Depletion of normal B cells with rituximab as an adjunct to IL-2 therapy for renal cell carcinoma and melanoma

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Background: We postulated that in patients with metastatic renal cell carcinoma (RCC) or melanoma, depletion of normal B cells using the anti-CD20 mAb rituximab before treatment with low-dose interleukin (IL)-2 would improve clinical outcome.

Patients and methods: Rituximab (375 mg/m²) weekly for 4 weeks. IL-2 [11 (million units) daily] s.c., 4 days a week for weeks 5–8, followed by a 2-week rest (weeks 9 and 10). Patients without disease progression continued on IL-2. Disease re-evaluation was performed after rituximab and after every course of IL-2.

Results: Fifteen patients with RCC and six with melanoma were enrolled. One patient had a partial response and seven patients had stable disease. Toxicities were similar to those expected with IL-2 alone, and there were no grade 4 events. Circulating B cells were depleted in all patients. The subsequent low-dose IL-2 increased absolute numbers of natural killer cells, activated CD4+ and activated CD8+ T cells. Expanded T cells produced interferon-γ, but not IL-4. Proliferation of peripheral blood lymphocytes to phytohemagglutinin was diminished following rituximab treatment, suggesting that B cells participate in this response in vitro.

Conclusions: Our results suggest that depletion of circulating B cells with rituximab does not increase the response rate, alter the toxicity profile or change the biological activity in response to low-dose IL-2 in patients with RCC or melanoma.

Key words: clinical trial, cytokines, immunoregulation, immunotherapy

Introduction

Interleukin (IL)-2 is a potent stimulator of T-cell and natural killer (NK) cell proliferation [1, 2] that has therapeutic efficacy in the treatment of renal cell carcinoma (RCC) and melanoma [3]. IL-2 has been administered as a continuous intravenous (i.v.) infusion, bolus i.v. infusion or subcutaneously (s.c.). Monotherapy with high-dose IL-2 in RCC and melanoma leads to response rates of ~15%, with durable complete responses seen in ~4% of patients, but with significant toxicity [4, 5]. A randomized phase III trial in RCC showed that low-dose intravenous IL-2 had similar response rates with an improved toxicity profile [6]. Low-dose subcutaneous IL-2 administered in an outpatient has shown similar response rates, with few side-effects [7]. However, disappointing overall response rates and long-term survival argue that improvements in the clinical response to IL-2 would be of benefit.

There is accumulating preclinical evidence that normal host B lymphocytes may interfere with T-cell-mediated tumor rejection. Brodt and colleagues reported that mice depleted of immunoglobulin (Ig)-bearing lymphocytes had increased antitumor immunity in vivo [8]. Schreiber and colleagues found that mice reconstituted to lack B cells showed slower tumor growth and higher rejection rates [9]. Qin et al. observed superior tumor rejection in B-cell-deficient mice compared with control mice [10]. Our laboratory has confirmed this notion using TCR transgenic T cells. 2C/RAG2−/− T cells that recognize the alloantigen Ld [11] were adoptively transferred into either RAG2-deficient mice (which lack T cells and B cells) or TCRα−/− knockout mice (which lack T cells but contain B cells). Upon subcutaneous challenge with Ld-expressing P1.HTR tumor cells [12], RAG2−/− but not TCRα−/− mice successfully rejected the tumor (Markiewicz and Gajewski, unpublished data). These observations support a negative regulatory role for B cells in countering T-cell-mediated tumor elimination.

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Previous studies in patients with lymphoma have shown that treatment with rituximab, in addition to causing regression of malignant CD20⁺ lymphomas, also results in a rapid and sustained depletion of circulating B cells, without significant toxicity [13–15]. These observations enabled the design of a phase II trial of B-cell depletion with rituximab in patients with RCC and melanoma before treatment with low-dose IL-2, in an attempt to eliminate the putative negative regulatory effect of B cells on T-cell-mediated tumor rejection.

