Imatinib mesylate reduces rituximab-induced tumor-growth inhibition *in vivo* on Epstein–Barr virus-associated human B-cell lymphoma

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We have reported earlier an increase of tumor-growth inhibition following chemotherapy combined with concomitant administration of imatinib mesylate. Conversely, the combination of imatinib and rituximab has been reported in very few cases of patients and remains controversial. To explore this particular combination of targeted therapies, we therefore investigated the in-vivo impact of rituximab plus imatinib on B-cell lymphoproliferation. Combination of the tyrosine kinase inhibitor imatinib mesylate (STI571) and the anti-CD20 monoclonal antibody rituximab was evaluated on an Epstein–Barr virus-associated B-cell lymphoproliferative disorder xenografted into severe combined immunodeficient or Rag2/$\gamma_c^{-/-}$ (B^-, T^- and NK^-) mice. Using severe combined immunodeficient mice, we found that STI571 diminished the efficacy of rituximab to inhibit tumor growth *in vivo*. Using alymphoid Rag2/$\gamma_c^{-/-}$ mice, we showed that the effect of STI571 was not dependent on the presence of natural killer cells. In contrast, serum complement administered after STI571 treatment reversed this inhibitory effect. Finally, using nonimmunodeficient mice, we observed an in-vivo decrease of CD4-positive T-cells and mature B-cell lymphocytes after imatinib administration. We found that STI571 decreased the in-vivo efficacy of rituximab via serum protein components that could influence complement-dependent cytotoxicity. In contrast, this effect was not dependent on the presence of natural killer cells. *Anti-Cancer Drugs* 18:1029–1037

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Introduction

Targeted therapy constitutes one of the most important advances in cancer therapy over the last decade. This approach uses specific characteristics of tumor cells, i.e. (1) exclusive expression or overexpression of membrane antigen/protein, (2) clonal enzymatic activity resulting from a genetic feature, (3) influence of environment and/or of neighboring cells on tumor-cell proliferation and apoptosis, and (4) immune tolerance. As it would be useful to identify the modalities of these targeted therapies, either alone or in combination with conventional chemotherapy and/or radiotherapy, close laboratory monitoring of combinations of these specific approaches is warranted. Furthermore, interruption or modification of a transduction pathway or an interaction between the tumor cell and its environment by a first targeted therapy might induce activation of resistance mechanisms that could be stimulated by a second specific therapy. Consequently, the principle that one biological effect combined with another induces additional or synergistic effects on tumor cells is not applicable to all clinical cancer situations.

Rituximab is a chimeric anti-CD20 monoclonal antibody [1] that has been extensively used in the treatment of B-cell non-Hodgkin's lymphomas, alone or in combination with chemotherapy. Various effects or mechanisms have been reported for rituximab: (1) complement-dependent cytolysis [2], (2) antibody-dependent cell-mediated cytotoxicity [2], (3) monoclonal antibody-triggered induction of B-cell apoptosis [3,4], (4) inhibition of cell proliferation [5], (5) inhibition of the nuclear factor (NF)-κB transduction pathway [6], and (6) a synergistic effect with cytotoxic agents [5,7] and interferon-α [8]. Rituximab combined with combination chemotherapy regimens induces a significant benefit, compared with chemotherapy alone, in terms of response rates, progression-free survival and overall survival in follicular [9] and diffuse large B-cell lymphomas [10]. The tyrosine kinase inhibitor, STI571 or imatinib, belonging to the 2-phenylaminopyrimidine class, selectively inhibits BCR/ABL [11], platelet-derived growth factor receptor (PDGFR) [12], c-kit [12], and c-fms/macrophage colony-stimulating factor receptor (MCSFR) [13] kinase activity. Commonly used in the treatment of chronic

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myeloid leukemia [14], c-kit-mutated gastrointestinal stromal tumors [15] and PDGFR-mutated tumors [16], imatinib is not clearly indicated in other cancers, particularly malignant lymphomas.

As imatinib is commonly used without chemotherapeutic agents, few reports have evaluated the therapeutic effect of concomitant administration of STI571 and chemotherapy in mice xenografted tumors and in cancer patients. Using two different human cancers xenografted into immunodeficient mice, a small cell lung cancer [17] and an Epstein–Barr virus (EBV)-associated B-cell lymphoproliferation [18], we reported an increase of tumor-growth inhibition following chemotherapy combined with concomitant administration of STI571 [17,19]. Conversely, combination of imatinib and rituximab was reported in very few cases of patients having two different tumors, yielding responses for both diseases [20,21], or for selection of preexistent resistant clones [22]. Moreover, few reports suggested a therapeutic effect of imatinib either on lymphoid tumors, as STIS71 (STI) inhibited the proliferation of multiple myeloma cells resistant to dexamethasone or melphalan and had an additive effect when combined with dexamethasone [23], or on a case of refractory mycosis fungoides, successfully treated by STI571 [24]. On the basis of these data, therefore, to explore other therapeutic combinations of targeted therapies in B-cell malignancies, we investigated the in-vivo impact of rituximab plus imatinib on an EBV-associated lymphoproliferation. We found that STI571 decreases rituximab-induced tumor-growth inhibition on both xenografted natural killer (NK)-positive and NK-negative mice. We also observed that normal mouse serum administered after STI571 treatment reversed this inhibitory effect. We therefore concluded that imatinib reduces rituximab efficacy via serum protein components that acted on complement-dependent cytotoxicity.

