Abstract

Objective: Conventional therapies for Graves’ disease, consisting of medical therapy or radioiodine are unsatisfactory, because of limited efficacy and adverse events. Interventions aimed at the underlying autoimmune pathogenesis of Graves’ disease may be worthwhile to explore. We therefore performed a prospective, 26-week phase II study with open-ended observational extension to assess the efficacy of rituximab in patients with recurrent Graves’ disease.

Design: We performed a prospective, 26-week phase II study with open-ended observations.

Methods: Thirteen patients with relapsing Graves’ disease (9 females and 4 males, age 39.5 ± 9.5 years) received 2 dosages of rituximab 1000 mg i.v. with a 2-week interval. Before administration and on several periods after the administration of TSH, free thyroxine (FT4), thyrotropin binding inhibitory immunoglobulins (TBII) and the proportion of CD19 and MS4A1 positive peripheral blood mononuclear cells were measured.

Results: The proportion of MS4A1 positive lymphocytes decreased in all patients from 5.8% at baseline to 1.4% at 26 weeks (P < 0.007). Four patients with high initial FT4 levels did not respond to treatment. All remaining patients had a decrease in FT4 levels at 26 weeks (P = 0.001) and an increase in TSH levels (P = 0.011). TBII decreased in all remaining patients (P = 0.003). At a follow-up time of 14–27 months, nine of these patients were still euthyroid with normal FT4 (P < 0.001) and TSH levels (P < 0.008).

Conclusions: The present study results suggest a beneficial role of rituximab in mild relapsing Graves’ disease. A subsequent randomized controlled trial with rituximab is recommended.

Introduction

Graves’ disease is the most prominent cause of hyperthyroidism, with a yearly incidence of 5/1000. Graves’ disease is a complex disease determined by multiple genetic, environmental and endogenous factors, which are responsible for the emergence of thyroid-stimulating antibodies, which bind to and activate the thyroid stimulating hormone (TSH) receptor on thyroid cells (1–4). Current therapies for Graves’ disease consist of antithyroid drugs, radioiodine and surgery (4). Antithyroid drugs, which are usually prescribed for at least 1 year, are associated with a sustained remission in only 30–40% of patients after 10 years (5, 6). In addition, these drugs are accompanied by rare side effects, the most important being agranulocytosis and hepatic failure (6). Radioiodine is the preferred initial treatment in North America, whereas it is indicated in relapsing disease in many other countries. The main side effect of radioiodine is hypothyroidism, with an estimated incidence of 30% in the first 2 years after therapy and a yearly incidence thereafter of 5% (7, 8).

Because of the imperfections of current therapies for Graves’ disease, treatments that are aimed at the underlying pathogenetic mechanism are warranted.

A promising strategy in autoimmune diseases is the elimination of activated B-lymphocytes. MS4A1 is highly expressed on the surface of pre-B lymphocytes as well as activated mature B-lymphocytes (9), which makes the MS4A1 antigen an attractive target for various disorders involving B-cell activation. In addition to abolishing immunoglobulin production, anti-MS4A1 therapy could also interfere with the antigen-presenting role of B-cells (10). B-cells are also able to modulate T cell activities. rituximab is a chimeric monoclonal antibody specific for human MS4A1 (9, 11, 12). rituximab was the first therapeutic monoclonal antibody used for the treatment of non-Hodgkin lymphoma (13, 14). It has subsequently been successfully applied in various autoimmune diseases (15), including idiopathic thrombocytopenic purpura (16), systemic lupus erythematosus (17, 18), haemolytic anaemia (19) and rheumatoid arthritis (10, 20, 21).
Recently, a few papers have been published on the effectiveness of rituximab in Graves’ ophthalmopathy and hyperthyroidism (22–24). In a study of nine patients with Graves’ ophthalmopathy, rituximab positively affected the clinical course without influencing thyroid function (22). El Fassi et al. (23) have recently published a study of 20 patients, with mostly a first episode of Graves’ disease. Rituximab in combination with methimazole induced a sustained remission in 4/10 patients, whereas all patients treated with methimazole alone relapsed. Because methimazole treatment in this study was ~4 months, which is unusually short, it is not clear whether rituximab would be more efficacious when compared with a full course of methimazole therapy.

