which
FIGURE No.4

**FIGURE 4.** Summary profile of the median levels of anti–citrullinated protein antibodies (ACPAs) in placebo-treated and atacicept-treated patients in cohort 6. Horizontal line represents baseline.

FIGURE No.5

**FIGURE 5.** Median percentage change in absolute counts of total B cells and mature B cells compared with baseline. The number of total B cells (CD19+) in A, the single-dose cohorts and B, the repeated-dose cohorts, and the number of mature B cells (CD19+, IgD+, CD27−) in C, the single-dose cohorts and D, the repeated-dose cohorts (based on absolute concentrations) are shown. Horizontal line represents baseline.

was apparently dose-independent during weeks 1–2. Thereafter, the nadir was dose-related, with the largest response obtained in the cohort 6 atacicept group. A greater reduction in IgM was observed with atacicept administered as 3 doses given over a month, as compared with administration of the same total dose as a single injection. In most treatment groups, IgM values had returned to near baseline levels by the end of the observation period; exceptions were the largest single dose (630 mg) and the longest treatment duration (7 doses of 420 mg).

**Rheumatoid factors.** Decreases in RFs were observed following atacicept administration, most consistently with 7 doses of 420 mg of atacicept, although baseline values differed considerably between groups, and the response in the placebo group was much more variable for the RFs than for the nonspecific immunoglobulins. In atacicept-treated patients in cohort 6, maximum decreases from baseline of 41–44% were observed for all 3 RF classes (Figure 3B). Particularly in cohort 6 patients receiving atacicept, RFs showed decreases consistently more often than were seen for nonspecific immunoglobulins.

Anti–citrullinated protein antibodies. Little change in the ACPA values and little difference between active treatment and placebo groups were noted for patients in cohorts 1 through 5. In cohort 6 patients receiving atacicept, ACPA levels consistently decreased, with a median percentage change from baseline of -25% on day 85, as compared with a 2.6% increase in patients receiving placebo (Figure 4).

**Markers of inflammation and cartilage degradation.** Up to 3 months of treatment with atacicept did not lead to any appreciable effects on the ESR, the CRP level, or the urinary HP:LP ratio. Slight overall decreases were observed, but these were difficult to interpret because there was high variability between patients for all of these measures.

**Findings of flow cytometry.** Atacicept treatment produced a biphasic response in total B cells (CD19+), mature B cells (CD19+, IgD+, CD27−), memory B cells (CD19+, CD20+, CD27+, CD38−), immature B cells (CD19+, IgD−, CD27−), as well as naive B cells (CD19+, CD20+, CD27−, CD38−), which was not seen among patients who received placebo. The initial phase consisted of a transient dose-related increase in cell concentrations observed within 2 weeks after a single (or the first) dose. Memory B cells, which accounted for most of this response, displayed the highest median percentage change, and mature B cells displayed the lowest median percentage change in this first phase. The second phase consisted of a sustained, dose-related reduction of B cell concentrations to below predose levels, which was most evident in the median percentage change from baseline among mature and total B cells (maximum decrease...
30–40% at 3 months) (Figure 5). Mature B cells accounted for most of the reduction phase of the total B cell response in terms of the percentage change from baseline.

Flow cytometric analyses showed no drug-related effects on total, helper, or cytotoxic T cells, NK cells, or monocytes (data not shown). Pre–germinal center B cells, plasma cells, plasmablasts, and Ig-secret ing cells were too sparse in the peripheral blood to allow meaningful interpretation.

This study was not powered to show statistically significant effects on clinical endpoints. However, pilot information on clinical outcomes was collected, including DAS28 scores and ACR20 responses. DAS28 scores indicated an improvement in RA signs and symptoms with atacicept treatment, particularly in cohort 6. The atacicept-treated patients in cohort 6 had a mean ± SD DAS28 score of 6.4 ± 3.9 at baseline, which had decreased to 5.1 ± 1.4 on day 85. The decrease persisted long beyond treatment cessation and had only slightly diminished by the follow-up visit. No change was seen in patients who received placebo.

During the 3 months of atacicept treatment, 6 of 19 patients (32%) attained an ACR20 response or better, 2 of whom attained an ACR70 response. Another 3 atacicept–treated patients (16%) attained an ACR20 response or better during the observational follow-up period, 1 of whom attained an ACR50 response. No responses were seen among the placebo-treated patients in cohort 6 at either time point, although ACR20 responses occurred among some placebo recipients in other cohorts.

Assessment of individual components of the ACR20 criteria for improvement showed some effect on the tender joint counts and possibly on the swollen joint counts at the end of the 3-month treatment period. Most of the effect on the ACR20 response came from the patient’s self-assessments of pain and overall disease activity. Patient-reported assessments showed improvements as early as 2 weeks after treatment initiation. No marked trend was evident for physical functioning, as assessed by the HAQ.