Materials and methods

Cell line, culture conditions and in-vitro rituximab treatment modalities

The human established LY-3 cell line was obtained from LY-3 tumors xenografted previously into severe combined immunodeficient (SCID) mice [18]. Histologic examination of the xenograft LY-3 tumor showed diffuse proliferation of large cells that indicated CD30 negativity, CD15 negativity, and high CD20, EBV LMP1 and EBV protein expression (EBNA-2), both in the morphologic and immunophenotyping analyses concluded on EBV-associated human B-cell lymphoma. Transplanted tumors were dissected and suspended in culture medium. After filtration, cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St Louis, Missouri, USA) supplemented with 10% fetal calf serum (FCS; Dutscher, Brumath, France), penicillin G (10^2 IU/ml) and streptomycin (50 μg/ml; Sigma–Aldrich). Cells were cultured in medium containing rituximab 10 μg/ml (Roche, Neuilly-sur-Seine, France) or medium alone in the presence or absence of 20% normal or decomplemented FCS, or 20% normal or decomplemented human serum, or 20% C57BL/6 mouse serum (control or collected after 5 days of treatment by one intraperitoneal injection of STI571 at a dosage of 70 mg/kg/day), or STI571 4 μg/ml (Novartis, Basel, Switzerland). Cell survival was assessed by cell counting of five wells with a Coulter automatic cell counter (Beckman Coulter, France). A survival rate was then defined as follows: mean number of treated cells/mean number of control cells.

In-vivo treatment modalities

STI571 (gift from Novartis, Rueil-Malmaison, France) was diluted in 150 μl of H_2O and administered at a total dosage of 70 mg/kg/day in one intraperitoneal injection on different days, as indicated. Rituximab was injected at a native concentration of 10 mg/ml and administered at a total dosage of 50 mg/kg/day in one intraperitoneal injection on different days, as indicated in the figure legends. The control group received 0.9% sodium chloride injections according to the same schedule as that of experimentally treated mice.

In-vivo experiments in immunodeficient mice bearing human tumors

Female SCID or Rag2/$\gamma_c^{-/-}$ mice [25], weighing 20–30 g, 6–8 weeks old, were bred in the animal facilities (Institut Curie, Paris, France), maintained under specific pathogen-free conditions with artificial lighting (12-h light/dark cycle), and fed with a regular diet and water *ad libitum*. The care, housing and handling of the mice were performed in accordance with the recommendations of the French Ethics Committee and under the supervision of authorized investigators. For curative therapeutic trials, the tumor-bearing mice were randomly divided into equivalent groups of 5–12 animals for the various experiments and mice were treated at different times after transplantation.

The human EBV-associated B-cell LY-3 lymphoma subcutaneously xenografted between the shoulder blades of immunodeficient mice was used for the experiments [18]. During and after treatment, all mice were weighed once a week. Tumor growth was monitored by measuring two perpendicular diameters with calipers. Tumor volume (V) and relative tumor volume (RTV) were calculated as follows:

$$V = ab^2/2,$$

where a is the width (large diameter) and b the length (small diameter) of the tumor (in mm).

$$\mathrm{RTV} = V_x/V_i,$$

where V_x is the mean tumor volume (in mm^3) at any given time and V_i is the mean initial tumor volume (in mm^3) at the start of treatment [26]. Mice were ethically killed when the tumor volume reached 2500 mm^3 in the control group.

Peripheral blood mononuclear cell count and immunophenotyping

Peripheral blood mononuclear cells (PBMC) were studied before and after administration of imatinib on C57 BL/6 mice. STI571 was administered at a total dosage of 70 mg/kg/day in one intraperitoneal injection from day 1 to 5. At day 5 after the last injection of imatinib, mice were killed and blood samples were collected. A blood count was then performed including total white cells, polymorphonuclear neutrophils, monocytes, red cells, and platelets. Thereafter, immunophenotyping of PBMC was performed by flow cytometry with a panel of monoclonal antibodies in four color combinations. The technique was a stain-then-lyse direct immunofluorescence technique, in which monoclonal antibodies were used in four stainings using fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC) and phycoerythrin–cyanin5 (PECy5). Indirect staining with a biotin-streptavidin combination was used for two monoclonal antibodies. Fifty microliters of blood was incubated with 10 µl of antibody at 4°C for 10 min, protected from light, followed by erythrocyte lysis (Pharmalyse; Pharmingen BD Biosciences, Le Pont de Claix, France) and washing. Acquisition of 10 000 events was performed on a Becton Dickinson (San Diego, California, USA) FACSCalibur flow cytometer using CellQuest Pro software.

The combinations of monoclonal antibodies (Pharmingen BD Biosciences,) were as follows:

(1) Tube 1: CD4APC/CD25PE/CD3FITC/βTCRbiotin PECY5.
(2) Tube 2: CD8FITC/CD4APC/βTCRxx/CD3PE.
(3) Tube 3: DX5FITC/CD3APC/CD14PE.
(4) Tube 4: CD19APC/CD21FITC/CD24PE/CD23biot-PECYA5.

