High efficacy of combined rituximab and gemcitabine on Epstein–Barr virus-associated human B-cell lymphoma obtained after Hodgkin's xenograft in immunodeficient mice

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The objectives were to characterize an Epstein–Barr virus-associated human B-cell lymphoma obtained from Hodgkin's xenograft, and to evaluate the in-vivo combination of rituximab and/or gemcitabine. A lymph node biopsy sample from a patient with Hodgkin's disease was xenografted into Rag $\gamma_c^{-/-}$ mice. Immunohistochemical, cytogenetic and genetic analyses were performed on both the human biopsy and xenografted tumor from severe combined immunodeficient mice. Tumor-bearing mice were then treated with rituximab and/or gemcitabine. Histologic features of the patient's biopsy concluded on classical CD15/CD30-positive Hodgkin's disease without expression of Epstein–Barr virus proteins. In contrast, morphologic and immunophenotypic examination of the xenograft showed diffuse proliferation of large B cells with high Epstein–Barr virus protein expression. Comparative genomic hybridization showed a normal pattern in the first case and a gain of chromosomal 12 in the xenografted tumor. Finally, polymerase chain reaction detected an immunoglobulin heavy chain rearrangement in the xenografted tumor. Altogether, these results indicate that the xenograft grew from the patient's Epstein–Barr virus-infected B-lymphoid cells and could be assimilated to posttransplant lymphoproliferative disease. In-vivo treatments of xenografted tumors showed significant tumor growth inhibition induced either by rituximab or gemcitabine alone and an impressive efficacy of combined treatment. This result therefore indicates that combined rituximab and gemcitabine could be an alternative approach in patients with posttransplant lymphoproliferative disease.
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Introduction

Preclinical experiments performed on animal models with xenografted human tumors constitute one way to improve the management of cancer patients, as in-vivo transplanted tumors are generally derived from biopsy samples from cancer patients. This approach requires good characterization of the xenograft that must be compared with the original human tumor, as the biopsy sample contains both malignant cells and bystander cells, which, in a few specific situations and in transplanted immunodeficient mice, can induce a clonal proliferation. For example, Epstein–Barr virus (EBV)-associated lymphoproliferations constitute a tumor cell expansion of EBV-positive bystander B lymphocytes that are present in cancer tissue samples, particularly lymph node biopsies. This situation mimics that reported in patients developing posttransplant lymphoproliferative disorders (PTLD) arising after allogeneic stem cell or solid-organ transplantations [1]. PTLD is often a fatal complication, in which the poor prognostic factors are hematopoietic stem cell transplant rather than solid-organ transplant, hematopoietic stem cell transplant for hematologic malignancy rather than immunodeficiency disorder and four or more sites of disease [2,3]. The main treatments proposed in patients developing such complications are (a) reduction of immunosuppression with an increased risk of graft-versus-host disease (GVHD) [4], (b) combination chemotherapy regimen, such as CHOP (cyclophosphamide, doxorubicin, vinvristine, prednisone) and Pro-MACE-CytaBOM (cyclophosphamide, doxorubicin, vinvristine, prednisone, etoposide, cytarabine, methotrexate, bleomycine), with a high infection and mortality rate [5,6], (c) anti-B-cell monoclonal antibodies such as rituximab [7–9], and (d) cellular immunotherapy,

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i.e. unselected therapy using donor lymphocyte infusion with a significant risk of GVHD [10] and more targeted cellular immunotherapy utilizing EBV-specific cytotoxic T cells [11] or autologous lymphokine-activated killer cells [12]. Among the various effective therapies that significantly increase the risk of GVHD, non-routine approaches and toxic combined chemotherapy with a high complication rate, rituximab appears to be an interesting treatment option for PTLD, as the overall response rate reported by Milpied *et al.* [8] was 69% with 62% of complete responses and a median duration of response of more than 1 year. As observed in certain other situations [13,14], a combination of both rituximab and chemotherapy should improve the overall and complete response rates, as well as the duration of remission. In a preliminary study including six PTLD patients, Orjuela *et al.* [15] reported an overall response rate of 100% (five complete responses and one partial response) after treatment with cyclophosphamide, prednisone and rituximab.

In this context, the objectives of this study were to (a) characterize an EBV-associated human B-cell lymphoma obtained from Hodgkin's xenograft in severe combined immunodeficient (SCID) mice, and (b) define a highly efficient combination of rituximab and chemotherapy. We first report immunohistochemical, cytogenetic and genetic analyses performed on both the human biopsy and xenografted tumor, and then demonstrate a highly effective combination of rituximab and gemcitabine on xenografted tumors. This result therefore indicates that combined rituximab and gemcitabine could be an alternative approach in patients with PTLD.

