

Featured Article

Neutrophils Contribute to the Biological Antitumor Activity of Rituximab in a Non-Hodgkin's Lymphoma Severe Combined Immunodeficiency Mouse Model

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Abstract

Purpose: Rituximab is a chimeric antibody (Ab) directed against the cluster designated (CD) 20 antigen found on normal and malignant B cells. Rituximab activity has been associated with complement-mediated cytotoxicity, Ab-dependent cellular cytotoxicity (ADCC), and induction of apoptosis. Recent studies performed in severe combined immunodeficiency (SCID) mouse models suggest that *in vivo* rituximab-associated ADCC is mediated via the Fc γ RIII receptor on effector cells. Despite low level expression of Fc γ RIII, neutrophils are also known to induce ADCC primarily via Fc γ RI receptor (CD64). The purpose of this work was to study the effect(s) of neutrophils on the *in vivo* antitumor activity of rituximab.

Experimental Design: To better characterize the biological activity of rituximab, we used a human non-Hodgkin's lymphoma animal model by injecting Raji cells *i.v.* into natural killer (NK) cell-depleted SCID mice. Disseminated disease involving liver, lung, and central nervous system developed, with subsequent death occurring approximately 3 weeks after tumor inoculation. Specifically, 6–8-week-old NK cell-depleted SCID mice were inoculated by tail vein injection with 1×10^6 Raji cells on day 0. The animals then were divided into three cohorts: (a) group A received placebo (PBS); (b) group B received rituximab administered via tail vein injection at 10 mg/kg on days 3, 5, 7, and 11; and (c) group C consisted of neutrophil-depleted SCID mice treated with rituximab at 10 mg/kg on the same schedule. Neutrophils were depleted by *i.p.* administration of 80 μ g of rat antimouse Ly-6G (Gr-1) Ab (BD PharMingen, Inc.) on days –1, 4, 9, and 14. The end point of the study was survival. Differences in outcome between treatment groups were analyzed by Kaplan-Meier methodology.

Results: Neutrophil- and NK cell-depleted SCID mice (group C) did not respond to rituximab, and the mean survival time was not significantly different from that of control mice. NK cell-depleted SCID mice with intact neutrophil function (group B) responded to rituximab, and 66% remained alive and appeared healthy after a mean follow-up period of 246 days. Overall, NK cell-depleted SCID mice with intact neutrophil function treated with rituximab had statistically longer mean survival as compared with mice in neutrophil-depleted and control groups (161 days *versus* 28 days *versus* 22 days, $P = 0.003$).

Conclusions: In the absence of neutrophils, rituximab was less effective in controlling lymphoma cell growth or prolonging survival in our B-cell lymphoma SCID mouse model. Neutrophil-induced ADCC appears to contribute to the *in vivo* antitumor activity of rituximab. Strategies that improve the function of neutrophils, such as granulocyte-macrophage colony-stimulating factor or G-CSF priming, may increase the antitumor effects of rituximab. Additional *in vivo* animal studies are warranted.

Introduction

The concept of using mAbs³ to treat cancer gained increasing popularity after the discovery of hybridoma technology in the 1970s (1). Initial clinical studies were disappointing, due to the observation of limited antitumor activity. Several factors contributed to such dismal results: (a) suboptimal antigen selection (*i.e.*, modulation of the antigen-Ab complex or highly shed target antigen); (b) mAbs used had ineffective *in vivo* biological activity (*i.e.*, ADCC, cCMC, direct apoptosis); and (c) development of human antimouse Abs by the host against murine protein (2).

Advances in molecular biotechnology and tumor immunology led to the development of chimeric and humanized mAbs with a longer half-life and a lesser degree of immunogenicity (3). Recent clinical trials testing newer mAbs have confirmed their improved *in vivo* antitumor activity (4).

Rituximab is an IgG κ chimeric mAb directed against the CD20 antigen that is present on normal B cells as well as the majority of NHLs (5). Clinical antitumor activity has been demonstrated in patients treated with rituximab as a single agent in Phase II and III studies (6–8). Furthermore, rituximab was

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³ The abbreviations used are: mAb, monoclonal antibody; SCID, severe combined immunodeficiency; CMC, complement-mediated cytotoxicity; ADCC, antibody-dependent cellular cytotoxicity; NHL, non-Hodgkin's lymphoma; NK, natural killer; G-CSF, granulocyte colony-stimulating factor; FcR, Fc receptor; PMN, polymorphonuclear cell; IL, interleukin; RPCI, Roswell Park Cancer Institute; CD, cluster designated; FSC, forward scatter; SSC, side scatter.

the first mAb to be approved by the United States Food and Drug Administration to treat patients with cancer (9).