CellQuest Pro software (Becton Dickinson) was used for data analysis; analysis was first performed in a lymphocyte gate according to forward and side scatter data, then analysis in other gates were performed in CD3- or CD19-positive cells to analyze T-cell and B-cell subpopulations.

Statistical analysis

A Mann–Whitney *U*-test was used to assess the in-vivo effect of the various treatments on the growth of xenografted tumors in nude or SCID mice. *U* values were considered significant when the probability of a difference was less than or equal to 0.05. Survival curves were estimated using the Kaplan–Meier method [27].

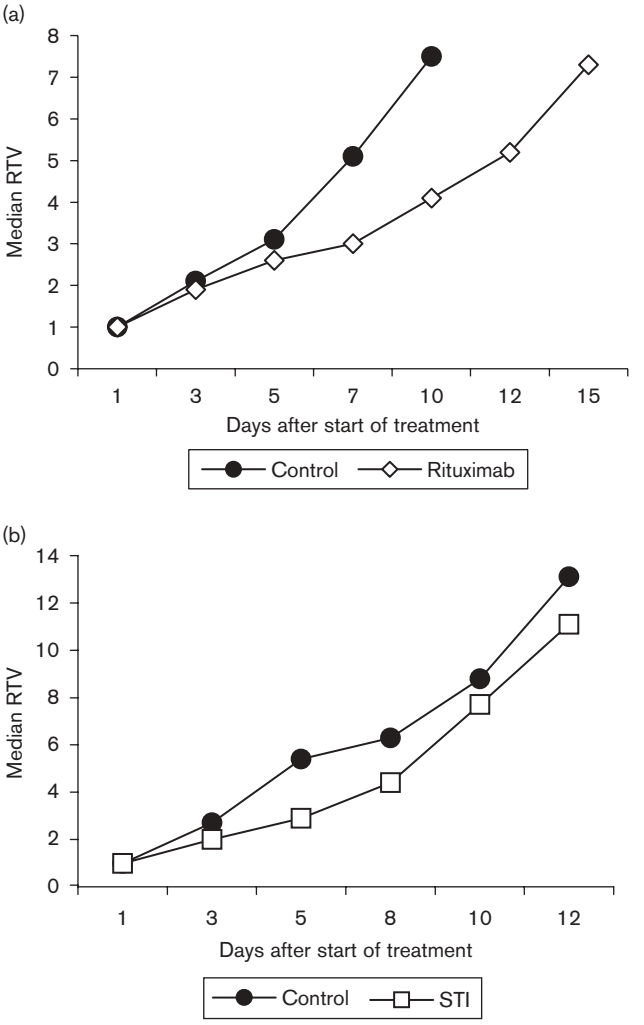
Results

In-vivo efficacy of rituximab or STI571 alone

SCID mice bearing LY-3 tumors measuring approximately 150 mm^3 were treated twice weekly by an intraperitoneal injection of 50 mg/kg of rituximab until the animals were killed. Significant tumor-growth inhibition was observed ($P < 10^{-2}$; Fig. 1a).

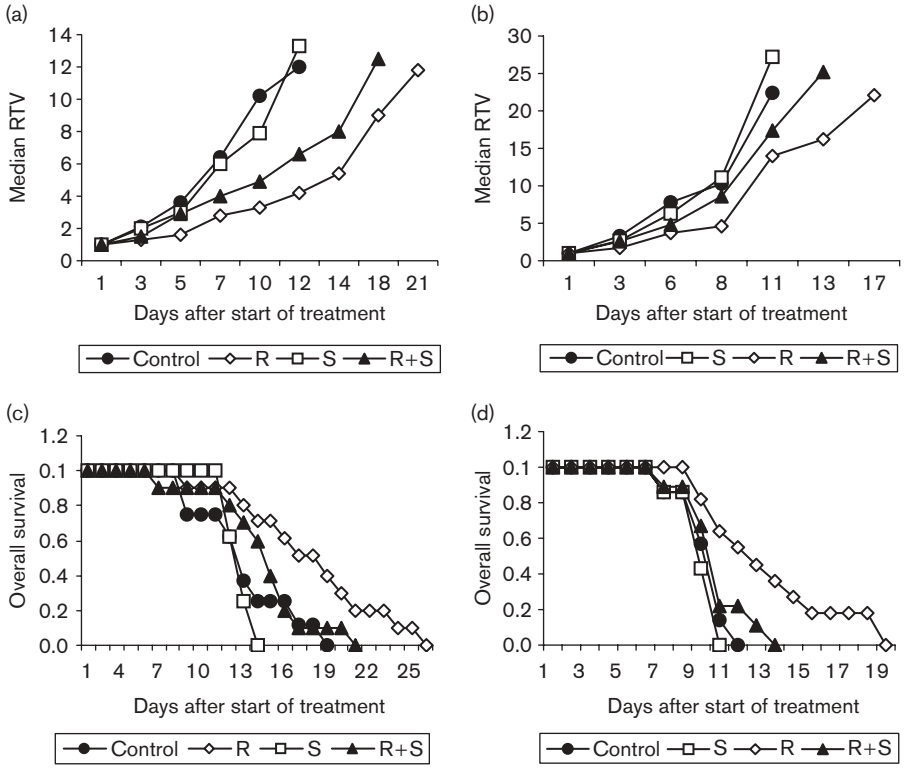
LY-3-bearing SCID mice received intraperitoneal injections of imatinib (70 mg/kg/day from a tumor of volume 150 mm^3 until the animals were killed). Early significant ($P < 0.05$ at day 5 after start of treatment) but transient tumor-growth inhibition was observed in treated mice (Fig. 1b). Overall, imatinib has no significant impact on the LY-3 tumor growth.

