CD20 monoclonal antibody therapy for B-cell lymphoma
van der Kolk, L.E.

Link to publication

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s),
other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating
your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask
the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam,
The Netherlands. You will be contacted as soon as possible.
Chapter 2

Treatment of relapsed B-cell non-Hodgkin’s lymphoma with a combination of chimeric anti-CD20 monoclonal antibodies (rituximab) and G-CSF: final report on safety and efficacy

L.E. van der Kolk¹, A.J. Grillo-López², J.W. Baars³ and M.H.J. van Oers¹

¹Department of Hematology, Academic Medical Center, Amsterdam, The Netherlands; ²IDEC Pharmaceuticals Corp., San Diego, CA, USA and ³Department of Medical Oncology, Antoni van Leeuwenhoek Hospital/Netherlands Cancer Institute Amsterdam, The Netherlands

Submitted for publication
Abstract

Possible mechanisms of action of the chimeric CD20 monoclonal antibody IDEC-C2B8 (rituximab) involve complement- and antibody-dependent cellular cytotoxicity (ADCC). Because granulocyte colony-stimulating factor (G-CSF) greatly enhances the cytotoxicity of neutrophils in ADCC, the clinical efficacy of rituximab might be enhanced by the addition of G-CSF. In a phase I/II clinical trial we investigated the safety and efficacy of the combination of rituximab and G-CSF (5μg/kg/day, administered for three days, starting two days before each infusion) in 26 relapsed low-grade non-Hodgkin’s lymphoma (NHL) patients. Adverse events occurred almost exclusively during the first infusion and mainly consisted of (grade I/II) fever, chills and allergic reactions. Toxicity was comparable to that reported for rituximab monotherapy. In the phase II (375 mg/m² rituximab + G-CSF), 19 patients were evaluable for efficacy. The response rate was 42% (8/19; 95% CI 20%-67%). The percentage of complete remissions (CR) and partial remissions (PR) were 26% (5/19) and 16% (3/19) respectively. The median duration of response has not been reached at a median follow-up of 23 months. Only one patient who achieved a CR showed progressive disease (after 18 months), the other 4 patients are still in CR (for 13+, 24+, 25+, 25+ months). We conclude that the combination of rituximab and G-CSF is well tolerated. Although the overall response rate seems comparable to that reported for rituximab monotherapy, CR rate is high and remission duration in this pilot phase II study is remarkably long. Randomized comparison with rituximab monotherapy should substantiate this promising finding.
Introduction

Despite the development of new (chemo-)therapy regimens, the median survival time in low-grade non-Hodgkin's lymphoma (NHL) has not changed over the past decades and cure remains elusive. Thus, the need for new treatment modalities has not abated. Chimeric anti-CD20 mAbs (IDEC-C2B8, rituximab) have been shown to be a promising new option.\textsuperscript{13} It has been demonstrated in B cell-lines \textit{in vitro} that rituximab can induce complement-dependent cytotoxicity (CDC)\textsuperscript{46} and antibody-dependent cellular cytotoxicity (ADCC), the latter with mononuclear cells as effector cells.\textsuperscript{4} Furthermore, ligation of CD20 inhibits B-cell proliferation\textsuperscript{7} and it has been described that CD20 mAbs can induce apoptosis in B-cell-lines, which is enhanced after crosslinking.\textsuperscript{8-10} Recently, the importance of FcyR-mediated mechanisms for \textit{in vivo} cytotoxicity of monoclonal antibodies, including rituximab, was clearly demonstrated.\textsuperscript{11} Furthermore, the absolute number of NK cells prior to onset of treatment with rituximab was found to be significantly correlated to the probability of response to rituximab.\textsuperscript{12} Hence, although the exact mechanism of action of the CD20 mAb \textit{in vivo} has not been established yet, these studies support an important role for Fcy-receptor dependent mechanisms in the efficacy of rituximab treatment.