We decided to perform a phase II pilot study with rituximab in patients with recurrent Graves’ disease. We chose to study recurrent Graves’ disease instead of a first episode of Graves’ disease because these patients are treated with radioiodine therapy that ultimately leads to hypothyroidism in most patients. We therefore considered any ability of rituximab to prevent radioiodine treatment an important health benefit.

Patients and methods

Subjects

Seventeen consecutive patients with relapsing Graves’ disease were recruited from patients who were referred to the Department of Nuclear Medicine of the Leiden University Medical Centre for radioiodine therapy because of relapsing Graves’ disease. Exclusion criteria were serious signs or symptoms of hyperthyroidism requiring immediate conventional therapy, other conditions or concomitant medication that could interfere with the study objectives, such as the use of corticosteroids for obstructive pulmonary disease, and contraindications for the use of rituximab such as previous treatment with murine monoclonal antibodies. Four patients refused rituximab treatment. Therefore, 13 patients (9 females and 4 males, age 39.5 ± 9.5 years) entered the study.

A prior episode of Graves’ disease had to be confirmed by documented elevated serum free thyroxine (FT₄) levels, suppressed serum TSH levels, a typical pattern of diffusely increased uptake of Technetium 99m pertechnetate at thyroid scintigraphy and/or elevated serum levels of binding thyrotropin inhibitory immunoglobulins (TBII). Patients had to be adequately treated for prior episodes of Graves’ disease with at least 1-year antithyroid drugs. Relapse of Graves’ disease after suspension of antithyroid drugs had to be confirmed by hyperthyroidism defined as suppressed TSH levels (<0.4 mU/l), coming from normal levels and increasing or elevated serum FT₄ levels.

Eleven patients had a first relapse. Two patients had a second relapse (nos. 7 and 10). Their first relapse was treated with radioiodine (no. 7, radioiodine 8 months before relapse; no. 10, radioiodine 3 years before relapse). Median (range) clinical activity score of Graves’ ophthalmopathy was 0 (0–2) points at baseline. Three patients had a NOSPECS score of 2a3040. Median (range) thyroid volume was 20 (20–40) cc. Median time since the first episode of Graves’ disease was 19 (14–66) months. The median (range) duration between withdrawal of antithyroid drugs and relapsing Graves’ disease was 2 months (1–24 months). Ten patients were treated temporarily with methimazole 30 mg/day after being diagnosed with relapsing Graves’ disease. These drugs were discontinued at least 4 weeks prior to rituximab administration to verify the existence of hyperthyroidism before treatment with rituximab. Four patients (nos. 1, 2, 8 and 10) had FT₄ levels within the reference range. However, FT₄ levels were higher than before relapse in all 4 patients (FT₄ before relapse, respectively, 12.1; 16.1; 12.2 and 19.5 pmol/l). Likewise, TSH levels were lower than before relapse in all 4 patients (respectively, 0.210; 0.341; 2.05 and 1.28 mU/l). Median serum levels of TBII were 4.1 IU/l (range 0.2–17.1 IU/l).

The local ethics committees approved the study as a preliminary phase II study, and written informed consent was obtained from all subjects.

Study design

The study was a prospective, open trial with a 26-week duration, with an open-ended observational extension afterwards. The primary endpoint was the absence of hyperthyroidism at the end of the study period (week 26 counted from the first rituximab infusion). The secondary endpoint was the relapse free survival time. Rescue therapy with antithyroid drugs followed by radioiodine was allowed when patients did not respond to rituximab, but this was considered a treatment failure.

Experimental protocol

Patients received two dosages of rituximab 1000 mg i.v. with a 2-week interval. This treatment schedule was based on the treatment schedule used in rheumatoid arthritis (20). To avoid allergic reactions, 10 mg dexamethasone and 2 mg clemastine was given intravenously before the rituximab injection. Study visits took place before the rituximab administration, 1 week after the first administration and at 2–4 week intervals after the second administration. At these visits, a physical examination was performed, including thyroid size estimation. Blood samples were taken for study parameters, including TSH, FT₄, TBII and the proportion of CD19 and MS4A1 positive peripheral blood mononuclear cells (PBMC). Safety parameters consisted of a haematological profile, serum levels of sodium, potassium and creatinine, lipids, renal and liver.
function and parameters of bone metabolism. Plasma and serum samples were handled immediately or stored at −80 °C in Sarstedt tubes.