Materials and Methods

Primary Hodgkin's disease sample and In-vivo LY-3 tumor xenograft

A 36-year-old man was referred to the Department of Clinical Hematology for multiple cervical lymph nodes. After surgical biopsy, the diagnosis of nodular sclerosis Hodgkin's lymphoma was established according to the World Health Organization classification [16]. Staging of the disease, including analysis of blood samples, computed tomography scans of the chest and abdomen, bone marrow biopsy, and Gallium scintigraphy, found stage IV disease with multiple lung sites, B-symptoms, accelerated erythrocyte sedimentation rate and a prognostic score of 2 [17]. At diagnosis, the patient had positive serum anti-VCA and anti-EBNA IgG antibodies indicating past EBV contamination. The patient received six courses of BEACOPP (cyclophosphamide, doxorubicin, vinvristine, prednisone, etoposide, bleomycine, procarbazine) regimen [18], inducing a good partial response, and a 30-Gy nodal involved-field radiotherapy. At the time of final evaluation performed 1 month after the end of irradiation, computed tomography scans showed axillary lymph nodes and multiple lung nodules. In order to formally eliminate an infectious complication and to confirm the Hodgkin's disease recurrence, an axillary lymph node biopsy was performed. After obtaining the patient's consent, a sample of the tumor biopsy was subcutaneously xenografted between the shoulder blades of Rag $\gamma_c^{-/-}$ mice [19]. After initial tumor take in Rag $\gamma_c^{-/-}$ mice for three passages, the tumor, named LY-3, was then maintained in SCID mice.

Histopathologic examinations of the patient's biopsy and LY-3 tumor xenograft

As for the first lymph node biopsy, the diagnosis of lymphoma was established on the recurrent adenopathy according to the World Health Organization classification [16]. Tissues were fixed in acetic acid, formalin, ethylic alcohol solution, processed by standard methods, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for histopathologic examination. Histopathologic examinations consisted of morphologic examination of the cellular proliferation (cellular appearance, architectural pattern, and bystander cells) combined with immunohistochemical staining. For immunohistochemical evaluation, tissues were analyzed using monoclonal anti-human antibodies against CD3 (clone F 7.2.38; Dako, Glostrup, Denmark), CD20 (clone L26; Dako), CD15 (clone C3D1; Dako), CD30 (clone BerH2; Dako), EBV-latency proteins LMP-1 (clone CS1-4; Dako) and EBNA-2 (clone PE2; Dako). Labeling was visualized with a commercially available streptavidin–biotin–peroxidase kit (LSAB 2 system; Dako) according to the manufacturer's instructions.

Cytogenetic and genetic analyses of the patient's biopsy and LY-3 tumor xenograft

Karyotyping was performed on both the axillary lymph node biopsy and the tumor xenografted into SCID mice, using a standard protocol and a RHG banding technique [20]. A xenografted tumor resected 9 months later was analyzed by comparative genomic hybridization (CGH) [21].

Immunoglobulin heavy chain (IgH) rearrangement was detected by polymerase chain reaction (PCR) and performed on both samples. DNA was extracted from 10–20 million cells using the phenol–chloroform method after K proteinase digestion. The first and second framework regions were amplified using the (a) FR1c (5′ AGG TGC AGC TGS WGS ATG CSG G 3′) with the JH primers (JH1245 5′ ACC TGA GGA GAC GGT GAC CAG GGT 3′, JH3 5′ TAC CTG AAG AGA CGG TGA CCA TTG T 3′, JH6 5′ ACC TGA GGA GAC GGT GAC CGT GGT 3′) and the (b) FR256 (5′ TGG RTC CGV CAG SCY CCN GG 3′, VH5 (5′ GAA AAA GCC CGG GGA GTC TCT GA 3′), VH6 (5′ GAA GAT CTC TCA CTC ACC TGT GCC ATC 3′) with the JH primers JH1245 (5′ ACC TGA GGA GAC GGT GAC CAG GGT 3′), JH3 (5′ TAC CTG AAG AGA CGG TGA CCA TTG T 3′), JH6 (5′ ACC TGA GGA GAC GGT GAC CGT

GGT 3′), as described [22]. Amplification of the EBNA-1 locus was performed using the following primers (5′ CCT GTA GGG GAA GCC GAT 3′ and 5′ CAA TGG TGT AAG ACG ACA TT 3′) [23]. PCR products were visualized after ethidium bromide staining.

Analyses of the human LY-3 cell line

The human LY-3 cell line was obtained from LY-3 tumor previously xenografted into SCID mice. Transplanted tumors were dissected and suspended in culture medium. After filtration, cells were grown in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St Louis, Missouri, USA) supplemented with 10% fetal calf serum (Dutscher, Brumath, France), penicillin G (10^2 IU/ml) and streptomycin (50 µg/ml; Sigma-Aldrich).