In a clinical trial in 166 relapsed low-grade NHL-patients treated with 375 mg/m\textsuperscript{2} rituximab weekly x 4, the overall response rate was 48% and the median time to progression 13 months.\textsuperscript{3} Thus, although clinical results with rituximab as single agent therapy are encouraging, they might still be improved. Therefore, several strategies to improve the clinical activity of rituximab are currently being investigated.\textsuperscript{13-17}

A possibility to improve the efficacy of unconjugated mAbs might be to enhance the efficacy of the effector cells involved in ADCC, one of the possible anti-tumor mechanisms of monoclonal antibodies. Granulocyte colony-stimulating factor (G-CSF) greatly enhances the cytotoxic capacity of neutrophils in ADCC-assays.\textsuperscript{18-20} It has been demonstrated \textit{in vitro} as well as \textit{in vivo} in healthy volunteers, that G-CSF induces the expression of Fcy-receptor type I (FcyRI) on neutrophils via an effect on myeloid precursor cells.\textsuperscript{19,21} This FcyR appeared to be the main FcyR involved in neutrophil-mediated ADCC-assays.\textsuperscript{18,19} Furthermore, G-CSF administration leads to a large increase in the number of circulating (FcyRI-positive) neutrophils. Therefore, adding G-CSF to rituximab therapy could theoretically enhance the efficacy of rituximab by exploiting the mechanism of ADCC.

We performed a phase I/II clinical trial to evaluate the safety (phase I) and efficacy (phase II) of the combination of rituximab and G-CSF. We hereby present the final data on safety and efficacy of this combination.
Materials and Methods

Study design
This was an open label, single arm phase I/II study. Patients were enrolled and treated in the Academic Medical Center and the Antoni van Leeuwenhoek Hospital, Amsterdam, the Netherlands. The study was approved by the ethics committee at each center and performed according to the guidelines of the Declaration of Helsinki.

Each patient received a total of 4 weekly intravenous (iv) doses of rituximab (IDEC-C2B8, Rituxan®, Mabthera®, IDEC Pharmaceuticals, San Diego, CA, USA) in combination with a standard dose of G-CSF (filgrastim, Neupogen; Amgen, Thousand Oakes, CA, USA; 5µg/kg/day subcutaneously) administered for three days, starting two days before each infusion of rituximab (fig. 1).

![Fig. 1 Treatment schedule](image)

Patients were treated with the combination of rituximab and G-CSF. Rituximab was given weekly for 4 weeks. G-CSF (5 µg/kg/day) was administered for 3 days, starting 2 days before each infusion of rituximab (i.e., the third injection of G-CSF was administered several hours before rituximab infusion). Follow-up was at 1, 2 and 3 months.

The phase I part of the study consisted of a dose escalation of rituximab, in combination with the standard dose of G-CSF. Cohorts of three patients were entered at each dose level of rituximab (125, 250 or 375 mg/m² rituximab weekly x4). Dose escalation was allowed only in the absence of serious toxicity, defined as grade 3 renal toxicity or any grade 4 drug-related toxicity according to the National Cancer Institute’s Adult’s Toxicity Criteria. Rituximab was administered according to the guidelines described in the investigational drug brochure. In brief, the first infusion was started at 50 mg/hr for the first hour and, if well tolerated, the infusion rate was escalated with increments of 50 mg/hr every 30 min, to a maximum of 300 mg/hr. Subsequent infusions were started at 100 mg/hr for the first hour, and increased with 100 mg/hr increments at 30 min-intervals, to a maximum of 400 mg/hr. Infusion was discontinued in case of hypotension (drop in systolic blood pressure of ≥30 mm Hg), rigors, bronchospasm or the occurrence of mucosal congestion or edema. If necessary, medication (consisting of an antihistamine (clemastine 2 mg iv), antipyretic (paracetamol 1000 mg) and/or corticosteroids (25 mg prednison iv) was administered. After resolution of the side effects, infusions were resumed at half of the previous rate, and, when tolerated, increased according to the described schedule.
After evaluation of the phase I, the dose of rituximab for the phase II study was established at 375 mg/m². In order to prevent the 'allergic reactions' and fever, which were observed frequently during the phase I (see 'Results'), it was decided to give premedication during the phase II, consisting of clemastine (2 mg iv) and paracetamol (1000 mg). Corticosteroids were never given as premedication. Since in phase I side effects were almost exclusively observed during the first infusion, premedication was only given before the first infusion.