Patients and methods

Protocol eligibility

Patients provided written informed consent to enroll in this trial, which was approved by the University of Chicago Institutional Review Board. Inclusion criteria included: histologically confirmed renal cell carcinoma or malignant melanoma with evidence of stage IV disease; unidimensional measurable disease; life expectancy of at least 12 weeks; and Eastern Cooperative Oncology Group (ECOG) performance status ≥2. Patients must have had adequate organ function, defined as absolute neutrophil count ≥ 1500/µl, hemoglobin ≥ 9 g/dl, platelets ≥ 100 000/µl, serum creatinine ≤ 1.5 times the upper limit of normal (ULN), aspartate aminotransferase ≤ 2 × ULN, bilirubin ≤ 2× ULN and calcium ≤ 11 mg/dl.

Exclusion criteria included: previous IL-2 therapy; use of chemotherapy, radiotherapy or biological therapy within 4 weeks before enrolment; concurrent systemic corticosteroids (except physiologic replacement doses) or other immunosuppressive medications; and clinically significant autoimmune disease. Patients with active infections including HIV, chronic active hepatitis, or known hepatitis B or C were excluded. Lactating or pregnant women were excluded and patients of reproductive age must have agreed to use an acceptable method of birth control.

Treatment

This was an open-label, non-randomized, single-institution study. Patients were treated in an outpatient basis. Treatment consisted of four, weekly doses of rituximab (375 mg/m² per dose, weeks 1–4), followed by IL-2 administration. IL-2 was given subcutaneously (11 × 10⁶ IU daily, 4 days a week) for 4 weeks (weeks 5–8), followed by a 2-week rest (weeks 9 and 10). If the total B-cell number recovered to 50% of pre-treatment numbers of T, B and NK cells, as well as of HLA-DR⁺ CD4⁺ and CD8⁺ T cells were analyzed by flow cytometry as described previously [16].

Immunologic monitoring

Immunologic assessments were performed before therapy, after the fourth dose of rituximab, and every 2 weeks while on IL-2. The clinical chemistry laboratory measured quantitative immunoglobulin levels. The numbers of T, B and NK cells, as well as of HLA-DR⁺ CD4⁺ and CD8⁺ T cells were analyzed by flow cytometry as described previously [16]. Numbers of CD4⁺ and CD8⁺ T cells capable of producing IL-2, IFN-γ and IL-4 were analyzed following stimulation with phorbol myristate acetate (PMA) + ionomycin and intracellular cytokine staining. Briefly, heparinized blood was centrifuged and plasma was removed. Cells were resuspended in RPMI-1640 medium with l-glutamine and monensin (2 µM). Half the sample was activated by the addition of PMA (40 ng/ml) and ionomycin (1.6 µg/ml); the other half was set aside as a control. Cells were incubated for 4 h at 37°C, stained with fluorescein isothiocyanate-conjugated anti-CD8 and PerCP-conjugated anti-CD3, and the red blood cells were lysed with fluorescence-activated cell sorter (FACS) Lysing Solution (BD Biosciences, San Jose, CA, USA). The remaining cells were washed, permeabilized and stained for intracellular cytokines using phycoerythrin-conjugated anti-IL-2, anti-IL-4 or anti-IFN-γ (BD Biosciences). Data were acquired using a FACSScan™ flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) and analyzed with FlowJo software® (Stanford University, 1995–1996; Tree Star, Inc., 1997–2002). The number of CD8⁺ T cells producing cytokine was determined after subtracting background staining.

Ex vivo proliferation in response to phytohemagglutinin (PHA) was measured in batch fashion on cryopreserved peripheral blood mononuclear cells (PBMCs). PBMCs were isolated using Lymphoprep™ (Axis-Shield, Oslo, Norway) and cryopreserved. For each patient, cryopreserved PBMCs from all time points were thawed and resuspended in RPMI-1640 medium supplemented with penicillin/streptomycin, l-glutamine and 10% fetal bovine serum. PBMCs (4 × 10⁶) were plated in triplicate on 96-well plates with PHA (Sigma, St Louis, MO, USA) at 5 µg/ml. After 72 h incubation at 37°C, the cells were pulsed with [³H]thymidine (1 µCi/well). After another 18 h, the cells were harvested using a Filtermate™ harvester and incorporated [³H]thymidine was detected using a TopCount® NXT™ microplate scintillation and luminescence counter (Packard BioScience, Meriden, CT, USA).