Strategies to improve the antitumor effects of rituximab have been evaluated. For example, the combination of standard doses of chemotherapy regimens and rituximab has been studied in patients with different subtypes of NHL (10, 11). Despite these promising results, not all of the patients respond to or relapse after rituximab alone or in combination immunotherapy. Approximately 50% of indolent NHL patients treated with single-agent rituximab fail to demonstrate an objective antitumor response [partial response, complete response (12)]. Furthermore, retreatment with rituximab at the time of relapse resulted in an overall response rate of 40%. Several mechanisms for tumor resistance to mAb therapy have been proposed. NHL-related factors postulated are: changes in CD20 antigen density expression; induction of complement-inhibitory protein expression by NHL cells; and high tumor burden (13–16). On the other hand, host-related factors such as pharmacokinetics and pharmacogenomics may play significant roles in patients that do not respond to rituximab (17–19).

The exact mechanisms involved in the *in vivo* antitumor activity of rituximab have not been completely elucidated. *In vitro* studies conducted primarily on EBV-transformed NHL cell lines suggest that rituximab induces antitumor activity by (a) CMC, (b) ADCC, and, to a lesser degree, (c) induction of direct apoptosis by a poorly defined signaling pathway (20–24).

Recently published *in vivo* studies have demonstrated that Fc γ receptor expression is necessary to eradicate NHL in a murine animal model, suggesting that ADCC plays a significant role in the activity of rituximab (25). Furthermore, polymorphisms in the Fc γ RIIIa gene have been associated with differences in clinical responsiveness to rituximab therapy in patients with indolent NHL (26).

FcRs mediate many of the cell-dependent functions of Abs, including phagocytosis of Ab-bound antigens, activation of mast cells, complement activation, and targeting/activation of NK cells. There are three types of FcRs and eight subtypes. Fc γ RI or CD64 (high affinity) mediates phagocytosis by macrophages and PMNs. Fc γ RIIb or CD32 (low affinity) transduces inhibitory signals in B cells. Finally, Fc γ RIIIa (CD16) is another low affinity receptor that mediates the activation of NK cells to induce ADCC (27).

Additional contributions to antitumor activity by neutrophils in tumor immunology may be underestimated. There is emerging evidence that PMNs are capable not only of migrating to and infiltrating cancerous tissues but also of inducing antitumor activity (28).

PMNs induce tumor destruction by several mechanisms. Tumor-recruited neutrophils produce several cytotoxic mediators such as reactive oxygen species, proteases, membrane-perforating agents, and soluble mediators of cell killing [tumor necrosis factor α , IL- β 1, and IFNs (29–33)]. A second mechanism of neutrophil-mediated antitumor activity is ADCC. PMNs express several subtypes of FcRs capable of inducing ADCC [Fc γ RIIIa, Fc γ RIIIa, and Fc γ RIIIb (34, 35)]. *In vitro* studies have demonstrated that granulocyte-macrophage colony-stimulating factor augments the normal PMN-mediated ADCC against melanoma and colon cancer cell lines (36). The interaction between neutrophils and rituximab against B-cell malignancies and its potential in augmenting mAb-associated antitumor activity deserve further evaluation.

In this report, we present data obtained from our SCID mouse model, which demonstrates that PMNs contribute significantly to the antitumor activity of rituximab.

Materials and Methods

Cell Lines. The Raji cell line is a well-characterized B lymphoblastic cell line (phenotype, CD20+/CD19+/CD22+) derived from a patient with Burkitt's lymphoma (obtained from the American Type Culture Collection, Manassas, VA). The DHL-4 cell line (a gift from Dr. Steven Treon; Dana-Farber Cancer Institute, Boston, MA) is a CD20+ B-cell transformed NHL known to be sensitive to complement lysis mediated by rituximab. The Raji and DHL-4 cells were cultured and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 5 mM HEPES, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The cultures were free of *Mycoplasma* and pathogenic murine viruses.

Animals. For the experiments with rituximab, 6–8-week-old SCID mice were bred and maintained at the Department of Laboratory Animal Resources facility at RPCI. Older SCID mice (12 weeks of age) were used for the production of the IL-2 receptor Ab (see "Abs").

The experiment design was approved by the Institutional Animal Care and Use Committee at RPCI under Protocol M821. All animals were housed and maintained in laminar flow cabinets or microisolator units and provided with sterilized food and water. Our laboratory facilities are certified by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulation and standards of the United States Department of Agriculture and United States Department of Health and Human Services.