Patients
Adults (>18 years) were eligible if they had measurable relapse or progression of histologically confirmed CD20-positive B-cell lymphoma. In the phase I part, patients with low and intermediate grade NHL were included (Working Formulation (WF) A-D). In the phase II part, only low-grade NHL patients were included (WF A-C). All patients had to meet the following inclusion criteria: expected survival of > 3 months; prestudy performance status of 0-2 according to the World Health Organization (WHO) scale; relapse or progression after at least one and no more than three prior systemic therapies; sero-negative for human immunodeficiency virus (HIV) and hepatitis-B surface antigen (HBsAg); serum IgG > 600 mg/dl; hemoglobin (Hb) > 5 mmol/L; white blood cell count (WBC) > 3.0 x10⁹/L; absolute granulocyte count > 1.5 x10⁹/L; platelet count (Pit) > 75 x10⁹/L; circulating tumor cells < 0.5 x10⁹/L. The number of circulating tumor cells allowed was initially very low (cells < 0.5 x10⁹/L) because of a possible relation between circulating tumor cells and side effects. During the study it became clear that side effects were generally mild and therefore this inclusion criterion was changed to 'circulating tumor cells < 2.5 x10⁹/L'.
Patients were ineligible if they had received other lymphoma treatment within three weeks prior to the scheduled first treatment; if they had a bilirubin level of > 1.5 mg/dl (26 μmol/L); alkaline phosphatase level of > 2x upper range of normal; AST (SGOT) level of > 2x upper range of normal; serum creatinine level of > 2.0 mg/dl (177 μmol/L) or if they had a serious non-malignant disease. All patients gave written informed consent before treatment was initiated.

Clinical parameters
Monitoring of safety was performed throughout the study and included medical history, vital signs, analysis of renal, hepatic and hematologic parameters and analysis of Ig levels. Toxicity was evaluated according to the National Cancer Institute's Adult's Toxicity Criteria. Response criteria were as follows: a complete remission (CR) required complete disappearance of all clinically detectable disease, including bone marrow infiltration, for at least 28 days; partial remission (PR) was defined as a ≥ 50% decrease in overall tumor size.
without any evidence of disease progression for at least 28 days; stable disease (SD) was defined as \( \leq 50\% \) decrease or \( \leq 25\% \) increase in overall tumor size, without any evidence of progression; progressive disease (PD) was defined as \( \geq 25\% \) increase in overall tumor size, or \( \geq 50\% \) increase in any single lesion, or the appearance of new lesions. Duration of response was measured from initial observation of response until the last date that the measurements satisfied the criteria of the response. Time to progression was measured from first study treatment until the first date when progressive disease was documented.

**Immunophenotypical analysis**

Mabs against CD11b (CLB-mon-gran/1, B2), CD13 (CLB-mon-gran/2), CD16 (CLB-FcRgran/1, 5D2), CD45 (CLB-T200/1, 15D9), CD64 (10.1), CD66b (CLB-B13.9) and the irrelevant control antibodies IgG1 and IgG2a were from the CLB (Amsterdam, The Netherlands). FITC-conjugated rabbit anti-mouse-Ig (F(ab')\(_2\) fragments) and Kappa and Lambda mAbs were from Dako (Glostrup, Denmark). Mean fluorescence intensity (MFI) was measured by flow cytometry (FACS-scan, Becton Dickinson, San Jose, CA, USA) and data were always corrected for the MFI in the presence of irrelevant control antibody.