DISCUSSION

Atacicept was generally well tolerated both systemically and locally. Infections were carefully monitored in this trial, since depletion of B cells and immunoglobulins with atacicept therapy, as with any other B cell–targeted therapy, may put patients at risk. In this trial, there was no notable difference in the rates of “infections and infestations” (according to the Med-DRA system) between atacicept–treated and placebo–treated patients. Future controlled trials will carefully monitor patients for infections and identify the optimal dose of atacicept as a potential treatment of RA.

More atacicept–treated patients experienced skin and subcutaneous tissue disorders (2 cases of urticaria, 1 case of macular erythema and pruritus, and 1 case of rash). One of the patients who experienced urticaria had a history of allergy to medicinal products, which was discovered upon investigation of the adverse event in the study, and treatment was discontinued. The remaining 3 patients did not experience a recurrence of symptoms with subsequent doses of atacicept. Although the limitations of this study (phase Ib trial, with a small study population and short treatment duration) must be taken into account, no potential safety concerns were identified.

Atacicept displayed nonlinear pharmacokinetics, characterized by a more than dose–proportional increase in free drug and a less than dose–proportional, saturated increase in atacicept–BLyS complex. Concentration–time profiles of free and composite atacicept displayed multiphasic pharmacokinetics, with fairly rapid absorption for this class of molecules, rapid distribution phases, and a prolonged terminal phase. The distribution phases appear to represent both the “true” distribution across tissues and, for free atacicept, the binding of the drug to its ligands. Indeed, the proportion of the overall area under the curve that corresponds to the distribution phase appears to be much higher for free atacicept than for composite atacicept; this difference is presumably largely explained by the binding. Very little, if any, distribution was seen for the atacicept–BLyS complex.

Minimal accumulation of free atacicept was observed with multiple doses. The accumulation of composite atacicept was moderate, while the accumulation of atacicept–BLyS complex continued throughout the entire dosing period (up to 7 biweekly doses in cohort 6). There were indications that a pseudo–steady-state would be achieved shortly after the seventh dose in this cohort.

Overall, the pharmacokinetics profiles of atacicept were consistent and predictable across the study doses and between the single and multiple doses. The results support the hypothesis that the pharmacokinetics of atacicept is mediated by its ligands.

Changes in pharmacologic biomarkers were consistent with the proposed mechanism of action of atacicept. Immunoglobulins and other biomarkers showed prompt reductions following the first dose of atacicept, which continued and showed greater reductions with repeated dosing. The greatest effects were seen with the 7 doses of 420 mg regimen of atacicept treatment. Reductions peaked within a few days of the last dose,
after which levels returned toward baseline. This treatment effect was particularly evident for IgM.

There was a biphasic response in the numbers of total, mature, memory, and immature B cells, comprising an initial increase within 2 weeks after a single (or the first) dose, followed by an approximately dosedependent reduction (maximum decreases 30–40% at 3 months). As expected from the preclinical findings, there were no drug-related effects on the numbers of total, helper, or cytotoxic T cells, NK cells, or monocytes. The results of these analyses indicate that concentrations of peripheral blood mature and total B cells are potentially useful markers of drug effects in patients with RA.

This study did not have sufficient power to evaluate clinical efficacy. However, positive trends were seen for effects on signs and symptoms of RA with 3-month atacicept treatment (DAS28 scores and ACR20 responses). Individual components of the ACR20 criteria for improvement showed some effect on the tender joint counts and perhaps on the swollen joint counts towards the end of the treatment period. However, ACR20 improvements were dominated by patient-assessed outcomes (pain, HAQ scores, and patient’s global assessment), which showed improvement after as little as 2 weeks of treatment, which was earlier than anticipated and was well before the maximal effect on pharmacodynamic markers was seen. The lack of a clear treatment effect on markers of inflammation (ESR, CRP, level, and tender and swollen joint counts) suggests that further study is necessary to understand the effect of atacicept on the pathology of RA and, specifically, whether a longer treatment period would demonstrate the anti-inflammatory effect of atacicept on the pathology of RA.

In summary, this exploratory phase Ib trial showed atacicept to be generally well tolerated both locally and systemically in patients with RA. Both the pharmacokinetics and pharmacodynamics profiles of atacicept were consistent with the proposed mechanism of action of the drug. These data will allow us to define dose ranges and regimens for the further trials that will be needed to characterize the safety profile of atacicept, enhance our understanding of its mechanism of action, and define its optimal clinical use.

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B Cells and B Cell directed therapies in Rheumatoid Arthritis

Within the past decade a number of novel effective treatments have become available for rheumatoid arthritis (RA), among which tumor necrosis factor (TNF) blockers, rituximab, abatacept and tocilizumab. Nonetheless, the response to these treatments differs between RA patients and disease remission is only achieved in a proportion of patients and patients need to be treated chronically with often relatively expensive drugs. There is therefore a need to further improve the treatment of RA. This could be achieved by combination of different strategies. First, more optimal use of currently available treatments is needed. The use of biomarkers could play a role in optimising therapy in individual patients. For this purpose, biomarkers need to be identified. Second, patients who benefit most from a specific treatment. We also need to understand which immune mechanisms contribute to the disease in patients who do not respond to current therapies. Subsequently, we can use this knowledge to develop novel therapies and to optimize current treatment schedules.