Abs. Human antimouse IL-2 receptor mAb was expanded in SCID mice. SCID mice (10–12 weeks old) were inoculated with 1×10^7 TM β 1 cells via i.p. injection. Ascites fluid was collected after 2 weeks and sterilized by ultrafiltration. Individual 100- μ l i.p. injections of sterile ascites containing anti-IL-2 receptor Ab induced NK cell inactivation before NHL tumor inoculation in SCID mice. The Ab produced by the TM β 1 cells recognizes the α chain of the IL-2 receptor. *In vivo* depletion of NK cells was confirmed by flow cytometric analysis of peripheral blood using a FITC-labeled rat antimouse DX5 Ab obtained from BD PharMingen, Inc. (San Diego, CA; data not shown).

The rat antimouse Gr-1 mAb (anti-Gr-1 Ab; BD PharMingen, Inc.) was used to deplete murine neutrophils. Flow cytometric studies with FITC-conjugated rat antimouse Gr-1 (BD PharMingen, Inc.) documented elimination of circulating PMNs. Gr-1 antigen is a specific marker for granulocytes and has been demonstrated to be present in neutrophils, eosinophils, and immature monocytes but is not expressed in mature monocytes.

Rituximab (IDEC/Genentech Inc., San Francisco, CA) was obtained from the RPCI Pharmacy Department at a stock concentration of 10 mg/ml. The Ab was dosed at 10 mg/kg and diluted in sterile PBS (200 μ g/100 μ l) for tail vein injection into

SCID mice. For *in vitro* testing, rituximab was used at a final dose of 10 µg/ml.

Immunophenotyping. Characterization of the phenotypic profile of the Raji cell line was performed with a fluorescence-activated cell sorter using a FACStar Plus (Becton Dickinson, San Jose, CA) flow cytometer. B-cell CD antigen phenotype was determined by direct immunofluorescence using several mAbs. Purified phycoerythrin-conjugated mouse antihuman CD19 and CD22 mAbs were obtained from Caltag (Burlingame, CA). Phycoerythrin-conjugated mouse antihuman CD20 and CD59 as well as Cy-conjugated mouse antihuman CD55 mAbs were purchased from BD Pharmingen, Inc.

Development of a Lymphoma Xenograft Mouse Model. To generate tumor, Raji cells were harvested from confluent cultures. Only cell suspensions with >90% viability were used for animal inoculation. Initial studies were performed to determine the most optimal and physiological route of inoculation. Twenty-four h before tumor implantation, murine NK cells were depleted by treating animals with 100 µl of ascites containing IL-2 receptor Ab. Subsequently, SCID mice were divided in three cohorts and received 1×10^6 Raji cells via i.v., s.c., or i.p. injections. A fourth group of healthy mice was used as control. The four groups of animals were observed daily for signs of disease (*i.e.*, tumor formation, ascites, or respiratory distress). On development of signs of distress or a tumor ≥ 20 mm in diameter, animals were killed by cervical dislocation, and pathological examination of involved organs confirmed the presence of lymphoma.

Immunohistochemistry of Tissue Organs from SCID Mice. Tissue and organs obtained (s.c. lung, liver, and brain lesions) from sacrificed tumor-bearing SCID mice were submitted for pathological examination. Unstained paraffin-embedded tissue sections were used for detection of CD20 antigen by immunohistochemistry. Sections (4–6-µm thick) were deparaffinized by incubation at 60°C for 1 h followed by immersion in xylene. Slides were then treated with serial dilutions of alcohol (100%, 90%, and 70% ethanol/distilled water) and rehydrated in PBS. No antigen retrieval was necessary. All samples were incubated with 3% hydrogen peroxide for 30 min to block endogenous peroxidase. Protein blocking was performed using horse serum for 20 min, followed by a 30-min incubation with mouse antihuman CD20 (DAKO, Carpinteria, CA) at a 1:50 dilution (stock, 200 µg/ml). A mouse antihuman cytokeratin (DAKO) at a 1:500 dilution (stock, 9.5 mg/ml) served as a negative control.

Sections were then incubated for 30 min with the corresponding horse antimouse biotinylated secondary mAb [dilution, 1:250 (v/v)] at room temperature. Positive reactions were visualized using the DAKO LSAB^{R2} System (DAKO). The slides were rinsed with distilled water, counterstained with hematoxylin for 1 min, and mounted with Universal Mount.