**Elastase/lactoferrin**

Plasma levels of elastase-\( \alpha_1 \)-antitrypsin complexes (further referred to as elastase) and lactoferrin were measured with radioimmunoassays (RIA) as described before.\(^{23}\) The levels are expressed as ng/ml using preformed complexes and purified lactoferrin as standards respectively. Levels of elastase and lactoferrin in healthy individuals are below 100 ng/ml and 400 ng/ml respectively.\(^{23}\)

**Statistics**

Statistical analysis was performed using the SPSS statistical package (SPSS for Windows, version 8.0). Data are expressed as means ± SEM, unless indicated otherwise. Serum concentrations were compared using the Wilcoxon Signed Ranks test. Correlations were assessed using Pearson’s correlation coefficient. Differences in response rates between groups were tested for statistical significance using Fisher’s exact test (two-sided). The duration of response was assessed using the method of Kaplan and Meier. A P-value ≤ 0.05 was considered significant.
Results

Patient characteristics

Nine patients were treated in the phase I part of the study, three patients at each consecutive dose level. Patient characteristics are listed in table 1. The median age was 53 years (range 32-59 yrs) and the histologic subtype was low grade NHL in 7 patients and intermediate grade NHL in 2 patients. In the phase II, 17 patients have been treated. The median age was 53 years (range 27-75 yrs) and all patients had a low-grade NHL (table 1). The median number of prior systemic therapies was 2, and regimens included CVP (n=19 patients), CHOP (n=8), chlorambucil (n=5) and fludarabin (n=6). One patient had undergone high dose chemotherapy with stem cell rescue 5 months before study entry.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th></th>
<th>phase I</th>
<th>phase II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=9</td>
<td>n=17</td>
</tr>
<tr>
<td>Age at study entry (year)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median</td>
<td>53</td>
<td>53</td>
</tr>
<tr>
<td>range</td>
<td>32-59</td>
<td>27-75</td>
</tr>
<tr>
<td>Classification (WF)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>1*</td>
<td>4**</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>No. of prior systemic regimens***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Stage at diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IE</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>IIA</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>IIb</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>IIIA</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>IIIb</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IVA</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>IVB</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>IVE</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

* malformation; ** 3 x immunocytoma, 1x marginal zone lymphoma, according to the Real/Kiel classification. *** One patient (phase I) had received 4 prior regimens
Toxicity

All but one patient received the 12 injections of G-CSF according to the study protocol. Administration of G-CSF did not induce significant toxicity. However, there were patients that experienced mild bone pain or flu-like symptoms. In those patients it was advised to take paracetamol (1000 mg) before G-CSF administration. One patient had experienced grade II side effects (bone pain) during the first cycle of G-CSF. At her request, she did not receive G-CSF preceding the subsequent rituximab infusions.

The nine patients included in the phase I all completed the four cycles of rituximab treatment and were evaluable for safety. No premedication was given. Adverse events are summarized in table 2. Toxicity during rituximab treatment was experienced by 8 (out of 9) patients and consisted mainly of infusion-related adverse events (total number of adverse events = 30). The most frequent adverse events were fever and ‘allergic reactions’ consisting of rhinitis, sneezing, itching of the oropharynx and a sensation of swelling of the throat. There was no apparent relation between the dose of rituximab and the amount or severity of the adverse events (see table 1). The infusion-related adverse events typically occurred one to two hours after onset of the infusion. Most events were classified as mild to moderate (toxicity grades 1-2) and occurred during the first infusion; only few patients experienced adverse events during subsequent infusions. One patient developed a viral stomatitis (2 weeks after completion of rituximab therapy), which was complicated by a bacterial superinfection.

<table>
<thead>
<tr>
<th>Table 2. Infusion related adverse events</th>
</tr>
</thead>
<tbody>
<tr>
<td>rituximab dose (mg/m²x4)</td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>fever</td>
</tr>
<tr>
<td>‘allergic reaction’</td>
</tr>
<tr>
<td>headache</td>
</tr>
<tr>
<td>pruritis</td>
</tr>
<tr>
<td>chills</td>
</tr>
<tr>
<td>pain**</td>
</tr>
<tr>
<td>rash</td>
</tr>
<tr>
<td>nausea</td>
</tr>
<tr>
<td>urticaria</td>
</tr>
<tr>
<td>hypotension</td>
</tr>
<tr>
<td>other</td>
</tr>
<tr>
<td>total adverse events</td>
</tr>
</tbody>
</table>