Multiple lines of evidence suggest RA is a heterogeneous disease with a variable response to targeted therapy. As discussed in chapter 1 this heterogeneity is observed at the clinical, genetic and immunological level. Detailed analysis of immunological mechanisms in RA and assessment of their relationship with genetic and environmental risk factors, clinical characteristics and treatment response may improve our understanding of the pathophysiologic heterogeneity in RA, yield biomarkers to predict response and help to design novel treatments. In this thesis we focussed on B cells and B cell-directed therapies. We investigated whether we could identify biomarkers predictive of the clinical response to TNF blockade and rituximab treatment. Furthermore, we evaluated repeated treatment in initial clinical responders versus non-responders to rituximab. Finally, we performed a phase I study with the novel B cell modulating agent atacicept.

Synovial lymphoid neogenesis is an inflammation-driven phenomenon in RA. The synovial tissue of RA patients is infiltrated predominantly by macrophages and lymphocytes, but also contains dendritic cells, mast cells, natural killer cells and neutrophile granulocytes. When focussing on lymphocytes, the level of infiltration differs between patients. In some patients the tissue contains a diffuse or scarce infiltrate of T cells, while in others B and T cells are organized in lymphocyte aggregates, which are surrounded by fields of plasma cells (1). Large lymphocyte aggregates may exhibit features of germinal centers of secondary lymphoid tissue, which has been termed synovial lymphoid neogenesis (SLN). This raises the question whether they refine and amplify local humoral autoimmune responses against for instance rheumatoid factor (RF) and anti-citrullinated peptide antibodies (ACPA).

It has been proposed that synovial tissue with SLN forms a pathophysiologic subtype of RA, characterized by amplified local autoantibody production, associated with a more severe, agressive disease course and differential response to therapy (2). In line with this hypothesis the presence of SLN was found to be associated with RF positive disease, the presence of erosions and rheumatoid nodules (3). However these observations were made in small patient cohorts using a selection of patients with end-stage disease. In this thesis we studied whether the presence of SLN can be used as a biomarker to identify clinically and immunological patient subsets and whether, as such, its presence can be used to predict clinical response to treatment.

In chapters 2 and 3 we analyzed the relationship between SLN, humoral autoimmunity, clinical disease characteristics and treatment response in a large and representative cohort of RA patients. First, we analyzed the relationship between SLN and disease characteristics. We found that the presence of SLN is related to increased local and systemic inflammatory parameters, but it is found equally frequent in seropositive and seronegative RA patients. Furthermore, SLN was not associated with increased clinical disease activity parameters or the presence of erosive or nodular disease. We recently confirmed and extended these findings in a prospective cohort of patients with early arthritis (4). The presence of SLN was neither associated with diagnosis nor with development of persistent or erosive disease. The presence of SLN decreased over time in individual patients, which probably reflected reduced synovial inflammation after anti-inflammatory therapy or self-limiting synovitis in this particular cohort.

The presence of SLN in both seronegative and seropositive RA, as well as other forms of arthritis, suggests that SLN is a phenomenon which is not necessarily associated with autoantibodies. In line with this we recently found that even in seropositive patients the presence of SLN is not associated with an increase in local ACPA or RF production compared to other antibodies (5). Taken together, despite the fact that SLN has germinal-center-like features, the data imply that SLN is rather an inflammation-driven humoral response phenomenon than a true germinal center reaction that refines and amplifies a local autoantibody response. SLN may contribute to inflammation by enhancing T cell activation, promoting local differentiation of plasma cells and by production of cytokines.

In chapter 3 we analyzed whether SLN predicts the response to TNF blockade. We found that patients with SLN overall responded well to TNF blockade and that SLN predicted clinical response independent of other known predictors, such as disease activity at baseline, the presence of ACPA and the level of synovial TNFα expression. Furthermore, we found that SLN is rapidly reversible after treatment. SLN tends to disappear already 48 hours after treatment, before a decrease in DAS28 occurs. These data suggest that TNF is involved in the formation of SLN. In line with this, previous studies showed that TNF blockade results in decreased expression of cytokines (6), chemokines (7), and adhesion molecules (8), which are required for secondary lymphoid organ formation.