Fig. 1



Antitumor activity of rituximab (a) and imatinib (b) in severe combined immunodeficient (SCID) mice bearing LY-3 tumors. Xenografted SCID mice were treated from a tumor of volume 150 mm^3 until the animals were killed (a) by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg (◇) or (b) by one daily intraperitoneal injection of tyrosine kinase inhibitor imatinib mesylate (STI571) at a dosage of 70 mg/kg (□). All control groups received injections of 0.9% NaCl according to the same schedule as that of experimentally treated mice (●). RTV, relative tumor volume.

Fig. 2



Antitumor activity of combined rituximab (R) and imatinib (S) in severe combined immunodeficient (SCID) mice bearing LY-3 tumors. Xenografted SCID mice were treated from a tumor of volume 150 mm^3 until the animals were killed, by one (a, c) or two (b, d) weekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg with (▲) or without (◇) one daily intraperitoneal injection of tyrosine kinase inhibitor imatinib mesylate (STI571) at a dosage of 70 mg/kg. Control groups received imatinib (□) and 0.9% NaCl (●) injections according to the same schedule as that of experimentally treated mice. Median relative tumor volume (RTV; a, b) and overall survivals (c, d) were evaluated, as described in Materials and methods.

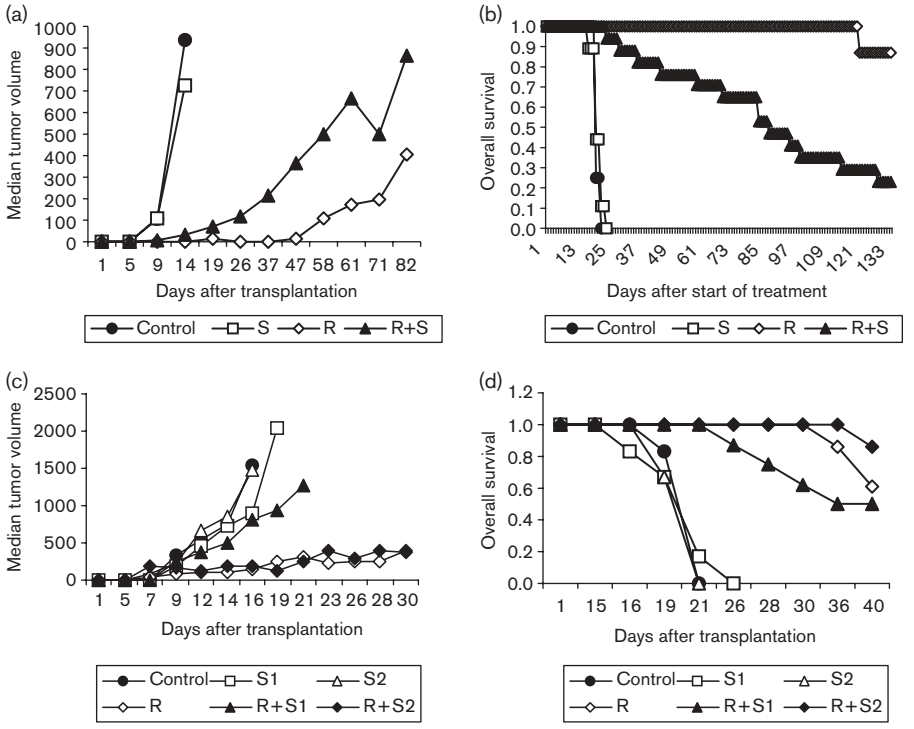
In-vivo efficacy of combined rituximab and STI571

SCID mice bearing LY-3 tumors measuring approximately 150 mm^3 were treated with daily intraperitoneal injections of imatinib at a dosage of 70 mg/kg/day with or without one (Fig. 2a) or two (Fig. 2b) weekly intraperitoneal injections of 50 mg/kg of rituximab until the animals were killed. In both experiments, tumor-growth inhibition was induced by rituximab alone ($P < 0.01$ and $P < 0.05$ for Fig. 2a, respectively). In contrast, this tumor growth inhibition was slightly reduced when rituximab was concomitantly injected with imatinib ($P < 0.05$ and $P =$ ns for Fig. 2a and b, respectively, based on a comparison of rituximab and rituximab + STI 571 curves). This effect was confirmed by the overall survival rates of SCID mice included in the two experiments (data not shown).