In Vivo Depletion of Neutrophils. PNMs were depleted from SCID mice using the anti-Gr-1 Ab. To determine the lowest effective dose, 8-week-old SCID mice were treated at single doses of 0, 80, or 100 µg of anti-Gr-1 administered via i.p. injection. Subsequently, blood samples were obtained on days 1, 3, and 5 from the retroorbital venous plexus. Fifty µl of heparinized mouse blood were incubated on ice with 0.4 µg of anti-CD16/CD32 (FcγIII/IIIR; BD PharMingen, Inc.) as a block-

ing step, followed by incubation on ice with 0.4 µg of FITC-conjugated anti-Gr-1 (BD PharMingen, Inc.). RBCs in the samples were then lysed with two rounds of $1 \times$ ack (15 mM NH₄Cl, 0.1 mM KHCO₃, and 0.01 mM Na₂EDTA), and all samples were washed before fixing in $1 \times$ PBS containing 2% paraformaldehyde. Samples were then run on a FACScan (Becton Dickinson) flow cytometer.

In addition, flow cytometric studies using FSC *versus* SSC profiles were performed from collected blood to study the effects of Gr-1 Ab on other circulating cell lineages such as monocytes and lymphocytes (*i.e.*, NK cells).

Characterization of Neutrophil Function on the Antitumor Activity of Rituximab. SCID mice (6–8 weeks old) were depleted of NK cells as described previously. The animals were divided into two cohorts; the first group (PMN-depleted group) received four i.p. injections of rat antimouse Gr-1 mAb (80 µg/dose) on days -1, 4, 9, and 14. The second group (PMN-intact group) received placebo i.p. injections. On day 0, 1×10^6 Raji cells were inoculated via tail vein injection. Animals were observed to note differences in tumor growth patterns between the two groups.

In the second set of experiments, three groups of NK cell-depleted SCID mice were inoculated with 1×10^6 Raji cells on day 0 (groups A1, B1, and C1). PMNs were depleted as described above in two groups of mice (groups A1 and B1). Subsequently, animals received either placebo (group A1) or rituximab (groups B1 and C1) for four doses of 10 mg/kg/dose administered via tail vein on days 3, 5, 7, and 11. Animals were observed daily for the development of limb paralysis, weight loss, and respiratory distress and sacrificed immediately if noted. The end point of the study was survival (*i.e.*, development of symptomatic visceral and/or central nervous system disease).

To better define the degree of importance that neutrophils may have on rituximab biological antitumor activity, we decided to design a third set of experiments in which NK cells were not depleted in our murine model. SCID mice (6–8 weeks old) with intact NK cells were divided into four cohorts (groups A2, B2, C2, and D2). Neutrophils were depleted in groups A2 and C2 as described above. Rituximab was administered i.v. to animals in groups C2 and D2 at a dose of 10 mg/kg/dose on days 3, 5, 7, and 11. The other groups of animals received placebo (groups A2 and B2). The end point of the study was survival. The experiments were repeated on three different occasions, and the results shown in Figs. 4 and 5 are representative of the cumulative results.

In Vitro Testing to Assess the Capacity of Rituximab to Induce Complement-Mediated Lysis Using Murine Versus Human Serum. Standard functional assays were performed in the complement-sensitive DHL-4 cell line to determine whether murine serum was capable of inducing cell lysis in the presence of rituximab. NHL cells (1×10^7) were labeled for 2 h at 37°C with 3.7 MBq of ⁵¹Cr (100 µCi). The radioactive excess was washed out three times in PBS, and the tumor cells were resuspended at a final concentration of 1×10^6 cells/ml in RPMI-10 media.

From the initial tumor cell suspension, 100-µl aliquots (1×10^5 cells/well) were placed in 96-well plates. Subsequently, NHL cells were treated with rituximab (at a final

concentration of 10 $\mu\text{g/ml}$, RPMI-10 media (background control), or 10 $\mu\text{g/ml}$ Trastuzumab (isotype control) in combination with human, SCID mouse, or BALB/c mouse serum (complement source) at a final dilution of 1:4. Human serum collected from healthy donors was obtained under protocol CIC 01-16, approved by the RPCI Institutional Review Board. The final volume per well was 200 μl . Treated cells were incubated at 37°C, 5% CO_2 for 6 h. A separate set of ^{51}Cr -labeled DHL-4 cells (1×10^5 cells/well) was incubated in RPMI-10 media and then treated with 50 μl of a 1% Triton solution to determine maximum chromium release. Finally, the 96-well plates were centrifuged at 1400 rpm ($300 \times g$) for 5 min at 4°C, the supernatant of each well was collected individually, and γ emission was measured by the Packard Auto-Gamma Cobra II series counting system (IBM). The percentage of specific ^{51}Cr release (lysis) was calculated using the standard formula: % lysis = [(test sample release – background release)/(maximum release – background release)] \times 100. All samples were run in triplicate.