In summary, we found that SLN does not define a clinically distinct disease phenotype. Furthermore, the data suggest that while SLN exhibits some features of germinal centers it does not function as such. As an inflammation driven humoral immune phenomenon it predicts a good response to infliximab. Nonetheless, the predictive value of SLN as a biomarker of response to TNF blockade in itself is not high enough to be used as a solitary biomarker, indicating the involvement of other, as yet unknown mechanisms. Future work should expand the search for other biomarkers and molecular networks, as well as combinations of clinical variables, to achieve an effective approach that will increase the percentage of patients exhibiting a robust response to TNF blockade.
Mechanism of Action of Rituximab and Biomarkers of Response. In chapter 4 to 7 we studied the effects of rituximab on synovial tissue and peripheral blood in relationship to clinical response. We identified biomarkers of response and immune mechanisms that remain active in patients who do not respond to therapy.

Rituximab induces a rapid, near complete depletion of CD20 positive B cells in peripheral blood. In chapter 4 we analysed to which extent rituximab depletes B cells in the synovial tissue of RA patients. We found that rituximab induces a significant decrease in synovial B cells within 4 weeks after administration, but that B cells persist in a proportion of patients. This variable depletion of synovial B cells has been confirmed by other groups. The percentage of patients in whom synovial B cells persist differed between these studies. As discussed in chapter 5 these differences may depend on the time point at which the second synovial biopsy was taken (i.e. at 4, 12 or 16 weeks after treatment) and the choice of the specific antibodies used to detect B cells by immunohistochemistry. Similar to synovial tissue, it was found that B cells, especially with a memory phenotype, are abundant in bone marrow of RA patients. Furthermore, B cells were found to persist in lymph nodes of patients with B cell non-Hodgkin lymphoma (B-NHL). In our other studies the persistence of synovial B cells did not predict clinical non-response to rituximab.

Conceivably, the clinical response to rituximab could be improved by achieving more complete depletion of B lineage cells in the tissues. This might be achieved by repeated administration of rituximab in patients with incomplete response, an approach we investigated in chapter 9 (see below). Alternatively, B cell depleting agents might be developed with increased efficacy. A number of these drugs are currently under development. Finally, rituximab could perhaps be combined with therapies that enhance B cell depletion. Up to date, no factors have been identified that play a role in protecting B cells from rituximab in synovial tissue. Roles have been suggested for Fc receptor polymorphisms, expression of the complement-inhibitory factors CD55 and CD59 and the B cell survival factors Blys and CXCL12, but definite prove for a protective effect has yet to be given.

In chapter 6 we analyzed the indirect effects of rituximab on synovial inflammatory cell populations and the differences in these populations between responders and non-responders to treatment. We found that rituximab induces a decrease in synovial T cells and disrupts the presence of SLN and follicular dendritic cells in both responders and non-responders. Furthermore, we found that rituximab induces a decrease in synovial macrophages, especially in the intimal lining layer. Finally, we found an indirect decrease (between 4 and 16 weeks after treatment) in synovial plasma cells in clinical responders. The decrease in plasma cells was correlated with the decrease in ACPA levels in peripheral blood. Of interest, we recently found that the serum levels of free light chains, plasma cell products with a half life of a number of days, also decline in responders to rituximab. Free light chain levels are not affected by infliximab, indicating a specific indirect effect of rituximab, through depletion of CD20 positive precursors, on short lived plasma cells that produce free light chains.

In contrast to observations in clinical responders, we found that synovial plasma cells and serum free light chains persist in non-responders to rituximab treatment. Persistence of synovial B lineage cells in non-responders to rituximab was also observed in other studies. These data suggest that patients may fail to respond to rituximab due to persistence of pathogenic B cells. We did not find baseline differences in the number of synovial B or plasma cells, the levels of RF, ACPA or free light chains in responders versus non-responders. Of note, all patients in our study were selected on the basis of being positive for RF and/or ACPA. Previous work has shown that autoantibody positive RA patients respond better to rituximab treatment than those who are autoantibody negative. Taken together, these data support the notion that autoantibody production by B cell-derived plasma cells plays an important role in promoting synovial inflammation. Conceivably, effectiveness of rituximab treatment may be increased in autoantibody positive non-responders by strategies aimed at suppression of plasma cells associated with autoimmunity in the tissues.

In chapter 7 we studied the relationship between the response to rituximab and the type I interferon (IFN) signature in peripheral blood mononuclear cells (PBMCs). The type I IFN signature is found in a subset of RA patients as the dominant signalling signature in PBMCs. It is also found in a proportion of patients with other autoantibody associated autoimmune diseases, such as Sjögren’s syndrome, multiple sclerosis, dermatomyositis, type 1 diabetes mellitus, systemic sclerosis and systemic lupus erythematosus. Rituximab has shown to be clinically efficacious in many of these conditions. In the two cohorts that we studied rituximab was less effective in RA patients with the IFN signature. These data suggest that the type I IFN signature might be used to predict beforehand which patients will benefit most from rituximab treatment. Still, the predictive value of the type I IFN signature in itself was not sufficient to be used as a solitary biomarker. Therefore, the type I IFN signature should be assessed in future studies in combination with other biomarkers for its validity to guide rituximab treatment decisions.