To confirm our observation, we therefore evaluated the in-vivo combination of rituximab and imatinib with other treatment regimens. We first treated SCID mice bearing LY-3 tumors with biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg with or without daily intraperitoneal administration of STI571 (50 mg/kg/day) from day 3 after transplantation. We then observed an impressive tumor-growth inhibition induced by the anti-CD20 monoclonal antibody rituximab ($P < 10^{-2}$). This inhibition was significantly reduced, but after a certain delay, in the case of concomitant administration of imatinib ($P < 0.01$ at day 61 after transplantation) (Fig. 3a). As before, this effect was confirmed by the overall survival rates of SCID mice included in the experiment (Fig. 3b).

Thereafter, we evaluated the impact of the interval between the first injection of imatinib and the first administration of rituximab. SCID mice bearing LY-3 tumors were treated by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg from day 6 after transplantation with or without daily intraperitoneal injections of imatinib at a dosage of 50 mg/kg/day from day 1 (S1) or day 6 (S2) after transplantation. As in the previous experiment, we observed marked tumor-growth rituximab-induced inhibition ($P < 0.01$) that was reduced when imatinib was administered at day 1 after xenografts ($P < 0.01$), but not when STI571 was administered concomitantly with rituximab at day 6 after xenograft

Fig. 3



Antitumor activity of combined rituximab (R) and imatinib (S) in severe combined immunodeficient (SCID) mice bearing LY-3 tumors. (a, b) Xenografted SCID mice were treated from day 3 after transplantation until they were killed, by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg with (▲) or without (◇) one daily intraperitoneal injection of tyrosine kinase inhibitor imatinib mesylate (STI571) at a dosage of 70 mg/kg. Control groups received imatinib (□) and 0.9% NaCl (●) injections according to the same schedule as that of experimentally treated mice. (c, d) Xenografted SCID mice were treated from day 6 after transplantation until they were killed, by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg with or without (◇) one daily intraperitoneal injection of STI571 at a dosage of 70 mg/kg administered from day 1 (S1) (▲) or day 6 (S2) (♦). Control groups received imatinib (S1) (□), (S2) (Δ) and 0.9% NaCl (●) injections according to the same schedule as that of experimentally treated mice. Median relative tumor volume (RTV; a, c) and overall survivals (b, d) were evaluated, as described in Materials and methods.

(Fig. 3c). This effect was confirmed by evaluation of overall survival rates of SCID mice included in the experiment (Fig. 3d).

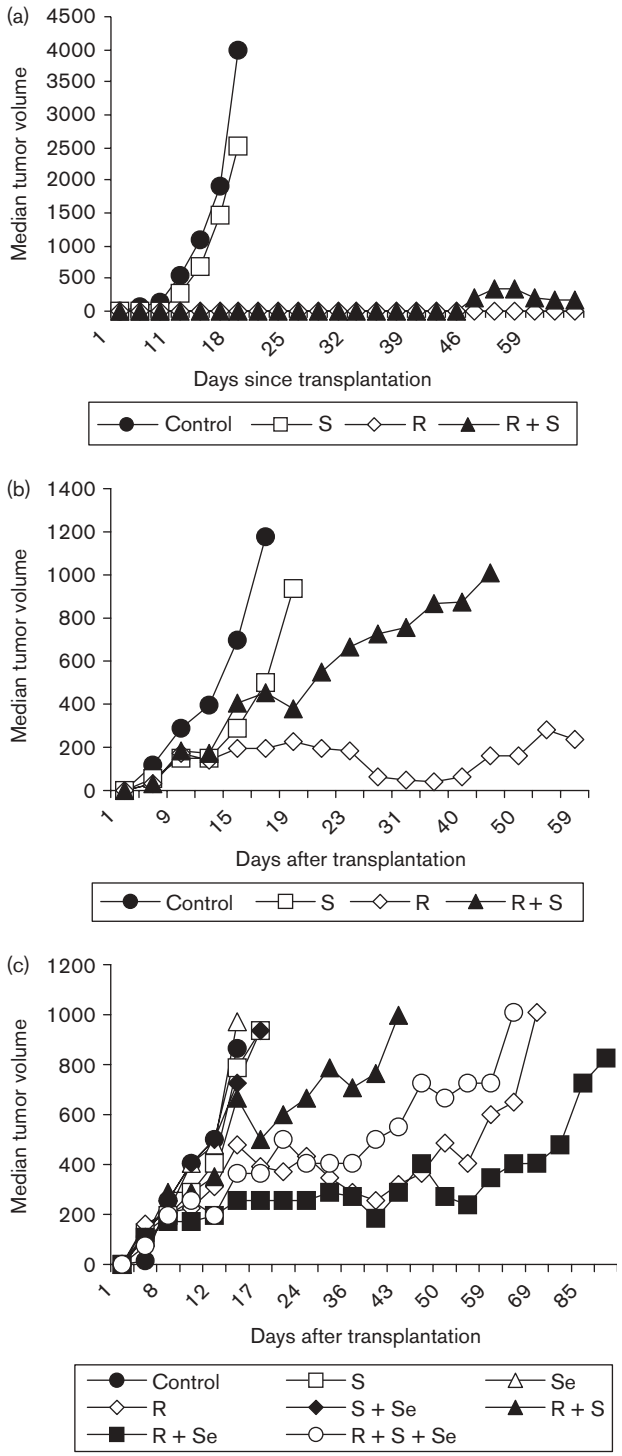
Mechanisms of the impact of imatinib on the in-vivo efficacy of rituximab