Statistical Analysis. Differences in survival between treatment groups were calculated using Kaplan-Meier curves (SPSS 11.0 for windows 2000 software). *P*s were calculated by log-rank and Breslow tests.

Results

Patterns of Tumor Growth in the SCID Mouse Model Differ According to Inoculation Route. *In vivo* tumor growth of the Raji cell lines in SCID mice differs significantly depending on the route of inoculation. Localized tumor nodules developed 3 weeks after s.c. tumor inoculation. Histological examination demonstrated the presence of CD20+ lymphoma cells and areas of central necrosis associated with inadequate blood supply to tumor. Necropsy of the entire animal failed to demonstrate the presence of systemic or disseminated disease. Inoculation of lymphoma cells via i.p. injection also failed to develop systemic tumors.

Inoculation of Raji cells via tail vein injection lead to a more “natural” (*i.e.*, nonlocalized disease, as more typically seen in the human host) development of systemic disease. Seventeen days after initial inoculation, untreated animals developed systemic metastasis in lungs, liver, and central nervous systems. Those animals exhibiting clinical signs of disease (*e.g.*, an increase in baseline respiratory rate and/or lower limb paralysis) were sacrificed. Histological examination demonstrated the presence of lymphomatous nodules in different organs such as brain, lungs, liver, spleen, and kidneys. Macroscopically, the lungs of these mice were filled with multiple tumor nodules. Mean lung weight was significantly heavier in mice inoculated via tail vein injection than in healthy animals (control) or in mice inoculated via s.c. or i.p. injection. Immunohistochemistry revealed that lymphoma cells expressed CD20 antigen similar to that expressed on parental cells (data not shown).

Rat Antimouse Gr-1 mAb Efficiently Depletes Neutrophils from Peripheral Blood in SCID Mice. Treatment of 6–8-week old SCID mice with a single i.p. dose of either 80 or 100 μg of rat antimouse Gr-1 mAb resulted in dramatic depletion of murine Gr-1+ granulocytes, predominantly PMNs (Figs. 1 and 2). The Gr-1 Ab specifically depleted granulocytes. On

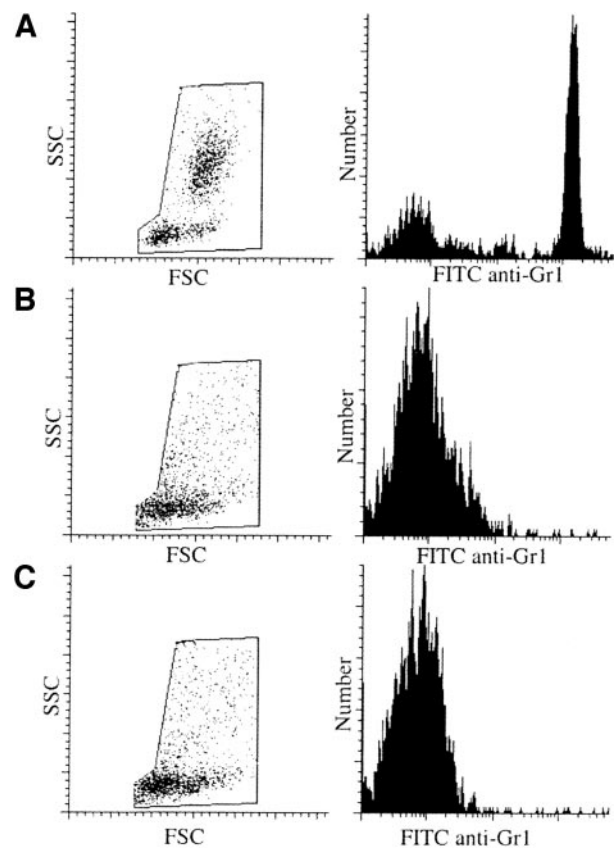


Fig. 1 Treatment of SCID mice with 80 (B) and 100 μg (C) of anti-Gr-1 mAb i.p. resulted in complete depletion of peripheral Gr-1+ granulocytes within 24 h compared with untreated control animals (A). Total peripheral blood leukocytes are analyzed for granulocytes using FSC versus SSC profiles on the left and for Gr-1+ cells on the right.

the other hand, circulating lymphocytes and monocytes were not affected by Gr-1 Ab administration. However, as the percentage of neutrophils decreased after Gr-1 Ab dosing, the relative percentage of lymphocytes (*i.e.*, NK cells) and monocytes increased (Fig. 2).