In chapters 8 to 10 we studied the current treatment schedule of rituximab, which consists of a course of 2 times 1,000 milligram of rituximab. In B-NHL patients rituximab is less efficacious in patients with a large tumor mass and rituximab levels are lower after therapy. In chapter 8 we analyzed whether we could find a similar relation in RA. We found that rituximab levels were not related to the extent of B cell depletion after treatment, or to clinical response. We also found that clinical response was not significantly lower in patients who formed anti-rituximab antibodies. These data indicate that the current rituximab treatment regimen results in drug levels that are in the therapeutic range in both responders and non-responders to treatment, even when patients form anti-rituximab antibodies.

In chapters 9 and 10 we studied the effects of repeated treatment with rituximab in initial responders and non-responders to treatment. In our cohort, initial non-responders did not respond to re-treatment, while clinical responders had an improved response to a second treatment course. Our findings have recently been confirmed in a large, randomized clinical trial. Other studies, however, have suggested that seropositive patients who fail to respond to a first course of rituximab may still respond to a second course. In light of the availability of other therapeutic options, for individual patients who are rituximab non-responders other treatment options should be considered.

Chapter 11 describes the results of a phase Ib trial with atacicept in RA patients. Atacicept is a recombinant fusion protein that binds and neutralizes the activity of the cytokines B lymphocyte stimulator (Blbs) and APRIL, a proliferation-inducing ligand (APRIL) and the heterodimer of these 2 cytokines. Blbs and APRIL enhance B cell survival, proliferation, antigen presentation, and class-switch recombination at various stages of B cell development. We found that atacicept is well tolerated with an in vivo biologic effect consistent with its mechanism of action. Treatment resulted in a decrease in serum RF and ACPA levels in parallel with total serum immunoglobulin levels. Future work will need to address whether this biological effect might translate into clinical efficacy.
B Cells and B Cell directed therapies in Rheumatoid Arthritis

The persistence of B lineage cells in the synovium of autoantibody positive RA patients who do not exhibit a robust response to the first therapy, and investigate the effect of systematic retreatment in initial responders versus non-responders. Finally, we conducted an exploratory study on the effects of atacicept in RA.

The results presented here support the notion that RA is a heterogeneous disease with a variable response to a given therapy and indicate that specific biomarkers may predict the response to therapy. Novel approaches may be needed to interfere with these cycles in initial non-responders.

CONCLUDING REMARKS AND FUTURE DIRECTIONS.

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B Cells and B Cell directed therapies in Rheumatoid Arthritis

Inflammatory mediators that work together to form a chronic inflammatory disease which is characterized by inflammation of the joints, but other tissues can be affected as well. The inflammatory process gradually results in progressive damage of cartilage and bone, resulting in increasing disability. RA occurs in around 1% of the population.

During the last fifteen years the diagnostics and treatment of RA have much improved. Tests have been developed facilitating early diagnosis, and patients are treated earlier with more effective drugs. Because of these reasons the disease process can currently be halted in an early phase in most patients. As a result, damage of cartilage and bone and severe disability occur less frequent. The basis for this progress has been formed by increasing knowledge of the inflammatory process in RA, which has resulted in the development of a number of new drugs which are all effective in a proportion of patients. Since every drug is only effective in part of RA patients it is important to be able to predict beforehand which patient will benefit from which treatment. This is called personalized medicine. To make personalized medicine a reality it is necessary to discover whether RA has a different pathogenetic mechanism in different patients. Furthermore, it is necessary to understand in detail the mechanism of action of new treatments. In this thesis I focused on treatment with rituximab. This is a drug that depletes B cells (B of bone marrow or bursa), white blood cells that are involved in RA in at least a proportion of the patients.

The first subdivision is made between ‘innate’ and ‘adaptive’ immunity. The innate immune system is evolutionary the oldest. It consists of white blood cells and inflammatory mediators that fight most infections very effectively. During evolution, however, bacteria and viruses evolved that can escape from the innate immune system. Therefore, adaptive immunity has developed. This consists of white blood cells and mediators of inflammation that direct the innate immune system and increase its effectiveness. Adaptive immunity has two characteristics: 1. It consists of white blood cells that have evolved to recognize unknown microorganisms much more effectively compared to the innate immune system; 2. After an infection with a microorganism small numbers of the white blood cells that have best recognized the microorganism persist in small numbers in the lymphoid tissue. In case of a new infection with the same microorganism, they will direct the innate immune system to attack it again, but with a faster, stronger response.

The adaptive immune system is divided into two parts: cellular and humoral immunity. Cellular immunity is directed against for instance infections of cells by viruses. Humoral immunity is directed against infections of tissues by bacteria, yeasts, fungi and parasites. Antibodies belong to the most important players in humoral immunity. They are produced by B cells and serve as mediators recognizing microorganisms very effectively. When they bind to a microorganism they can direct the innate immune system to kill it.