‘Allergic reaction’ refers to a number of reactions including rhinitis, sneezing, itching of oropharynx and a sensation of swelling of the throat. * The number of adverse events during the first infusion is given in parenthesis. ** Pain at tumor sites
This infection resolved upon treatment with acyclovir and antibiotics. Though no serious infusion-related toxicity was observed during the phase I, it was decided to treat patients participating in the phase II study prophylactically with clemastine (2 mg iv) and paracetamol (1000 mg) in order to attenuate the allergic reactions that were frequently observed during the phase I study. Infusion related side effects observed in the phase II were comparable to those documented in the phase I. However, the allergic reactions as described in the phase I patients appeared to occur less frequently in these patients (9/61 during phase II versus 8/30 during phase I) and were clinically less pronounced than during phase I. Only mild hematologic toxicity was observed (grade 1-2) and parameters usually recovered at follow up. No renal or hepatic toxicity was observed (data not shown).

Neutrophil activation upon G-CSF-administration

Neutrophil activation was assessed 1) by analysis of phenotypic changes of neutrophils and 2) by measuring plasma levels of elastase and lactoferrin as markers for neutrophil degranulation. The rationale of adding G-CSF to the treatment with rituximab was to induce the expression of the FcγRI on the circulating neutrophils, while at the same time increasing their number. As shown in fig. 2, directly prior to the start of the first rituximab infusion the number of neutrophils was strongly increased (from $4 \pm 0.4 \times 10^9$ to $19 \pm 2 \times 10^9$, $P<0.001$). Moreover, FcγRI-expression on neutrophils rose from $26 \pm 9$ (MFI) at baseline to $68 \pm 8$ ($P=0.001$) after three days of G-CSF. Three days after treatment, the mean expression level of FcγRI on circulating neutrophils was still elevated. The increase in neutrophil count

**Fig. 2 Neutrophil counts and expression of activation markers**

A) Absolute number of neutrophils ($x10^9$) and expression of CD64 (FcγRI) and B) expression of CD11b, CD13 and CD16 (FcγRIII) during treatment with rituximab and G-CSF. Values are mean ± SEM of 19 patients (i.e. all patients that received 375 mg/m² rituximab in combination with G-CSF). Time points are related to rituximab infusion: **48**: 48 hrs before rituximab infusion, before first administration of G-CSF; **-1**: 1 hour before rituximab infusion, after 3 injections of G-CSF; **72**: 72 hours after both rituximab infusion and the third G-CSF injection. Asterisks indicate a significant difference (**: $P<0.05$, **: $P<0.01$) as compared to baseline value (-48 hours).
and induction of FcγRII-expression by G-CSF showed a comparable pattern during the first week and the fourth week of treatment (fig. 2A). CD11b (C3bi receptor), CD13 (aminopeptidase N), CD45 (membrane associated tyrosine kinase) and CD66b (CEA/like antigen, mainly localized in the specific granules) are antigens considered to be associated with neutrophil activation. Expression levels of these antigens were determined by flow cytometry, at different timepoints during in the first and fourth week of treatment and all values were compared to baseline levels (week I, timepoint -48 hrs, see legend fig. 2). CD13, CD16 (FcγRIII) and CD11b decreased upon G-CSF administration (fig. 2B). However, expression returned to normal at day three after rituximab treatment. There was no significant change in the mean expression of CD45 and CD67 (not shown).

The concentration of elastase was 49 ± 7 ng/ml at baseline (t=-48hrs). After 3 injections of G-CSF, the concentration increased to 129 ± 20 ng/ml (t=-1 hr, just before rituximab infusion; P=0.018). Three days later (t=+72 hours), levels had returned to normal (<100 ng/ml). The same pattern was observed during the fourth week of treatment (fig. 3A).

Lactoferrin levels rose from 179 ± 35 ng/ml at baseline, to 395 ± 112 ng/ml after 3 days of G-CSF (t=-1 hr; not significant (NS)), which is just within normal limits. At 72 hours after G-

![Fig. 3 Levels of elastase and lactoferrin](chart.png)

**A, B** Elastase (A) and lactoferrin (B) measured at different time points (see legend fig. 2). Values are mean ± SEM of 7 patients. **C** Elastase and lactoferrin measured in serial samples during the first infusion of rituximab. Timepoints are minutes after start infusion. Values are mean ± SEM of 5 patients. Asterisks indicate a significant difference (*: P<0.05, **: P<0.01) as compared to baseline value (A, B:-48; C: t=0).
CSF, lactoferrin levels further increased to 432 ± 100 ng/ml (t=72 hrs, NS) (fig. 3B). During the fourth week of treatment, lactoferrin levels were significantly increased when compared to baseline values (p<0.05 at all time points, fig. 3B). In two control patients, treated with rituximab (375 mg/m²) only, levels of elastase or lactoferrin did not change at these time points, indicating that the observed increases were due to G-CSF pretreatment.