Immunophenotyping of the LY-3 cell line was performed by flow cytometry with a panel of monoclonal antibodies in triple-color combination. The technique was a stain-then-lyse direct immunofluorescence technique, in which monoclonal antibodies were used in triple stainings using fluorescein isothiocyanate (FITC) and phycoerythrin (PE) and PerCP or PerCP-Cy5. After two washings, 500 000 cells were incubated with 10 µl of antibody at 4°C for 30 min protected from light. Acquisition of 15 000 events was performed on a Becton Dickinson (San Diego, California, USA) Facscalibur flow cytometer using Cell Quest Pro software. The monoclonal combinations were CD3FITC–CD19PE–CD45PerCP, CD5FITC–CD10PE–CD19PerCP–Cy5, KFITC–LPE–CD19PerCP–Cy5 and CD22PE–CD20PerCP–Cy5.5 (Oncomark reagents from Becton Dickinson). The Cellquest Pro software program (Becton Dickinson) was used for data analysis; the analysis was first performed on the CD45 population to quantify the percentage of B and T cells, and the analysis was then performed on the CD19 or CD20 gate to complete the profile of B cells.

In-vivo experiments in severe combined immunodeficient mice bearing LY-3 tumors

Female SCID mice, 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/12-h 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 seven to 10 animals and were treated as soon as the xenografted tumors reached a diameter of 5 mm (or a tumor volume of approximately 60 mm^3).

To test the sensitivity of this tumor, SCID mice bearing the xenografted tumor were treated with either gemcitabine (Lilly France, Suresnes, France) 60 mg/kg weekly until sacrifice of animals or rituximab (Roche, Neuilly-sur-Seine, France) 50 mg/kg twice weekly until sacrifice. Gemcitabine was diluted in 150 µl of 0.9% sodium chloride. Rituximab was injected at a native concentration of 10 mg/ml. The control group received 0.9% sodium chloride injections according to the same schedule as experimentally treated mice. All treatments were administered by intraperitoneal injection.

All mice were weighed once weekly. The tumor growth was monitored by measuring two perpendicular diameters with calipers. Tumor volume (V) and relative tumor volume (RTV) were calculated as follows:

$$V = a^2 \times b/2$$

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

$$\mathrm{RTV} = V_x/V_\mathrm{i}$$

where V_x is the mean tumor volume in cubic millimeters at any given time and V_i is the mean initial tumor volume in cubic millimeters at the start of treatment [24]. Mice were ethically sacrificed when the tumor volume reached 2500 mm^3 in the control group.

Statistical analysis

Mann–Whitney U-test was used to assess the *in vivo* effect of the various treatments on the growth of xenografted tumors in SCID mice. U values were considered significant when the probability of a difference was less than or equal to 0.05.