To explain the in-vivo reduction of rituximab-induced tumor-growth inhibition, we used an alymphoid (B^-, T^-, and NK^-) mouse strain (Rag2/$\gamma_c^{-/-}$), particularly suitable to assess the implication of NK cells in this strain, compared with that observed in xenografted SCID mice, and performed experiments similar to those reported previously. Rag2/$\gamma_c^{-/-}$ mice bearing LY-3 tumors were treated with biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg with or without daily intraperitoneal administration of STI571 at a dosage of 50 mg/kg/day from day 3 after transplantation. We observed an impressive efficacy of rituximab ($P < 0.01$) that was not inhibited by concomitant administration of STI571 (Fig. 4a). We then treated Rag2/$\gamma_c^{-/-}$ mice bearing LY-3 tumors by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg from day 6 after transplantation with or without daily intraperitoneal injections of imatinib at a dosage of 50 mg/kg/day from day 1 after transplantation. We found an identical efficacy of rituximab ($P < 10^{-2}$) that was inhibited when imatinib was administered on day 1 ($P < 10^{-2}$) (Fig. 4b).

Finally, we evaluated the role of serum proteins on the reduction of rituximab-induced tumor-growth inhibition induced by imatinib. Serum from SCID mice was collected earlier and frozen before use. SCID mice bearing LY-3 tumors were treated by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg from day 6 after transplantation, with or without daily intraperitoneal injections of imatinib at a dosage of 50 mg/kg/day from day 1 after transplantation, and with or without injections of SCID-mice serum at a total volume of 150 µl at day 6 and 50 µl at day 9. As shown previously, we observed an impressive rituximab-induced tumor-growth inhibition that was significantly reduced by concomitant administration of STI571 ($P < 0.05$)

(Fig. 4c). Although serum from SCID mice had no impact on tumor growth when it was injected alone or combined with imatinib, we observed that it partially maintained the tumor-growth inhibition induced by rituximab when administered with STI571 (*P* not significant for the comparison of rituximab alone and rituximab + STI571 + serum groups).

Fig. 4



(a, b) Antitumor activity of combined rituximab (R) and imatinib (S) in Rag 2/$\gamma_c^{-/-}$ mice bearing LY-3 tumors. (a) Xenografted Rag2/$\gamma_c^{-/-}$ mice were treated from day 3 after transplantation until they were killed, by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg, with (▲) or without (◇) one daily intraperitoneal injection of tyrosine kinase inhibitor imatinib mesylate (STI571), at a dosage of 70 mg/kg. (b) Xenografted Rag2/$\gamma_c^{-/-}$ mice were treated from day 6 after transplantation until they were killed, by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg with (▲) or without (◇) one daily intraperitoneal injection of STI571 at a dosage of 70 mg/kg administered from day 1 (S1). Control groups received imatinib (□) and 0.9% NaCl (●) injections according to the same schedule as that of experimentally treated mice. (c) Antitumor activity of combined rituximab, imatinib and mouse serum (Se) in severe combined immunodeficient (SCID) mice bearing LY-3 tumors. Xenografted SCID mice were treated from day 6 after transplantation until they were killed, by biweekly intraperitoneal injections of rituximab at a dosage of 50 mg/kg, with or without (◇) one daily intraperitoneal injection of STI571 at a dosage of 70 mg/kg administered from day 1, and with (○) or without (▲) SCID mice serum being administered by one intraperitoneal injection at a total volume of 150 µl at day 6 and 50 µl at day 9. Control groups received imatinib (□), SCID mice serum (△), rituximab + serum (■), imatinib + serum (♦) and 0.9% NaCl (●) injections according to the same schedule as that of experimentally treated mice.

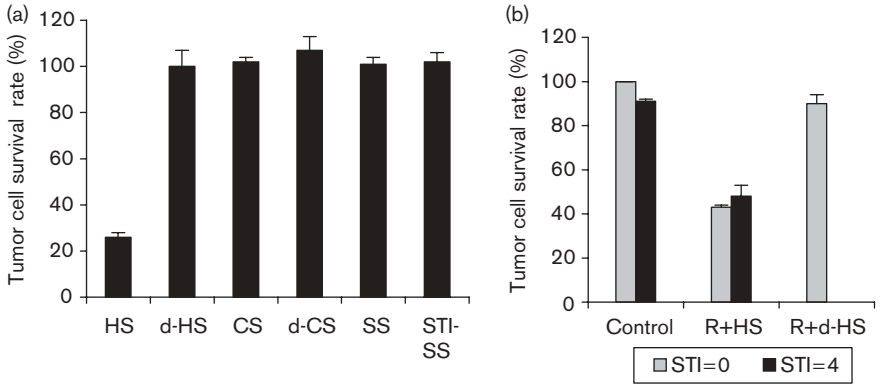
In-vitro efficacy of combined rituximab and serum