There were no statistically meaningful differences between the two doses of Gr-1 mAb tested. The effect of the Ab on peripheral neutrophils was observed as early as 24 h after administration and lasted for up to 4 days. A small population of Gr-1+ cells began to be seen in the peripheral blood at day 5 (Figs. 2 and 3). Based on these results, administration of rat antimouse Gr-1 mAb was scheduled every 5 days for 25 days in our experiments.

Depletion of Both Neutrophils and NK Cells Results in a Complete Loss of Rituximab Antitumor Effects *in Vivo*. Among untreated NHL-bearing SCID mice (NK cell depleted), tumor growth and survival (time to development of limb paralysis) did not differ significantly between neutropenic mice and nonneutropenic mice in the placebo group (data not shown). The median survival time for NHL-bearing neutropenic mice was 20 days versus 22 days for mice with intact neutrophil function (*P* = nonsignificant).

Depletion of neutrophils with rat antimouse Gr-1 mAb

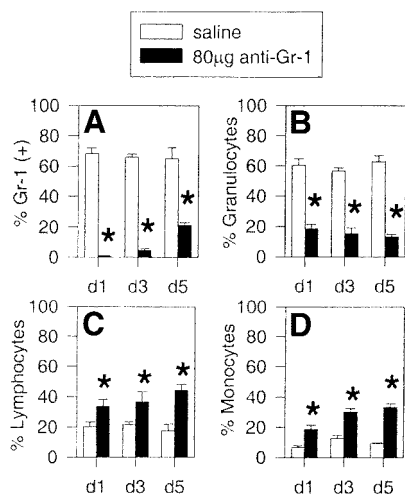


Fig. 2 Treatment of SCID mice with rat antimouse Gr-1 mAb preferentially depleted granulocytes for up to 5 days. The percentages of Gr-1+ cells (A), granulocytes (B), lymphocytes (C), and monocytes (D) were determined within the total peripheral blood leukocyte population of SCID mice 1, 3, and 5 days after a treatment with 80 µg of rat anti-Gr-1 mAb i.p. Granulocyte, lymphocyte, and monocyte populations were determined based on their FSC versus SSC properties by flow cytometry. *, $P < 0.02$ when comparing saline-treated and anti-Gr-1-treated groups ($n \geq 5$ for each group) at each time point using unpaired Student's t test.

before rituximab therapy significantly impaired the outcome of NHL-bearing SCID mice (Fig. 4). The median survival for neutrophil-depleted mice treated with rituximab (group B1) was similar to that of untreated animals (group A1; 28 versus 22 days). On the other hand, NK cell-depleted, nonneutropenic mice treated with rituximab (group C1) had the longest median survival (155 days), when compared with the two other groups described ($P = 0.003$).

Depletion of Neutrophils Alone Results in a Partial Loss of the Antitumor Effects of Rituximab *in Vivo*. In an attempt to determine the degree of antitumor activity that neutrophils contribute to rituximab therapy, we conducted studies similar to those described previously in non-NK cell-depleted mice. Once again, no differences in survival were noticed between tumor-bearing mice with or without intact neutrophils in the placebo groups. The median survival for animals in group A2 (untreated, neutrophil-depleted mice) was 21 days versus 23 days in group B2 (untreated, with intact neutrophils; $P =$ nonsignificant). Rituximab-treated animals have a longer overall survival when compared with controls. Depletion of neutrophils resulted in a partial loss of the antitumor effects of rituximab (Fig. 5) in NK cell-intact animals. Neutropenic mice treated with rituximab (group C2) had a shorter survival as compared with similarly treated nonneutropenic animals (group D2). The median survival for SCID mice within group C2 was 97 days versus 180 days for animals in group D2 ($P = 0.0001$).

Contribution of CMC to Rituximab-Associated Antitumor Activity. *In vitro* CMC assays using the complement-sensitive DHL-4 cells and serum [as a source of complement from SCID mice (immunodeficient), BALB/c mice (immunocompetent), and human donors] were performed. Whereas sig-

nificant CMC activity against DHL-4 cells was seen using human serum, no significant CMC was demonstrated using either mouse sera (Fig. 6).

Discussion

Our preclinical *in vivo* model has attempted to simulate the clinical behavior of disseminated human lymphoma by inocu-

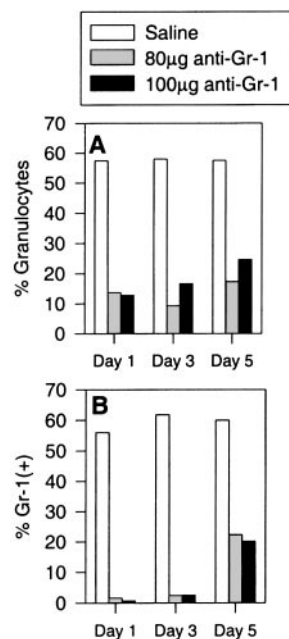


Fig. 3 Treatment of SCID mice with 80 and 100 µg of anti-Gr-1 mAb i.p. resulted in complete depletion of peripheral Gr-1+ granulocytes within 24 h compared with untreated control animals. Total peripheral blood leukocytes are analyzed for granulocytes using FSC versus SSC profiles in A and for Gr-1+ cells in B.