Statistical considerations

Previous studies had documented response rates of ~15% with IL-2 in melanoma and RCC. A total of 15 melanoma patients and 15 renal cell carcinoma patients were intended to be treated. If two or more of the 15 patients in either group responded, the regimen would be considered active, warranting further study in that disease. However, when only a single clinical response was seen in the first total of 21 patients, the study was closed to accrual. Estimates of time to progression and survival were determined using the Kaplan–Meier method. Immunologic parameters were characterized with descriptive statistics.

Results

Patient demographics

From August 2000 to April 2002, 15 RCC and six melanoma patients were enrolled. Patient characteristics are summarized in Table 1. The majority of patients had received no previous therapy for metastatic disease. All patients were evaluable for response and toxicity. Three patients with RCC were lost to follow-up after they were removed from the study.
Patient treatment and clinical response

All patients received the planned four doses of rituximab and re-dosing was not required for subsequent cycles for any patient. The 21 patients received a total of 48 courses of therapy; the median number of treatment courses was two (range 1–4).

No objective responses were observed after treatment with rituximab alone. Among the 21 evaluable patients, there was one partial response following IL-2 administration in a patient with RCC. That response remained durable for at least 28 months with no further therapy. Seven patients with RCC and two patients with melanoma had stable disease.

The median time to progression was 15.4 weeks (range 3.3–115.7 weeks) and the median overall survival was 77 weeks (range 14.9–135 weeks). The median duration of stable disease was 26.7 weeks (range 8.9–71 weeks) (data not shown).

Adverse events

Table 2 lists the observed adverse events. The majority of adverse events were grade 1 or 2, and there were no grade 4 toxicities. Anemia was the most common hematologic toxicity and grade 2 neutropenia was observed in two patients (10%). The most common non-hematological toxicity was grade 1–2 fever and/or chills. Other common toxicities were fatigue, anorexia and injection-site reactions. None of the 21 patients developed opportunistic infections. The observed toxicities were similar to those seen in other clinical trials using similar doses of IL-2. No significant toxicities were observed after treatment with rituximab.

Immunologic monitoring

Flow cytometry on peripheral blood was used to quantitate the number of CD19+ cells. After four weekly doses of rituximab, durable B-cell depletion was achieved in all patients (Figure 1).

![Figure 1](image-url)
Serum immunoglobulin levels (IgG, IgM, IgA) were not affected by B-cell depletion with rituximab (data not shown). T-cell subsets and NK cells were enumerated to determine if IL-2 successfully expanded these populations, despite the absence of circulating B cells. Treatment with rituximab did not alter numbers of T or NK cells (Figure 2). Following administration of low-dose IL-2, the number of CD3+/CD8+ and CD3+/CD8− (CD4+) cells increased as expected, returning near pretreatment values when the IL-2 was discontinued (Figure 2A). A similar pattern of expansion was observed

![Figure 2](image1.png)

**Figure 2.** Expansion of activated T cells and natural killer (NK) cells by IL-2, despite B-cell depletion. (A) The absolute numbers of activated T-cell subsets detected in the peripheral blood were determined by flow cytometry at the indicated time points. CD3−CD8−HLA-DR+ cells (black circles), CD3−CD8−HLA-DR+ (gray squares), and total CD3+HLA-DR+ (inverted triangles) are shown. The CD8− cells represent predominantly CD4+ cells. (B) The numbers of circulating NK-cell subsets were determined at the indicated time points by flow cytometry. CD56−CD3+ cells (black circles), CD56−CD3+ cells (gray squares) and total CD56+ cells (inverted triangles) are shown. The CD56−CD3+ cells likely represent NKT-lineage cells. Error bars represent standard deviations.