To evaluate the impact of serum on the in-vitro efficacy of rituximab, we cultured LY-3 cells with or without 10 µg/ml rituximab in the presence or absence of 20% normal or decomplemented human serum, or 20% normal or decomplemented FCS, or 20% C57BL/6 mouse serum, which was obtained from control mice or mice previously treated for 5 days by one daily intraperitoneal injection of STI571 at a dosage of 70 mg/kg/day. The combination of normal human serum and rituximab induced a significant inhibition of tumor-cell proliferation and this effect disappeared after serum decomplementation (Fig. 5a). Conversely, no inhibition of tumor-cell proliferation was observed after the in-vitro combination of rituximab plus normal calf serum or rituximab plus mouse serum obtained after in-vivo STI administration (Fig. 5a). Previous exposure of normal human serum to 4 µg/ml STI571 did not reverse the inhibition of tumor-cell proliferation induced by the combination of rituximab and normal human serum (Fig. 5b). Finally, in-vitro administration of 1 or 10 µmol/l of imatinib for 2.5 days did not induced an inhibition of tumor-cell proliferation (data not shown).

Analysis of peripheral blood mononuclear cells after imatinib administration

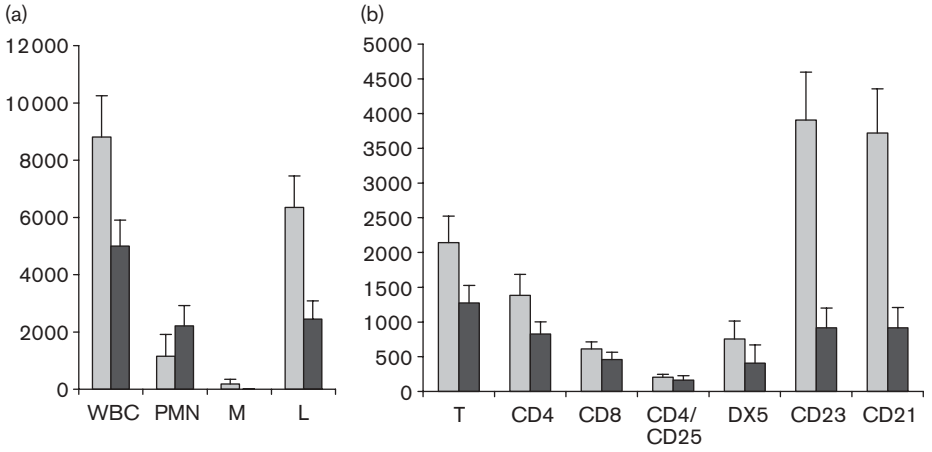
Finally, we evaluated the impact of imatinib on PBMC. After 5 consecutive days of treatment by intraperitoneal injections of 70 mg/kg of STI571, C57BL/6 mice were killed and blood samples were collected. A blood count was then performed including total white cells, polymorphonuclear neutrophils, lymphocytes and monocytes. A significant decrease of white blood cell count was observed after imatinib administration, affecting lymphocytes but not polymorphonuclear neutrophils or mono-

Fig. 5



(a) LY-3 cells were cultured with or without 10 μg/ml of rituximab in the presence or absence of 20% normal or decomplemented human serum (HS), or 20% normal or decomplemented calf serum (CS), or 20% C57BL/6 mouse serum obtained from control mice or mice previously treated for 5 days by one intraperitoneal injection of 70 mg/kg/day tyrosine kinase inhibitor imatinib mesylate (STI571). (b) LY-3 cells were cultured with or without 10 μg/ml of rituximab in the presence or absence of 20% normal human serum or human serum previously incubated for 28 h with 4 μg/ml of STI571.

Fig. 6



(a) Peripheral blood mononuclear cells (PBMC) after imatinib administration. C57BL/6 mice were treated by one daily intraperitoneal injection of tyrosine kinase inhibitor imatinib mesylate (STI571) at a dosage of 70 mg/kg administered from day 1 to day 5. Blood samples were then collected and PBMC counts were evaluated. (b) Immunophenotyping of PBMC after imatinib administration. As before, blood samples were collected after 5 consecutive days of STI571 treatment and immunophenotyping was then performed, as described in Materials and methods. WBC, white blood cells; PMN, polymorphonuclear neutrophils; M, monocytes; L, lymphocytes.

cytes (Fig. 6a). Immunophenotyping of the PBMC performed by flow cytometry before and after STI571 injections showed a significant decrease of T-cell lymphocytes affecting CD4-positive but not CD8-positive and CD4/CD25-positive T cells (Fig. 6a and b), and a significant decrease of CD21-positive and CD23-positive B mature cells (Fig. 6b). In contrast, despite the fact that the total number of NK cells (DX5-positive cells) was not modified by imatinib administration, a dramatic increase of the intensity of membrane uptake was observed (data not shown).