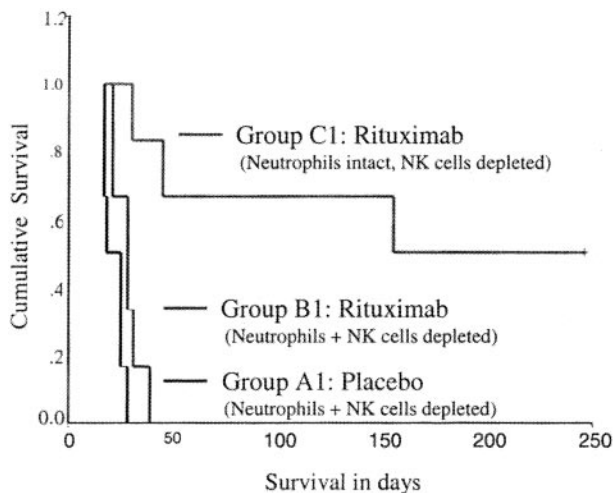


Fig. 4 Kaplan-Meier survival curves demonstrate that neutrophil depletion dramatically reduces the efficacy of rituximab in the NK cell-depleted SCID mouse model ($P = 0.003$).

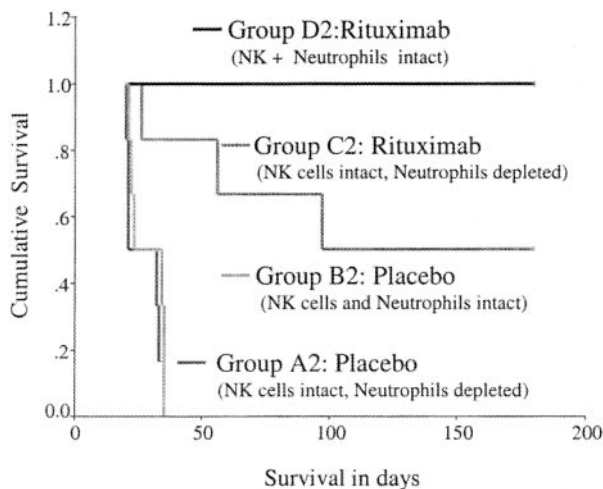


Fig. 5 Kaplan-Meier survival curves demonstrate that neutrophil depletion dramatically reduces the efficacy of rituximab in the non-NK cell-depleted SCID mouse model ($P = 0.0001$).

lating Raji lymphoma cell lines into SCID mice via tail vein injection. The development of systemic disease was reproducible in all experiments described above. The antitumor activity of rituximab observed in NHL-bearing SCID mice was directly related to the amount and type of immune effector cells present in a given animal group. Our data strongly suggest that Gr-1+ cells significantly contribute to the *in vivo* biological efficacy of rituximab.

In our murine system, Gr-1+ cells represent predominantly neutrophils. The Gr-1 Ab recognizes primarily the Ly6-G antigen and, to a lesser degree, Ly6-C (37). The expression of Ly6-G antigen is restricted to granulocytes (neutrophils and eosinophils; Ref. 37). In addition, cross-reactivity to the Gr-1 Ab has been demonstrated recently in a subpopulation of murine dendritic cells isolated from immunocompetent mice (C57BL/6; Refs. 38 and 39). Moreover, recent evidence suggests that certain subtypes of dendritic cells can activate NK cells by producing cytokines (40, 41). However, whether these new populations of dendritic cells exist and possess similar functions in SCID mice remains to be determined.

Dendritic cells are known to be necessary for the development of adoptive immunity, and the activation of the innate immune effector cells (NK cells; Ref. 42). However, in general, dendritic cells lack Fc and complement receptors and cannot induce ADCC by themselves. Some reports describe the expression of FcRs at early stages of dendritic cell maturation or in certain subsets of peripheral blood human dendritic cells. However, FcRs are down-regulated on activation (42–44).

Our experiments demonstrated that Gr-1+ cells are necessary for the antitumor activity of rituximab. In the absence of NK cells, lymphoma-bearing SCID mice with intact neutrophils only partially respond to rituximab and have a longer survival as compared with rituximab-treated placebo mice.