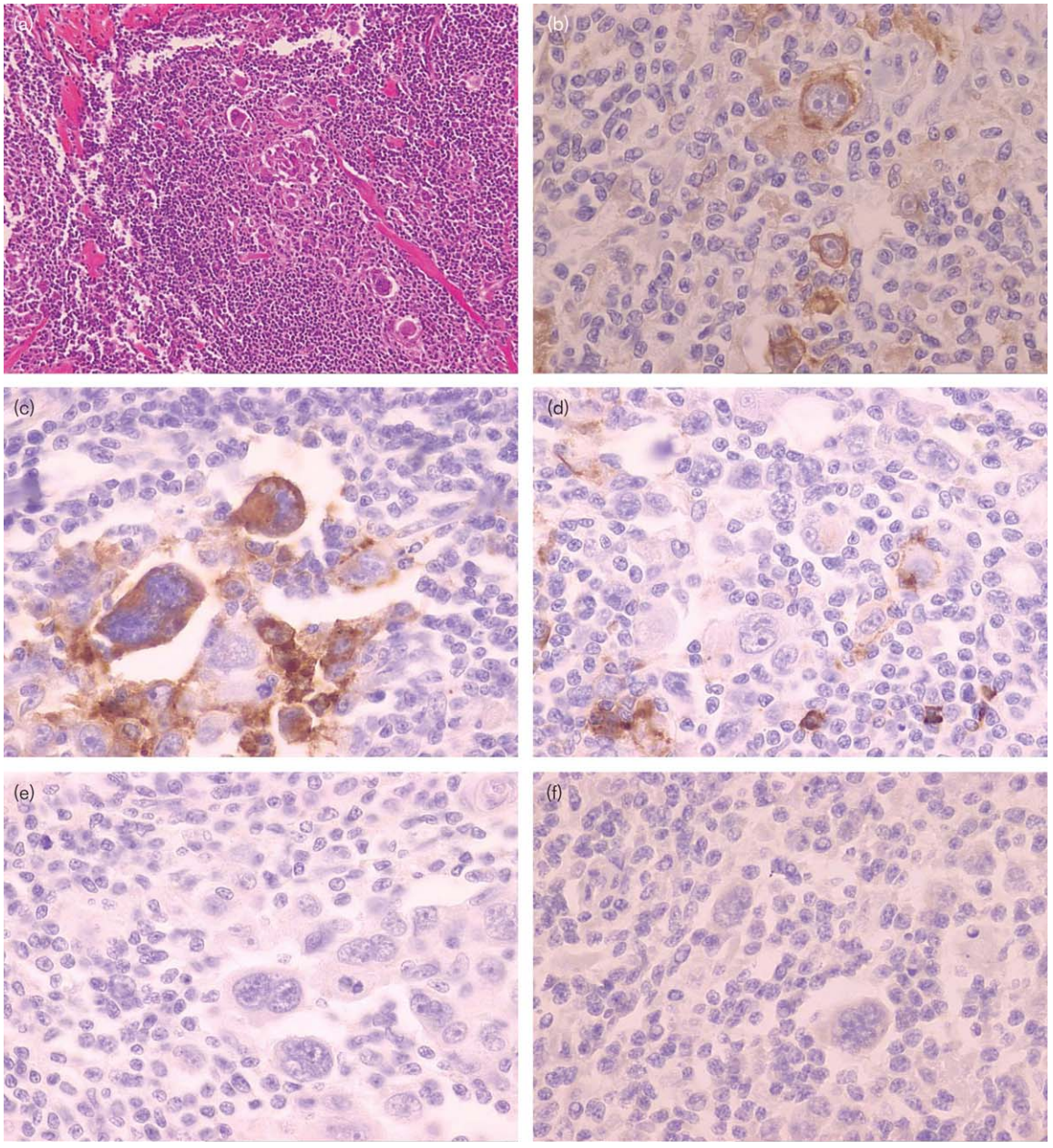
Results

Xenografted LY-3 tumor analyses

Morphologic examination of the patient's lymph node biopsy showed nodular proliferation of large Reed–Sternberg (RS) cells mixed with small lymphocytes, plasma cells and eosinophils. Tumor nodules were separated by large bands of fibrosis (Fig. 1a). Immunophenotyping showed CD30 (Fig. 1b) and CD15 (Fig. 1c) positivity, without expression of CD3 (data not shown) and CD20 antigens (Fig. 1d), nor expression of either EBV LMP1 (Fig. 1e) or EBNA-2 (Fig. 1f) proteins. Taken together, morphologic and immunophenotyping analyses concluded on classical nodular sclerosis Hodgkin's disease. Histologic examination of the xenograft LY-3 tumor showed diffuse proliferation of large cells with a high mitotic index (Fig. 1a′). Immunophenotyping indicated CD30 negativity (Fig. 1b′), CD15 negativity (Fig. 1c′), and high CD20 (Fig. 1d′), EBV LMP1 (Fig. 1e′), and EBNA-2 (Fig. 1f′) protein expression. Taken together, morphologic and immunophenotyping analyses concluded on EBV-associated human B-cell lymphoma.

Cytogenetic analysis performed on the lymph node biopsy showed a clone with a highly rearranged near-