**Blood chemistry**

FT4 was measured on a Modular Analytics E-170 (Roche Diagnostic Systems; intra-assay variability: 2.47–7.57%, inter-assay variability: 5.6–12.4% at different levels, normal range 10–24 pmol/l). TSH was determined with a Modular Analytics E-170 (Roche Diagnostic Systems), intra-assay variability: 0.88–10.66%, inter-assay variability: 0.91–12.05%, normal range 0.4–4.8 mU/l). FT3 was measured on a Modular Analytics E-170 (Roche Diagnostic Systems, reference range 3.9–6.7 pmol/l). TBII were measured with a TRAb enzyme-immunoassay, using coated recombinant human TSH receptor and biotin labelled human TSH (Medizym T.R.A. kit, Medipan, Berlin, Germany; functional assay sensitivity is 0.2 IU/l). TBII levels above 2.0 IU/l and below 1.0 IU/l indicate positive and negative results according to the manufacturer.

**Flow cytometric analysis**

PBMC were prepared by centrifugation over Ficoll-Hypaque gradients and cryopreserved in liquid nitrogen. Cells were labelled for 30 min at 4 °C, using anti-MS4A1 fluorescein isothiocyanate (FITC) (clone 2H7), anti-CD19PE (clone H1B19) and anti-CD3 allophycocyanin (APC) (clone UCHT1) (Beckton Dickinson, San Diego, CA, USA). All stained cells were analysed with a FACS calibur (Becton Dickinson) flow cytometer and the associated software program FlowJo (Tree Star Inc., Ashland, OR, USA) was used to calculate frequencies within the lymphocyte population. B cells were identified as CD3− and CD19+ cells.

**Sample size calculation**

To calculate the required sample size, we used the algorithm proposed by Simon et al. for phase II trials (25). Adopting a \( p_0 \) (absence of efficacy) of 0.20 and a \( p_1 \) (presence of efficacy) of 0.40. With \( \alpha \) and \( \beta \) values of 0.05 and 0.80, respectively, the treatment is rejected if 3 or fewer out of 13 patients respond to the treatment. In this setting, the likelihood of rejecting the treatment in case \( p_0 \) is true is 75%. In other words, if four or more patients responded, the treatment was considered worthwhile to further explore. Response was defined as euthyroidism at the end of the study period (primary objective, 26 weeks) or relapse-free survival (secondary objective).

**Statistical analysis**

Values are presented as mean ± s.d. or as median (range). Data between groups are analysed by one-way ANOVA and independent sample tests for nonparametric data. Data within groups are analysed by repeated measures ANOVA. SPSS 12.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for all analyses. Differences were considered statistically significant at \( P<0.05 \).

**Results**

All patients underwent the rituximab treatment without important side effects. Two patients (nos. 1 and 4) had temporary joint complaints without clinical signs or laboratory abnormalities. No laboratory side effects were observed. The effect of rituximab on lymphocyte populations was verified by the assessment of CD19 and MS4A1 subsets. All patients had a clear decrease in CD19 (data not shown) and MS4A1 subpopulations (\( P=0.007 \) versus baseline, Fig. 1). One patient (no. 1) appeared to have a low percentage of MS4A1 positive lymphocytes of 0.74% at baseline, despite a normal total lymphocyte count.

Four patients (nos. 6, 7, 9 and 13) had to be excluded from the study because of rituximab failure (Table 1). They received subsequent therapy with radioiodine. In all remaining nine patients, FT4 levels were decreased versus baseline at 26 weeks (\( P=0.001 \)) whereas TSH levels increased (\( P=0.011 \)), and were within the normal range in five patients. Median (range) FT3 was 8.2 (3.9–24.3) pmol/l at baseline. Median FT3 at 26 weeks was 4.8 (3.5–5.9) pmol/l for responders.
Table 1 Study results.