The capacity of rituximab to induce tumor growth arrest and improve the survival was abolished on depletion of neutrophils in NK cell-depleted SCID mice. In this particular group of

animals, the time to limb paralysis development was not different from that seen in untreated mice. It is important to note that no apparent direct antitumor activity via induction of Ab-associated CMC or direct apoptosis was observed in our murine system [*i.e.*, no significant antitumor activity was seen in rituximab-treated animals that are neutrophil and NK cell depleted as compared with placebo (group B1 *versus* A1)]. The effect of immune effector cells appears to be specific to rituximab and not a xenograft rejection, given the fact that no significant differences were noticed between neutropenic and nonneutropenic untreated mice.

The antitumor activity of rituximab was decreased in NK cell-intact lymphoma-bearing SCID mice when neutrophils were depleted. In this setting, the therapeutic loss was only partial, most likely because NK cells were left intact. The longest survival in our experimental groups was observed in rituximab-treated mice with both neutrophils and NK cells intact. Our studies conclusively demonstrate that neutrophil function is necessary for optimal ADCC activity of rituximab *in vivo*.

The degree of antitumor effects that neutrophils contribute to rituximab biological activity in our model appears to be at least partial (especially in the concurrent presence of NK cells). Extrapolation to a more “immunocompetent or humanized system” must be done with caution because other host mechanisms such as T-cell-mediated antitumor activity, as well as CMC, may contribute significant roles to the antitumor activity of rituximab.

Many mechanisms have been proposed to explain the ability of neutrophils to mediate antitumor reactions (45). Release of oxygen radicals or inflammatory cytokines in the tumor bed, complement activation, and ADCC are some of the postulated mechanisms of neutrophil action against malignancies/infections (29–35). Based on the fact that no demonstrable antitumor activity was observed in untreated nonneutropenic mice, we postulate that neutrophils induce antitumor activity in the presence of rituximab via ADCC in our SCID mouse model.

Neutrophils express several Fc γ receptors needed for cell-cell interactions, induction of ADCC, and phagocytosis (34). Fc γ RI (high affinity) and Fc γ RIII (low affinity) are present in the surface of PMNs at different degrees. Furthermore, the importance of Fc γ RIII expression for the antitumor activity (*i.e.*, ADCC) of rituximab and Trastuzumab has been demonstrated *in*

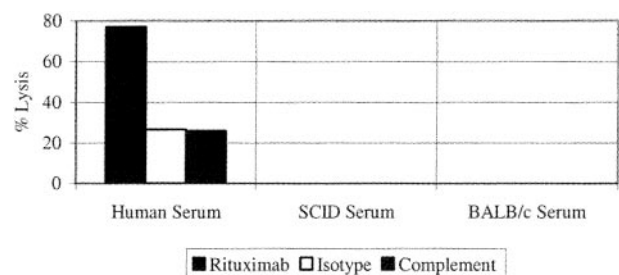


Fig. 6 *In vitro* studies conducted in the DHL-4 cell line demonstrated that rituximab mediated cell-specific lysis only in the presence of human serum. No CMC was detected when either SCID or BALB/c mouse serum was used as complement source.

vivo (25). Our results are consistent with the studies recently published by Clynes *et al.* (25). Macrophages and monocytes are other effector cells that possess the capacity to mediate cellular cytotoxicity and ADCC. In our lymphoma murine model, both macrophages and monocytes cells were preserved. No significant antitumor activity was seen in neutrophil- and NK cell-depleted mice with intact macrophages/monocytes treated with rituximab (group B1). This finding suggests that their role in the biological activity of rituximab may be limited.

Strategies to enhance the antitumor activity of mAbs using cytokines that stimulate neutrophils and monocytes have been explored by our laboratory and other groups of investigators (46–48). *In vitro* studies have shown that up-regulation of neutrophils using G-CSF can enhance the antitumor activity of rituximab or Abs directed against class II antigens (46, 47). Recently, we have demonstrated that the administration of G-CSF before *in vivo* rituximab therapy results in improved antitumor activity and survival in NHL-bearing SCID mice (48).

In conclusion, the *in vivo* biological activity of rituximab in our SCID mouse model appears to be mediated by activation of the innate immune system and requires intact neutrophils and NK cells for optimal biological activity. The data presented here are the first to address the potential role of neutrophils in the antitumor activity of rituximab in an *in vivo* animal model. Moreover, because neutrophils appear to play a significant role in the biological activity of mAbs, strategies that improve the quantity and/or quality of neutrophils, such as by granulocyte-macrophage colony stimulating factor or G-CSF priming, may potentially increase the antitumor effects of rituximab in human clinical trials.

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