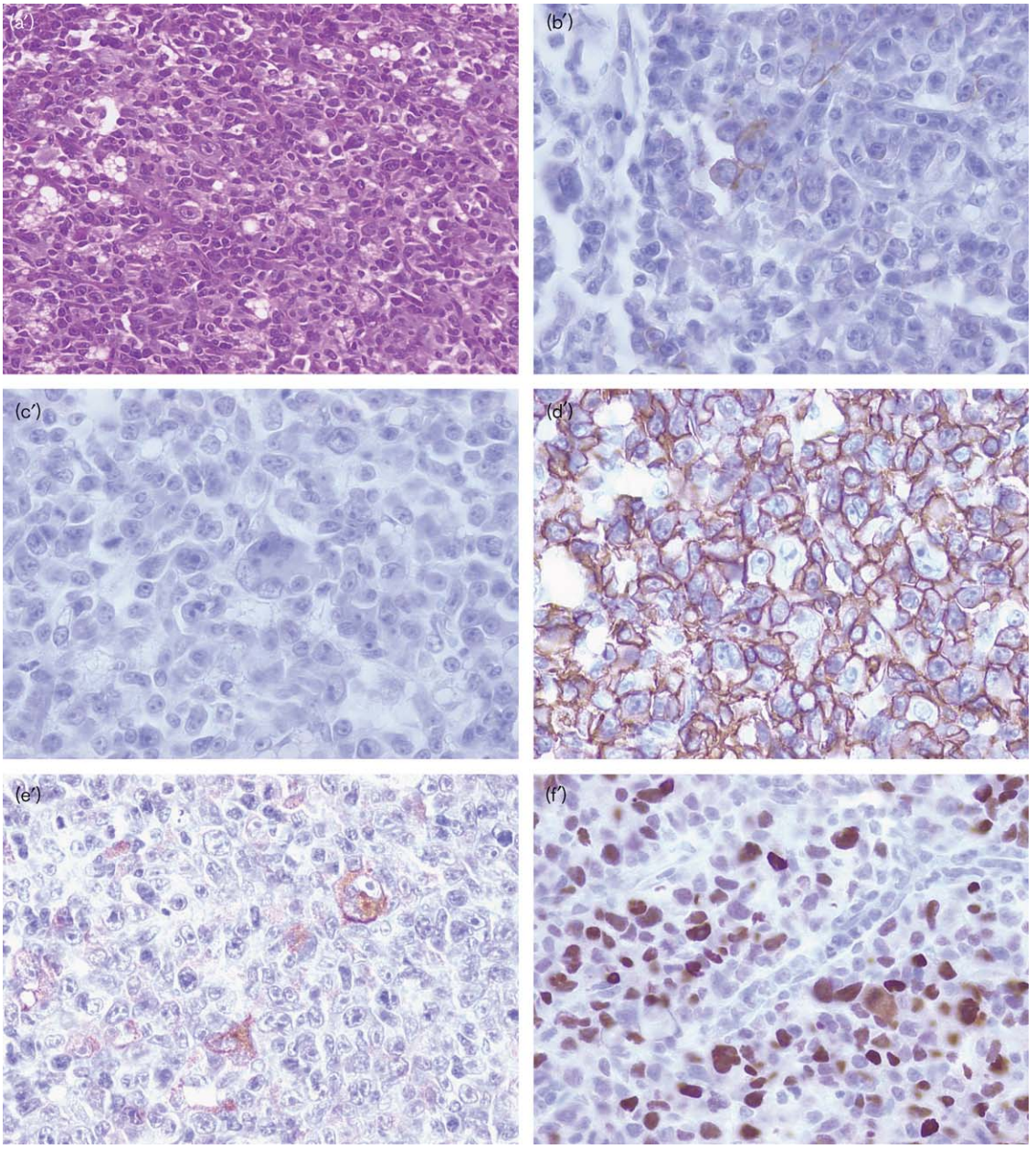
Fig. 1



(A) Histopathologic examination of the patient's biopsy. Morphologic examination and immunophenotyping concluded on classical nodular sclerosis Hodgkin's disease with nodular proliferation of large Reed–Sternberg cells mixed with small lymphocytes, plasma cells and eosinophils separated by large bands of fibrosis (hematoxylin–eosin–safran × 200; a), and CD30 positivity (b), CD15 positivity (c), CD20 negativity (d), LMP1 negativity (e), and EBNA-2 negativity (f) of tumor cells. (B) Histopathologic examination of the LY-3 tumor xenograft. Morphologic examination and immunophenotyping concluded on Epstein–Barr virus (EBV)-associated human B-cell lymphoma with diffuse proliferation of large cells with a high mitotic index (hematoxylin–eosin–safran × 200; a′), and CD30 negativity (b′), CD15 negativity (c′), CD20 positivity (d′), LMP1 positivity (e′), and EBNA-2 positivity (f′) of tumor cells.

diploid karyotype, while the xenografted LY-3 tumor presented a normal human karyotype (data not shown). CGH performed on the xenografted tumor resected 9 months later showed a gain of region 11q23–25 and all of chromosome 12 (Fig. 2a). Finally, PCR performed on both samples detected a clonal rearrangement of the IgH only in the xenografted tumor. Altogether, these results indicated that the in-vivo LY-3 xenograft did not grow from Hodgkin's disease-derived cells, but from the patient's EBV-infected memory B-lymphoid cells, as the

Fig. 1 (Continued)



EBNA viral DNA was amplified by PCR from both the patient tumor and the xenografted tumor (Fig. 2b). Clonal proliferation in mice led to the gain of chromosome 12 and a segment of 11q.