![Figure 3](image2.png)

**Figure 3.** A predominant Th1 cytokine phenotype is preserved following B-cell depletion. The cytokine-producing phenotype of circulating T cells was assessed at the indicated time points. The absolute number of cells producing each cytokine in response to PMA+ionomycin was determined. The number of cells producing interferon-γ (IFN-γ) (A), interleukin-2 (IL-2) (B) and IL-4 (C) is shown. Error bars represent standard deviations.
Figure 4. Diminished phytohemagglutinin (PHA)-stimulated proliferation following rituximab treatment. Cryopreserved peripheral blood mononuclear cells were thawed in batch fashion for each time point for each patient, and stimulated with PHA in vitro. Thymidine incorporation was assessed, and a mean and standard deviation for all patients was calculated and is shown. Error bars represent standard deviations.

with CD56+ cells (Figure 2B). The magnitude of expansion is similar to what has been seen in previous studies using IL-2 alone [16]. Thus, depletion of B cells did not appear to result in greater IL-2-induced expansion of T cells or NK cells.

As antigen presentation by B cells may preferentially activate Th2 cells [17], it was conceivable that the cytokine-producing profile of T cells would change following B-cell depletion with rituximab. This was examined by intracellular flow cytometry on cells stimulated with PMA + ionomycin. As shown in Figure 3, preferential expansion of cells producing IFN-γ and IL-2 was observed both before and after rituximab. Thus, B-cell depletion with rituximab did not alter the cytokine-producing phenotype of activated T cells analyzed ex vivo.

In vitro stimulation of peripheral blood lymphocytes with PHA is often used as a general indicator of T-cell responsiveness. However, analysis of PHA-driven proliferation in the present study was diminished in samples obtained after treatment with rituximab (Figure 4). These results suggest that B cells likely participate as accessory cells in vitro to support T-cell proliferation in response to PHA.

Discussion

In the present study, we pursued the hypothesis that depletion of normal B cells with rituximab would improve the response rate to IL-2 in patients with RCC and melanoma. As expected, rituximab depleted circulating B cells; yet, no objective responses were observed with B-cell depletion alone. Furthermore, the toxicity profile, expansion of T/NK cells and response rate of the combination of rituximab and IL-2 were similar to what has been reported with low-dose IL-2 alone. The cytokine profile of the majority of T cells indicated that a global Th1 phenotype was preserved and was not augmented, despite circulating B-cell depletion.

Although multiple experimental preclinical models were encouraging, our clinical trial did not show any benefit of B-cell depletion with rituximab as an adjunct to IL-2 therapy. Rituximab is directed against the CD20 antigen expressed by normal pre-B- and mature B-lymphocytes. CD20 is not found on the surface of plasma cells, and whether rituximab effectively depletes tissue-based B cells is unclear. The lack of diminution in circulating Ig levels suggests that incomplete depletion of B-lineage cells is achieved with rituximab. The potentiation of antitumor immunity noted in murine models by the absence of B cells may be mediated by a feature of B-lineage cells not negated by rituximab.

Several models have been proposed to explain the inhibitory effect of B cells on antitumor immunity in murine studies. These include regulation by antigen–antibody complexes [18], linkage of specific IgG to latent transforming growth factor-β (TGF-β) [19], secretion of IL-10 and consequent inhibition of Th1 responses [20], and Th2 promotion through antigen presentation by B cells [17]. Of note, B-cell depletion with rituximab did not alter the predominant Th1 cytokine profile seen, arguing against the latter mechanism.

Although the use of rituximab to deplete B cells did not augment clinical activity in response to IL-2, it could theoretically potentiate responses to other immunotherapeutic interventions, such as vaccines or adoptive T-cell therapy. B-cell depletion might favor antigen presentation by dendritic cells, and also could facilitate homeostatic proliferation of transferred T cells. Future studies will be required to explore these possibilities.

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