Neutrophil activation upon rituximab infusion
To investigate whether neutrophils were additionally activated during infusion of rituximab we assessed kinetics of plasma levels of elastase and lactoferrin during the first infusion of the antibody in five patients. At the start of the infusion, levels were already slightly increased due to G-CSF pretreatment (see previous paragraph). Upon infusion of rituximab, elastase rose from 165 ± 38 ng/ml to a maximum of 301 ± 36 ng/ml at 180 minutes; lactoferrin rose from 254 ± 36 ng/ml at baseline to a maximum of 630 ± 172 at 90 minutes. At the end of the infusion, elastase and lactoferrin had almost returned to pre-infusion values (fig. 3C).

Response
Twenty patients have been treated with the highest dose of rituximab (375 mg/m²) in combination with G-CSF, 3 in the phase I and 17 in the phase II part of the study. Since one patient did not receive all G-CSF injections, 19 patients were evaluable for response (table 3). The response rate was 42% (95%CI 20%-67%) (40% on intent-to treat basis). The percentage of complete remissions (CR) was 26% (5/19) and partial remissions (PR) was 16% (3/19). In two patients, the complete remission could not be confirmed by bone marrow biopsy (due to patient refusal). The median duration of response has not been reached at a median follow-up of 23 months (table 4). Only one patient who achieved a CR relapsed (after 18 months remission duration), the other 4 patients are still in CR. The duration of partial remissions was shorter: 4, 10 and 4+-months respectively. In 4 (from 8) patients,

Table 3. Response to the combination of rituximab (375 mg/m²x4) and G-CSF

<table>
<thead>
<tr>
<th>response</th>
<th>n=19*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR**</td>
<td>5</td>
</tr>
<tr>
<td>PR</td>
<td>3</td>
</tr>
<tr>
<td>SD</td>
<td>7</td>
</tr>
<tr>
<td>PD</td>
<td>4</td>
</tr>
<tr>
<td>OR (CR+PR)</td>
<td>42% (8/19)</td>
</tr>
</tbody>
</table>

* of 20 patients treated, 19 patients were evaluable for efficacy.
** 2 CR’s are not confirmed by bone marrow biopsy
the duration of response to rituximab treatment was already considerably longer than the response to last systemic regimen (table 4).

Although the sample size is rather small, factors that have previously been reported to influence the response to rituximab treatment were analyzed in our patient group as well. Patients that achieved a CR or PR to their last prior chemotherapy regimen (n=11) seemed to have a better response to rituximab therapy than patients that did not respond to last prior therapy (n=8) (response rate 63% (7/11) vs 13% (1/8) respectively, P=0.059). The response rate in patients who had previously received only one systemic treatment (n=10) was similar to the response in those who had received >1 regimens (n=9) (response rate 50% (5/10) vs 33% (3/9) respectively, P=0.65). No difference in response rates were observed between patients with bulky disease (defined as lesions >7 cm) versus (vs) patients without bulky disease (P=0.17), patients with bone marrow infiltration vs without bone marrow infiltration (P=0.37), and patients with extranodal lesions vs without extranodal lesions (P=1.0). In previous studies, WF A lymphoma was found to be correlated with a lower response to rituximab treatment, as compared to WF B and/or WF C lymphoma. Among the 19 evaluable patients in the present study, there were 4 with WF A lymphoma (3 patients with immunocytoma (Kiel classification) and one with marginal zone lymphoma (Real classification)). These patients were all non responders.