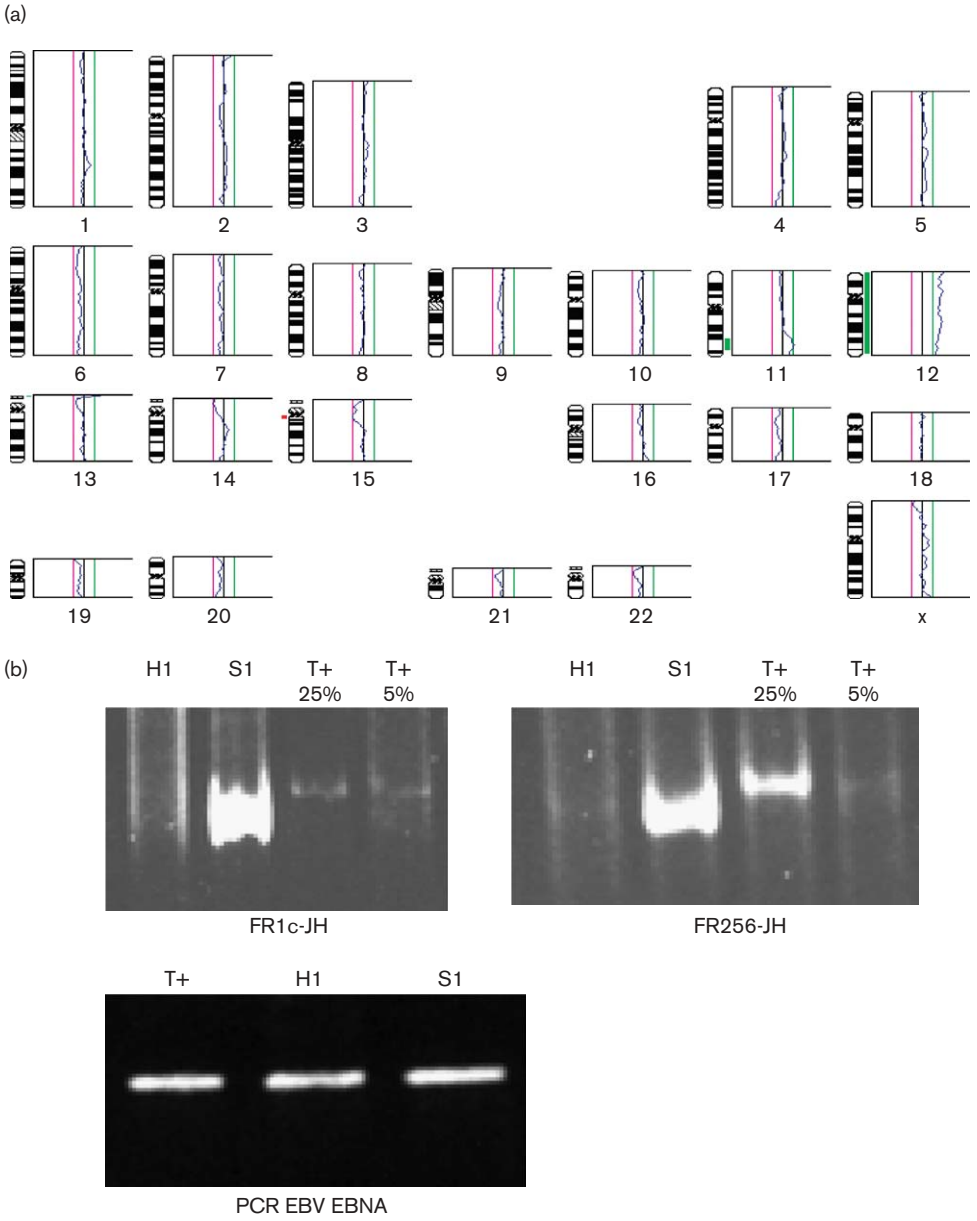
Analyses of the LY-3 cell line

Immunophenotyping of the LY-3 cell line showed a B-cell lymphoid proliferation with CD19 positivity (Fig. 3b–d), CD20 and CD22 positivity (Fig. 3e), CD3 negativity (Fig. 3b), CD5 negativity (Fig. 3c), CD10 negativity (Fig. 3d), and absence of expression of κ-light and λ-light chain immunoglobulins (Fig. 3f).

In-vivo experiments in severe combined immunodeficient mice bearing LY-3 tumors

Mice bearing tumors measuring approximately 60 mm^3 were treated twice weekly by an intraperitoneal injection of 50 mg/kg of rituximab, until sacrifice of the animals. Significant tumor growth inhibition was observed ($P < 10^{-2}$; Fig. 4a). LY-3-bearing mice received intraperitoneal injections of gemcitabine (60 mg/kg weekly until sacrifice of animals). As with rituximab, significant tumor growth inhibition ($P < 10^{-3}$) was observed in gemcitabine-treated mice (Fig. 4b). Finally, SCID mice bearing LY-3 tumors were treated by intraperitoneal injections of gemcitabine