During life B cells are continuously being formed in the bone marrow and they circulate through the blood and the lymph nodes. When they encounter a microorganism, the B cells that recognize the microorganism the best start to divide and the daughter cells produce large amounts of antibodies. These differentiated cells are called plasma cells.

There are various lines of evidence indicating that B cells are involved in the inflammatory process in the joints of RA patients. First, in around 70% of RA patients antibodies are present that recognize substances which are present in the inflamed joint. These antibodies are called rheumatoid factors and anti-citrullinated protein antibodies (ACPA). It is currently not known why these specific antibodies are involved in RA, but it has been suggested that ACPA can be formed in response to non-specific stimuli (such as bacteria found in the gingiva or smoking) in individuals with a specific genetic predisposition. Moreover, the inflamed joint tissue of RA patients contain clusters of B cells and plasma cells. Intriguingly, these clusters are only formed in a subset of the patients. In other patients the joint tissue does not contain B or plasma cells.

In chapter 2 we show in a cohort of 100 RA patients that patients with B cell clusters in the joint tissue do not have a more severe disease subtype, characterized by for instance more joint damage. Furthermore, B cell clusters are observed equally in the joints of patients with or without rheumatoid factors or ACPA. This indicates that there is a different cause for the presence of B cell clusters in the synovial tissue in the joints of a proportion of the patients. Possibly, these clusters are formed because of a certain genetic predisposition. Interestingly, as we show in chapter 3, patients with B cell clusters in their synovial tissue have increased synovial inflammation and they are enriched in their clinical response the anti-TNF antibody infliximab.

**B Cell Directed Treatments:** Rituximab is an antibody that has been developed to deplete the B cells during a period of several months. Rituximab has originally been developed as a treatment for non-Hodgkin’s lymphoma, a type of B cell cancer, but it also turned out to be an effective treatment for RA patients. It is currently not completely understood why rituximab improves synovial inflammation, and whether the current treatment schedule is optimal for treatment of RA. Rituximab is currently given as a course consisting of 2 infusions during a 2-week period.

In the blood rituximab destroys B cells within hours. In chapter 4 and 5 we show that the B cells do not disappear from the inflamed synovial tissue of all RA patients. In chapter 6 we show that plasma cells, which are derived from B cells and produce antibodies, only disappear in patients who exhibit reduced synovial inflammation after rituximab treatment. This suggests that there are two possible explanations for why some patients do not respond to rituximab: 1. B cells and plasma cells persist in these patients because the administered rituximab dose is too low or the antibody should be administered more frequently. The data presented in chapter 8 suggest that the dosage is probably sufficient; 2. Rituximab is not able to deplete the specific B cells driving the disease process in these patients due to the presence of protective factors. We are currently investigating why B cells may be resistant to rituximab in a proportion of the RA patients.

In chapter 7 we investigate whether there are differences in the biological mechanisms driving the inflammatory process in RA between patients who respond compared to patients who do not respond to rituximab treatment. The white blood cells in the peripheral blood are highly...
activated in a subset of the RA patients. When analyzing this activation pattern it seems like these white blood cells are activated by interferon I. This is a protein playing a role in viral infections. Of interest, interferon I has been shown to be involved in several rheumatological diseases, including Sjögren’s syndrome, systemic lupus erythematosus and dermatomyositis. The precise role of interferon I in these conditions is currently unknown, but interestingly, interferon I stimulates survival of B cells. In chapter 7 we show that patients with a strong interferon I activity overall respond less well to rituximab treatment than those with less IFN I activity. This suggests that rituximab might perhaps be combined with drugs that block interferon I or reduce B cell survival in these patients.

Another possibility could be to re-administer rituximab before B cells return. We have not formally tested this possibility, but data presented in chapter 9 and 10 suggest that this approach may only work in patients who have responded to some extent to the first rituximab treatment course. When these patients receive a second course 6 months after the first course their synovial inflammation continues to diminish. Patients who have not responded at all to the first treatment course (around 30% of patients) do not exhibit robust clinical improvement after the second course either. These patients are candidates for treatment with drugs with a different mechanism of action.

In chapter 11 we conduct a clinical trial with a new B cell directed drug, atacicept, which was tested for the first time in RA patients. Atacicept blocks survival factors of both B cells and plasma cells. In this trial in 73 RA patients we show that atacicept can be given safely to RA patients. Furthermore, it induces clear effects on B cells, rheumatoid factors and ACPA. Some of the patients experienced decreased joint inflammation. Larger clinical trials are currently being conducted to investigate the right dose, safety and effectiveness atacicept treatment.
SUMMARY IN DUTCH

REUMATOIDE ARTRITIS (RA) is een chronische ontstekingsziekte waarbij met name de gewrichten van handen en voeten ontstoken zijn, maar die ook gepaard kan gaan met ontsteking in andere organen. Deze ontstekingsleid geledelijk tot toenemende schade aan kraakbeen en bot, wat zich uit in toenemende invalidering. RA komt voor bij ongeveer 1% van de bevolking.