<table>
<thead>
<tr>
<th>Time of measurements</th>
<th>Relapse free survival</th>
<th>Treatment failure</th>
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<tbody>
<tr>
<td>After cessation of ATD for first episode of Graves' disease</td>
<td>Baseline</td>
<td>26 Weeks</td>
</tr>
<tr>
<td>Relapse Interval after cessation of ATD (months)</td>
<td>FT4 (pmol/l)</td>
<td>TSH (mU/l)</td>
</tr>
<tr>
<td>1 Patient numbers FT4 (pmol/l)</td>
<td>TSH (mU/l)</td>
<td>FT4</td>
</tr>
<tr>
<td>1</td>
<td>12.1</td>
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</tr>
<tr>
<td>2</td>
<td>16.1</td>
<td>0.540</td>
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<tr>
<td>3</td>
<td>17.9</td>
<td>1.69</td>
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<tr>
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<tr>
<td>7</td>
<td>19.5</td>
<td>1.280</td>
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<tr>
<td>8</td>
<td>18.0</td>
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<tr>
<td>9</td>
<td>13.5</td>
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<tr>
<td>10</td>
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</tr>
<tr>
<td>11</td>
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</tr>
<tr>
<td>12</td>
<td>19.6</td>
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</tr>
<tr>
<td>13</td>
<td>20.6</td>
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</tr>
<tr>
<td>Mean</td>
<td>17.1</td>
<td>0.976</td>
</tr>
<tr>
<td>s.d. or median</td>
<td>2.6 (0.022–2.500)</td>
<td>8.7 (&lt;0.005–0.008)</td>
</tr>
</tbody>
</table>

ATD: Anti-thyroid drugs; Non-parametric paired t-test: *P=0.001 versus baseline; †P=0.011 versus baseline; ‡P<0.001 versus baseline; §P=0.008 versus baseline. 

*Patients received temporary methimazole 30 mg/day. This was discontinued 4 weeks before rituximab treatment.

†Patients received radioiodine therapy at earlier relapse.
(P=0.011 versus baseline), whereas median FT₃ for non-responders when they were withdrawn from the study was 13.21 (9.6–18.7) pmol/l (P=0.457 versus baseline). The median relapse free survival in these patients is now 18 months (range 14–20), with normal FT₄ levels (P<0.001 versus baseline) and normal TSH levels (P=0.008 versus baseline) in all patients. According to the criteria for success as defined in the Patients and methods section, the criterion of more than three patients being euthyroid was reached. We therefore considered the treatment worthwhile.

The number of patients with positive TBII levels is presented in Table 2. No differences were observed between responders and non-responders. TBII levels decreased significantly in the nine responders (P=0.003, Fig. 2), the median level at baseline being 4 IU/l (0.2–6.3) and at 26 weeks 1.9 IU/l (0–3.2). No correlation was found between proportions of CD-20 + lymphocytes and TBII levels.

Baseline FT₄ levels were significantly higher in non-responders versus responders (P<0.001), whereas TBII levels did not differ significantly. No differences were observed in thyroid size between responders and non-responders.

**Discussion**

The present study was performed to investigate the efficacy of rituximab in relapsing Graves’ disease. From the 13 patients included, 9 patients remained euthyroid after a median follow-up duration of 18 months. Four patients did not respond and had to be treated with radioiodine. One patient (no. 1) who afterwards appeared to have a low proportion of MS4A1 positive lymphocytes at baseline may not have been a suitable candidate for rituximab.

According to the criteria for success as defined in the Patients and methods section, the criterion of more than three patients being euthyroid was reached. We therefore consider the treatment worthwhile to further explore.

The most obvious question is whether the effects observed can be attributed to rituximab therapy. The natural course of relapsing Graves’ disease is not known, as most patients with relapsing Graves’ disease will receive treatment. The generally accepted notion that therapy with antithyroid drugs does not provide a definite cure in relapsing Graves’ disease is the base for definite therapies consisting of radioiodine or surgery. This notion makes it very unlikely that nine patients, in whom radioiodine therapy was indicated, would resolve spontaneously. In addition, all patients had increasing serum FT₄ and decreasing serum TSH levels at the time of treatment. It is unlikely that this course would have changed spontaneously in all patients. We therefore believe that the effects observed can be attributed to rituximab. These findings are in agreement with the conclusions of El Fassi et al. (23) who found a beneficial effects of rituximab in patients who in the majority had a first episode of Graves’ disease, but differs from the observations in patients with Graves’ ophthalmopathy, where no effect on hyperthyroidism was found (22, 24).