### Table 4. Duration of response to rituximab treatment

<table>
<thead>
<tr>
<th>patient</th>
<th>response and duration of response* to rituximab**</th>
<th>last prior regimen***</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>PR 4 (6)</td>
<td>PR 27</td>
</tr>
<tr>
<td>12</td>
<td>CR 25+ (29+)</td>
<td>PR 2</td>
</tr>
<tr>
<td>14</td>
<td>CR 18 (24)</td>
<td>CR 13</td>
</tr>
<tr>
<td>15</td>
<td>CR 25+ (27+)</td>
<td>CR 3</td>
</tr>
<tr>
<td>16</td>
<td>CR 24+ (26+)</td>
<td>CR 12</td>
</tr>
<tr>
<td>17</td>
<td>PR 9 (12)</td>
<td>NC NA</td>
</tr>
<tr>
<td>23</td>
<td>CR 13+ (16+)</td>
<td>CR 47</td>
</tr>
<tr>
<td>28</td>
<td>PR 4+ (6+)</td>
<td>CR 26</td>
</tr>
</tbody>
</table>

Abbreviations: CR: complete remission; PR: partial remission; NC: no change; NA: not applicable. * in months ** the time to progression is given in parenthesis *** last systemic chemotherapy regimen
Discussion

In the present study, we evaluated safety and efficacy of the combination of rituximab and G-CSF in relapsed low-grade NHL patients.

Side effects of G-CSF were mild and consisted mainly of bone pain and flu-like symptoms, which is in line with previous studies on G-CSF administration. Side effects during rituximab-infusion were mild (toxicity grade 1-2) and included mainly fever and ‘allergic reactions’, consisting of sneezing, rhinitis and itching of the oropharynx. Thus, the combination therapy of rituximab and G-CSF was well tolerated and toxicity appeared to be similar to that reported for rituximab monotherapy. The response rate was 42% (95% CI 20%-67%), and comparable to the response rate achieved with rituximab monotherapy. Interestingly, the CR rate was high and the duration of responses was remarkably long. At a median follow up of 23 months, 5 (from 8) responders were still in remission, 3 of them for >24 months. Thus, the median time to progression has not been reached yet. Duration of CR appeared to be longer than of PR and, furthermore, in 4 (from 5) patients that achieved a CR the duration was already considerably longer than remission duration achieved on last previous systemic therapy. Thus, although the overall response rate was not increased, addition of G-CSF to rituximab may be beneficial by increasing response duration.

Still, results were not as favorable as hoped for. Theoretically, possible explanations might be: 1) neutrophils did not express FcγRI; 2) FcγRI-positive neutrophils were not capable of mediating CD20-dependent ADCC; 3) competition of rituximab with circulating monomeric IgG for the ligand binding site of FcγRI; 4) FcγRI-positive neutrophils did not reach tumor sites.

The expression of FcγRI on neutrophils was significantly increased after three injections of G-CSF (i.e. just prior to rituximab infusion) (fig. 2). Previously, the capacity of CD20 mAbs in mediating ADCC has been questioned. We found however, that chimeric CD20 mAbs were capable of inducing ADCC in B cells with G-CSF-primed neutrophils as effector cells, whereas unstimulated neutrophils could not induce CD20-dependent ADCC (van der Kolk et al, manuscript in preparation). Thus, as intended, G-CSF administration resulted in FcγRI-positive, cytotoxic neutrophils.

It has been suggested that circulating monomeric IgG inhibits the function of FcγRI in vivo. However, several observations challenge this suggestion. First, from a study by Heijnen et al it can be deduced, that although a competition between chimeric antibodies and endogenous IgG for FcγRI might exist, this can be overcome when adequate amounts of antibody are administered. Notably, concentrations of rituximab reached during treatment
are high, with maximum levels up to 660 μg/mL, easily exceeding the maximum concentration used in the described study.\textsuperscript{2,27} More important however is the observation that FcyRIII-deficient mice still develop autoimmune hemolytic anemia (AIHA) upon administration of monoclonal antibodies against red blood cell antigens in vivo. This AIHA was found to be mediated by FcyRI (and not FcyRII), indicating the functional activity of FcyRI in vivo.\textsuperscript{28,29} Taking these arguments together, we do not think that competition with circulating IgG for FcyRI-binding has hampered the clinical efficacy of rituximab in the present study.