Tijdens de laatste vijftien jaar zijn de diagnostiek en behandeling van RA sterk verbeterd, omdat er testen zijn ontwikkeld waarmee de diagnose eerder gesteld kan worden en vanwege snellere, agressievere behandeling met effectievere medicijnen. Vanwege deze redenen kan het ziekteproces momenteel bij de meeste patiënten in een vroege fase worden geremde, waardoor kraakbeen- en botschade bij steeds minder patiënten voorkomen. Tegelijkertijd heeft toenemende kennis over de ontstekingsleiding van RA geresulteerd in een aantal nieuwe behandelingen die ieder bij een gedeelde van de patiënten erg effectief zijn. Omdat elke medicijn bij slechts een gedeelde van de patiënten goed werkt is het belangrijk om van te voren te kunnen voorspellen welke persoon baat zal hebben bij welk medicijn. Dit wordt therapie op maat of ‘personalized medicine’ genoemd. Om ‘personalized medicine’ mogelijkt te maken is het nodig om er achter te komen of RA bij verschillende mensen een verschillende oorzaak heeft en om de precieze werking van medicijnen beter te begrijpen. In dit proefschrift heb ik mij gefocust op één van de nieuwe behandelingen voor RA, rituximab. Dit is een medicijn dat specifiek gericht is op het tijdelijk uitschakelen van één soort witte bloedcel die een rol speelt in de gewrichtsontsteking bij RA patiënten. Dit is de B cel (B van beemmerg of bursa).

B cell gerichte medicijnen. Rituximab is een medicijn dat ontwikkeld is met het doel om alle B cellen gedurende enkele maanden uit het lichaam te verwijderen. Rituximab werd oorspronkelijk ontwikkeld voor patiënten met non-Hodgkin lymfoom, een vorm van B cel lymfoom, maar bleek ook te werken voor RA patiënten. Het is momenteel onbekend waarom rituximab precies leidt tot een vermindering van de gewrichtsontsteking bij een gedeelte van RA patiënten en of het behandelingseffect van rituximab ook te werken voor RA patiënten. Het is mogelijk dat RA, rituximab en verschil- len momenteel gegeven in losse kuren van 2 infusionen die herhaald worden als de gewrichten ontstoken blijven of opnieuw ontstoken raken na een eerdere behandeling.

In het bloed worden alle B cellen binnen enkele uren door rituximab vernietigd. In hoofdstuk 2 en 5 tonen we aan dat dit in het gewrichtsweefsel de B cellen niet bij alle patiënten verdwijnen en in hoofdstuk 6 tonen we aan dat plasmacellen, de eindstadium B cellen, alleen verdwijnen in patiënten die reageren op therapie. Dit suggereert dat er twee mogelijke verklaringen zijn waarom patiënten niet reageren: 1. Bij patiënten die niet reageren blijven B en plasmacellen aanwezig omdat deze een te lage dosis krijgen. In hoofdstuk 8 tonen we echter aan dat hier geen aanwijzingen voor zijn; 2. Rituximab slaagt...
er in sommige patiënten niet in om de B cellen te vernietigen. Waarom deze B cellen resistent zijn tegen rituximab is onderwerp van vervolgonderzoek.

In hoofdstuk 7 onderzoeken we of er verschillen zijn in de biologische mechanismen die de ontsteking bij RA onderhouden tussen patiënten die reageren en patiënten die niet reageren op rituximab. In een gedeelte van de RA patiënten zijn de witte bloedcellen in het bloed sterk actieveerd. Als je dit activatiepatroon bestudeert lijkt het alsof ze door de stof interferon I zijn actieveerd. Dit is een ontstekingsstof die normaal een rol bij virale infecties heeft. Interferon I blijkt een belangrijke te spelen in veel reumatische ziekten, waaronder de ziekte van Sjögren, lupus en dermatomyositis. Wat nu precies de rol van interferon I in deze ziektes is onbekend, maar interessant genoeg kan interferon I de overleving B cellen sterk stimuleren. In hoofdstuk 7 tonen we aan dat patiënten met een sterke interferon I activiteit gemiddeld genomen minder goed op rituximab reageren. Dit suggereert dat het effect van rituximab versterkt kan worden door dit te combineren met medicijnen die B cel overleving remmen.

Een andere mogelijkheid is om rituximab na enkele maanden opnieuw te geven, voor dat de B cellen terugkeren. In hoofdstuk 9 en 10 tonen we aan dat dit alleen werkt bij patiënten die al in enige mate op de eerste kuur met rituximab hadden gereageerd. Als bij deze patiënten een half jaar na de eerste kuur een tweede kuur wordt gegeven neemt hun gewrichtsontsteking wat verder af. Patiënten die helemaal niet op een eerste kuur hebben gereageerd (circa 30% van de RA patiënten) reageren ook niet goed op vervolgbaandeling met een tweede kuur. Waarschijnlijk reageren deze patiënten het beste op combinatietherapie of behandeling met een heel ander medicijn.