The four patients who did not respond had highly elevated FT₄ levels. It seems therefore likely that the beneficial effect of rituximab in our study was limited to those patients with relapsing Graves’ disease in an early phase. In the study of El Fassi et al. (23), FT₄ levels did not differ between responders and non-responders, however, in that study, all patients were pretreated with methimazole. The explanation for differences in the efficacy of rituximab in patients with relatively low versus high serum FT₄ levels is complicated as the working mechanism of rituximab in the context of Graves’ disease is incompletely understood. Aspects relevant for the efficacy of rituximab include pharmacokinetic effects (rituximab half-life may be influenced

<table>
<thead>
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<th>Time (weeks)</th>
<th>Response</th>
<th>No response</th>
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<tbody>
<tr>
<td>T=0</td>
<td>8/9</td>
<td>4/4</td>
</tr>
<tr>
<td>T=2</td>
<td>7/9</td>
<td>4/4</td>
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<tr>
<td>T=4</td>
<td>6/9</td>
<td>3/4</td>
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<tr>
<td>T=10</td>
<td>4/9</td>
<td>a</td>
</tr>
<tr>
<td>T=18</td>
<td>4/9</td>
<td></td>
</tr>
<tr>
<td>T=26</td>
<td>3/9</td>
<td></td>
</tr>
</tbody>
</table>

TBII levels >2.0 IU/l indicates a positive result.

*Four patients withdrawn from study, because of persistent high FT₄ levels.
by the degree of hyperthyroidism), pharmacodynamic aspects (differences in dose-effect relationships and susceptibility at the level of developing B-cells, differences in half-lives of activated B-cells, differences in tissue penetration of rituximab and differences in the degree of plasma cell and T-lymphocyte activation, given the fact that rituximab does not directly affect plasma cells or T-lymphocytes). It could furthermore be hypothesized that the response to rituximab was dependent on the interval between withdrawal from antithyroid drugs and relapse. However, this relationship was not obvious from our data, although the small number of patients prevents a definite conclusion.

We observed a clear decrease in TBII levels in patients with a favourable outcome, whereas TBII levels remained elevated in non-responders. However, a clear relationship between serum TBII levels and outcome could not be studied because of the small number of patients in our study. It would be useful to study this further to define a subgroup of patients likely or not to be responders. El Fassi et al. found a relation between baseline TBII levels and rituximab effectiveness, patients with lower TBII levels responding more favourably (23). However, they did not find an effect of rituximab on TBII in comparison with methimazole. In addition, no correlation was found between proportions of CD-20+ lymphocytes and TBII levels. This is in agreement with the study of Salvi et al. (24) and El Fassi et al. (23). In fact, it is well documented that there is no linear relationship between serum TBII concentrations and serum FT4 levels in patients with Graves’ disease (1), as TBII are a heterogeneous pool of both stimulating and blocking antibodies.

All patients showed a marked decrease in CD19— and MS4A1 positive lymphocytes, irrespective of outcome. Apparently, no clear relation exists between the degree of depletion of MS4A1 positive cells from peripheral blood and the therapeutic effect. The relation between circulating levels of MS4A1 positive lymphocytes, serological markers of autoimmune disease and effectiveness of rituximab in autoimmune disease is complex, but a number of studies suggest an inverse relationship between proportions of B-cells, autoantibody titres and therapeutic effect in rheumatoid arthritis and lupus (10, 17, 26, 27). In our study, the proportion of CD-20 positive lymphocytes was not associated with TBII levels. As discussed by Salvi et al. (24) and El Fassi et al. (23) synthesis of new antibodies occurs after rituximab treatment indicated that the half-life of human IgG is ~3 weeks. This and the absence of a beneficial effect of rituximab in some patients may be related to the fact that an important source of auto antibodies is plasma cells that do not express MS4A1. In addition, a fraction of plasma cells have a very slow turnover (28). In addition, B cell depletion may be less pronounced in lymphoid cells in germinal centres in the thyroid (29) as discussed by El Fassi and Salvi (23, 24).

However, El Fassi found complete intrathyroidal B-lymphocyte depletion (30).

In summary, we found indications for a beneficial effect of rituximab in patients with relapsing Graves’ disease with mild hyperthyroidism. As this was a phase II study, these results need to be confirmed in a randomized study. In addition, the issue of safety is still a concern given a recent report by the FDA, which reported an increased risk of progressive multifocal leucoencephalopathy (http://www.fda.gov/CDER/Drug/InfoSheets/HCP/rituximab.pdf). Although the high cost of rituximab may limit its use in first episodes of Graves’ disease, preventing hospital admission for radiiodine therapy may grant rituximab a more prominent place in the treatment of relapsing Graves’ disease, when the efficacy can be confirmed.

Declaration of interest
The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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