In order to emigrate to lymphatic tissue or sites of inflammation, neutrophils need to be attracted (by chemoattractants, e.g. cytokines, histamine, C5a) and migratory capacity should be adequate. Though B-cells in lymph nodes were coated with rituximab (data not shown) as was also observed in previous studies\textsuperscript{30}, the degree of inflammation induced by rituximab at tumor sites may be insufficient to initiate neutrophil migration. Furthermore, compared to unstimulated neutrophils, in vivo G-CSF-stimulated neutrophils show certain differences that may influence the migratory capacity in vivo. First, expression of L-selectin (CD62L), an adhesion molecule playing an important role in the initial interaction between neutrophils and endothelial cells (rolling)\textsuperscript{31,33}, and found to be essential for leukocyte recruitment to lymphatic tissue and inflammatory sites\textsuperscript{34,37}, was shown to be downregulated after in vivo G-CSF-administration.\textsuperscript{38-41} Second, upon G-CSF administration we found a decrease in the expression of CD11b, a β2-integrin that plays an important role in the adhesion of neutrophils to endothelium (fig. 2).\textsuperscript{31,32} This was in contrast to previous reports in which upon in vivo G-CSF-administration an increase in CD11b was found\textsuperscript{42-44}, accompanied by an increase in adhesion of neutrophils to various cells and coatings.\textsuperscript{42,43} Although it is known that conformational changes in integrins may be more important for adhesion than the actual expression level\textsuperscript{45,46}, and G-CSF-stimulated neutrophils were found to have a normal capacity to upregulate CD11b in response to activating stimuli\textsuperscript{47}, the decrease in CD11b may still have influenced the migration of neutrophils in this study. Finally, the directed migration (chemotaxis) of in vivo G-CSF-stimulated neutrophils was found to be decreased.\textsuperscript{42,47,48}

In the present study, lymph node biopsies were taken one day after the last rituximab infusion in three patients. Although the lymphocytes were coated with rituximab, only few neutrophils were observed (data not shown). Thus, at least at the described time point, neutrophils did not migrate to tumor sites in large numbers. This might suggest that insufficient migration has hampered the beneficial effects of G-CSF. However, since only three biopsies were taken, we can not draw definite conclusions as to this possibility.

Respiratory side effects during treatment with monoclonal antibodies have been attributed to the accumulation of activated neutrophils in the lung vasculature.\textsuperscript{49} Interestingly, although our patients had very high numbers of neutrophils that subsequently became activated
during rituximab infusion (fig. 3C), only one patient showed respiratory problems. In analogy to the study by Pajkrt et al., G-CSF-induced downregulation of L-selectin may have prevented neutrophils from accumulation in the lungs. Thus, the same mechanism that theoretically might have decreased the beneficial effect of G-CSF may at the same time have protected patients for potential side effects.

Recently, results of a clinical trial evaluating the safety and efficacy of the combination of rituximab and interferon-α-2a (IFN-α) were published. IFN-α is known to stimulate the ability of NK-cells in mediating ADCC. Thus, combining rituximab with G-CSF or IFN-α is a similar approach intended to increase the efficacy of rituximab.

The overall response rate of the combination of rituximab and IFN-α was 45% (i.e. similar to rituximab monotherapy), but response duration appeared to be longer than for rituximab monotherapy. However, side effects observed during treatment with rituximab and IFN-α were more severe, including grade 3 adverse events in 12 patients. The majority of the side effects were attributed to IFN-α. Hence, although the potential beneficial effects of IFN-α and G-CSF on the response duration of rituximab may be similar, the safety profile of the combination with G-CSF is clearly more favorable.

In conclusion, the combination of G-CSF and rituximab was well tolerated in relapsed low grade NHL-patients. Although the response rate was comparable to that reported for rituximab monotherapy, CR rate was high and remission duration in this small sized phase II study was remarkably long. Randomized comparison with rituximab monotherapy should substantiate this promising finding.
Reference List

Safety and efficacy of the combination of rituximab and G-CSF