In hoofdstuk 11 verrichten we tenslotte een onderzoek met een nieuw B cel gericht medicijn, atacicept, dat we voor het eerst aan RA patiënten hebben gegeven. Bijzonder aan atacicept is dat het zowel B als plasmacel gerichte overlevingsstoffen die door het gewrichtsweefsel worden gemaakt blokkeert. Het vormt hierdoor in theorie een aanvulling op rituximab. In dit onderzoek in 73 RA patiënten tonen we aan dat atacicept veilig gegeven kan worden en een duidelijk effect heeft op B cellen, reumafactoren en ACPA in deze patiënten. Bij een gedeelte van de patiënten trad ook een vermindering van de ziekteactiviteit op. Grotere vervolgonderzoeken (eventueel in combinatie met rituximab) zijn nodig om de veiligheid en effectiviteit van atacicept verder aan te tonen.
DANKWOORD

Ik wil iedereen bedanken die heeft bijgedragen aan de totstandkoming van dit proefschrift. Ik wil hiernaast een speciaal woord van dank richten tot:

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Roche Nederland, Roche international, Merck Serono, Millennium Pharmaceuticals: many developments in rheumatology have been made possible by the interest of pharmaceutical and biotechnological companies in developing innovative therapies for rheumatic diseases. Thank you for the excellent collaboration in various clinical studies.
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**CURRICULUM VITAE**

Rogier Thurlings was born in Alkmaar, the Netherlands, on the 2nd of April 1978. He graduated cum laude from the Murmellius Gymnasium in Alkmaar in 1996. During his school years he was an active member of the International Model United Nations of The Hague. He started medical school in 1996 at the University Medical Center of Utrecht where he graduated from medical school in 2004.

During his studies he worked as a student teacher in epidemiology, physiology, anatomy and medical skills. He was an active member of student society Unitas S.R., directed plays, was an editor for student’s magazine Arts & Fiets, and worked as a tour guide in China and Tibet. In 1998 he performed a clerkship at the department of Radiology at the Queen Mary hospital in Hong Kong. In 2000 he performed his research elective at the Department of Neurology at the University Medical Center of Utrecht, where he investigated neuronal plasticity after spinal cord injury. In 2004 he worked as an intern in Internal Medicine at the Academic Medical Centre (AMC) and subsequently started his PhD project at the Department of Clinical Immunology and Rheumatology at the AMC/University of Amsterdam (Promotor: Prof. dr. P.P. Tak). He received the Best Poster Award at the 2nd European Workshop on Immune-Mediated Inflammatory Diseases in Nürnberg 2007. In 2010 he started his training in internal medicine (supervisor: Prof. dr. P. Speelman) and rheumatology (supervisor: Prof. dr. P.P. Tak) at the AMC. He lives with his future wife Sarah Heijse in Amsterdam.
B Cells and B Cell directed therapies in Rheumatoid Arthritis


COLOR PRINT SECTION
Different patterns of lymphocyte infiltration in representative synovial tissue specimens from patients with rheumatoid arthritis. In some patients, mixed infiltration of aggregates of T and B cells was present (A and C), together with a high number of infiltrating macrophages (C). In other patients, there was diffuse or scarce infiltration of CD3+ T cells (B), and few or no B cells (D), while macrophages were the dominant infiltrating cell population (F). (Original magnification x 20.)
CHAPTER 4 FIGURE. Change in the number of CD22+ B cells in representative serial synovial tissue samples obtained from 2 different rheumatoid arthritis patients before (A and C) and 4 weeks after (B and D) initiation of rituximab treatment. Different patterns of depletion were identified. In some patients, there was complete B cell depletion (compare A and B), while in other patients, few B cells were depleted (compare C and D). (Original magnification x 20.)

CHAPTER 2 FIGURE. Follicular dendritic cells (FDCs) expressing the CD21 long isoform (A), detected in CD22+ B cell–containing lymphocyte aggregates (B). Synovial tissue samples from 8% of the rheumatoid arthritis patients contained lymphocyte aggregates with CD22+ B cells surrounding FDCs. (Original magnification x 20; x 40 in inset.)
CHAPTER 6 FIGURE 5. Change in the number of CD138+ plasma cells in representative serial synovial tissue samples obtained at 4 (A and C) and 16 (B and D) weeks after initiation of rituximab treatment. Different patterns of response were identified. In patients who responded to treatment we observed a reduction in plasma cells between 4 and 16 weeks after treatment (compare A and B), while in patients who did not fulfil the response criteria, plasma cells persisted (compare C and D) (Original magnification x20). Linear regression analysis revealed a significant relationship between the decrease in plasma cell numbers and the decrease in 28-joint Disease Activity Score (DAS28) at week 24.
B Cells and B Cell directed therapies in Rheumatoid Arthritis