Fig. 2



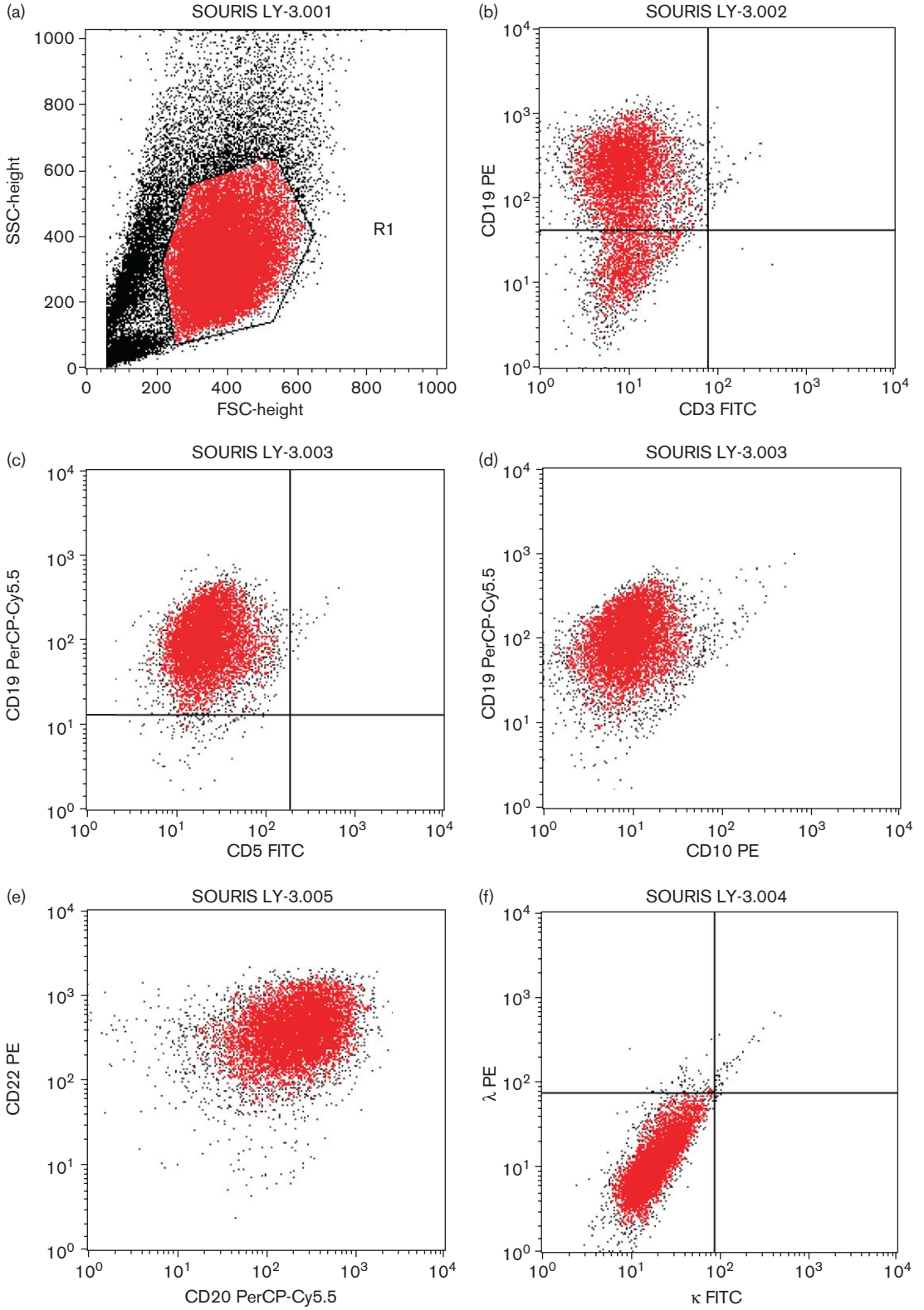
(a) Comparative genomic hybridization (CGH) of LY-3 tumor xenograft. CGH performed on the xenografted tumor resected 9 months later showed a gain of region 11q23–25 and all of chromosome 12. (b) Genetic analyses of the patient's biopsy and LY-3 tumor xenograft. Immunoglobulin heavy chain (IgH) rearrangement was detected by polymerase chain reaction (PCR) in both Hodgkin's lymphoma (H1) and LY3 tumor xenografted (S1). A clonal rearrangement using both FR1c-JH and FR256-JH primers was detected in the S1 tumor only. Dilutions of the positive control (T+) was needed to assess the sensitivity of the PCR experiment. A PCR amplification of the EBNA-1 locus was detected in H1 and S1.

(60 mg/kg weekly until sacrifice) and rituximab (50 mg/kg twice weekly until sacrifice). We observed an impressive effect of combined therapy compared with rituximab or gemcitabine alone ($P < 10^{-3}$; Fig. 4c).

Discussion

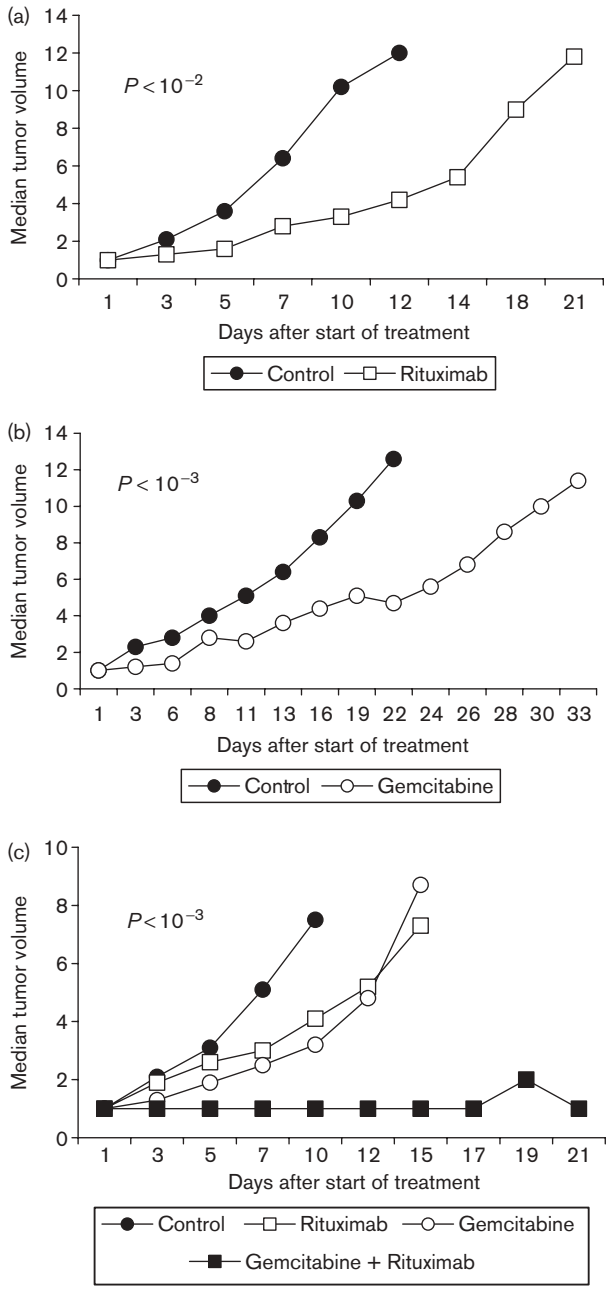
In this report, we describe an in-vivo model of EBV-associated lymphoproliferative disorder obtained from a Hodgkin's biopsy sample xenografted into immunodeficient mice. After morphologic, phenotypic and cytogenetic characterization and comparison with the primary biopsy, we evaluated various therapeutic approaches combining the monoclonal anti-CD20 antibody rituximab and single-agent chemotherapy that could be proposed in the treatment of posttransplant lymphoproliferative disorders. We observed a very high efficacy of the combination of rituximab and gemcitabine in SCID mice