Discussion

In conclusion, we have shown that STI571 reduces rituximab-induced in-vivo tumor-growth inhibition on a CD20-positive EBV-associated lymphoproliferation. This effect was mainly observed when imatinib was administered before monoclonal antibody therapy, both on xenografted NK-positive and NK-negative mice. We also observed that serum decomplementation performed after STI571 treatment reversed this inhibitory effect and that mouse serum collected after STI571 injections did not reverse the in-vitro inhibition of cell proliferation induced

by rituximab and normal control serum. Altogether, these observations argue in favor of inactivation of complement by the STI571 tyrosine kinase inhibitor.

The main mechanisms by which rituximab induces in-vivo tumor-growth inhibition and human antilymphomatous effect are antibody-dependent cell-mediated and complement-dependent cytotoxicity [2]. To determine the pathway by which imatinib reduces rituximab-induced tumor-growth inhibition, we first studied the efficacy of combined STI571 and rituximab on NK-positive (SCID) and NK-negative (Rag2/$\gamma_c^{-/-}$) mice and showed that, on both types of xenografted animals, STI571 inhibited the efficacy of the anti-CD20 monoclonal antibody. This finding therefore suggests that this inhibitory effect of imatinib was not mediated via NK-cell activity. Our result could appear to contradict the report by Borg *et al.* [28] showing that imatinib promotes NK-cell activation via dendritic cells. As we have shown that inhibition of the rituximab effect by STI571 was not dependent on the presence of NK cells, this effect was not mediated by inhibition of antibody-dependent NK-cell-mediated cytotoxicity. We then studied the impact of serum proteins collected in mice before and after treatment by imatinib and showed that normal serum was able to reverse the in-vivo inhibitory effect of STI571. Conversely, neither normal mouse serum nor mouse serum collected after in-vivo STI571 treatment induced inhibition of in-vitro tumor-cell proliferation. This last observation was also confirmed with normal calf serum, suggesting that an efficient combination of rituximab and serum requires specificity between tumor cells and the concomitantly administered serum. Altogether, these observations suggest that the inhibitory effect of imatinib on in-vivo tumor-growth inhibition induced by anti-CD20 monoclonal antibody is mediated by serum proteins and possible complement activation, particularly in view of the rituximab-induced complement-dependent cytotoxicity. This point is supported by the fact that complement activation clearly determines the therapeutic activity of rituximab *in vitro* [29,30] and *in vivo* [31]. This observation is also supported by the fact that STI571 predominantly decreased the efficacy of rituximab to inhibit tumor growth *in vivo* when mice were treated previously by imatinib after tumor transplantation. The possibility, however, that imatinib interferes with mechanisms leading to the growth of transplanted tumors in immunodeficient mice, such as induction of neovascularization, cannot be excluded. Further mechanisms of rituximab efficacy have been reported and evaluated in our study. A direct monoclonal antibody-induced inhibition of cell proliferation, as published previously [5], which was reduced by STI571, was formally excluded owing to the absence of an in-vitro effect of rituximab alone on the lymphomatous-cell proliferation. Finally, we showed that STI571 did not influence the absolute neutrophil count in mice, as neutrophils have been shown to contribute to the biological antitumor activity of rituximab in a non-Hodgkin's lymphoma SCID mouse model [32].

After having demonstrated the inhibitory effect of STI571 on rituximab-induced tumor-growth inhibition on SCID and alymphoid (B^-, T^- and NK^-) Rag2/$\gamma_c^{-/-}$ mice, we studied the impact of imatinib on lymphoid subpopulations in immunocompetent mice. We observed a dramatic decrease of the absolute lymphocyte count affecting CD4-positive T-cells and predominantly mature B-cells. No decrease of the total number of NK cells was observed in our experiments, but a marked increase of DX5 expression was demonstrated by cytofluorometry. Various and sometimes contradictory immunological activities of imatinib have been reported: (1) increased [33–35] or decreased [36,37] dendritic cell antigen presentation function, (2) inhibition of the activation and proliferation of normal CD4-positive and CD8-positive T lymphocytes [38], (3) reversion of tumor-induced CD4-positive T-cell tolerance [35], and (4) in-vitro inhibition of hematopoietic progenitor-cell growth [39]. In contrast, very few data are available on the effects of imatinib on lymphoid cells. A recent report showed that STI571-induced improvement of autoimmune nephritis in MRL/*lpr* mice via inhibition of PDGF signaling associated with a decrease of concomitant lymphadenopathy and salivary-gland inflammation [40]. Despite the impressive decrease of the total number of lymphocytes and mature B-cells induced by imatinib, this effect could not explain the inhibitory effect on rituximab-induced tumor-growth inhibition for two main reasons: (1) this effect was observed on B-cell-negative mice and, (2) rituximab does not act via B-cell lymphocyte depletion. Our observation therefore supports the hypothesis that imatinib reduces the efficacy of rituximab via serum protein components such as hemolytic complement.

In conclusion, we have shown that STI571 decreases in-vivo rituximab-induced tumor-growth inhibition and that this effect could be mediated by inhibition of complement-dependent cytotoxicity. This observation underlines the fact that concomitant targeted therapies should be cautiously administered, particularly in the context of an extensive biological overview of each approach and each combination studied. Preclinical pharmacological experiments, therefore, are one way of exploring such associations before human phase I or phase II trials.

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