Fig. 3



Analyses of the LY-3 cell line. Immunophenotyping of the LY-3 cell line was performed by flow cytometry with a panel of monoclonal antibodies in triple-color combination. Immunophenotyping of the LY-3 cell line showed a B-cell lymphoid proliferation with CD19 positivity (b–c), CD20 and CD22 positivity (e), CD3 negativity (b), CD5 negativity (c), CD10 negativity (d), and the absence of expression of κ-light chain and λ-light chain immunoglobulins (f).

Fig. 4



In-vivo experiments in severe combined immunodeficient (SCID) mice bearing LY-3 tumors. (a) Antitumor activity of rituximab in LY-3 xenografted tumors. Rituximab was administered by 2 weekly intraperitoneal injections at a dose of 50 mg/kg from day 1 until sacrifice of the animals (□); (b) SCID mice bearing the xenografted tumor were treated with intraperitoneal injections of gemcitabine 60 mg/kg weekly until sacrifice (○); (c) xenografted tumors were treated by 2 weekly intraperitoneal injections of rituximab at a dose of 50 mg/kg (□), one weekly intraperitoneal injection of gemcitabine at a dose of 60 mg/kg in one daily intraperitoneal injection (○), and combined rituximab and gemcitabine according to the same schedule as single-agent therapies (■), from day 1 until sacrifice. All control groups received injections of 0.9% NaCl according to the same schedule as experimentally treated mice (●). Tumor growth was evaluated by measuring the relative tumor volume, as described in Materials and methods. A Mann–Whitney *U*-test was used to assess the effects of rituximab and/or gemcitabine on LY-3 xenografted tumor growth.

transplanted with EBV-associated lymphoma, indicating that combined rituximab and gemcitabine could be an alternative approach in patients with PTLD.

The LY-3 tumor obtained in SCID mice grafted with lymph node invaded by Hodgkin's disease resembles tumors described in immunodeficient mice grafted with peripheral blood lymphocytes from EBV-seropositive donors [25], as the histologic features, immunophenotype (CD3 negativity, CD20 positivity, and CD15 negativity) and viral antigen expression (LMP1 and EBNA-2 gene products) of the LY-3 xenograft were the same as those reported in the literature. This tumor was also comparable to human EBV-positive lymphoproliferations observed in posttransplant patients [26,27]. In agreement with a previous report [25], EBV clonality was correlated with clonal IgH rearrangement as determined by PCR. Overall, this observation is concordant with that reported by Meggetto *et al.* [28] showing that 10 EBV-associated tumors but no Hodgkin's tumors were obtained in a series of 25 Hodgkin's disease lymph node tissue samples xenografted into SCID mice. Our results and those of other studies show that RS cells isolated from fresh lymph nodes involved by Hodgkin's disease are not tumorigenic in SCID mice. In contrast, some viral proteins, such as EBNA-2 not expressed by RS cells, as well as the lack of EBV-specific T-cell response in SCID mice observed in patients with acquired severe immunosuppression, such as posttransplant status, confer a growth advantage to bystander EBV-positive lymphocytes present in the Hodgkin's disease patient's biopsy sample. This finding is reinforced by a correlation between the number of EBV-positive small lymphocytes detected in Hodgkin's disease and the percentage of EBV-positive tumors obtained in SCID mice [28]. Altogether, these data establish a new in-vivo model of posttransplant lymphoproliferative disorder in severely immunodeficient mice that could be used to determine new therapeutic approaches.

We observed that the chimeric monoclonal anti-CD20 antibody rituximab induced tumor growth inhibition in our in-vivo EBV-associated CD20-positive lymphoma. Rituximab is a chimeric monoclonal antibody directed against CD20 antigen [29]. It acts through complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity [30], which constitute the two main mechanisms of its action, and through direct antiproliferative and proapoptotic activity [31]. The rituximab-induced tumor growth inhibition that we observed is in line with previous reports showing regression of posttransplant lymphoproliferative disorders after rituximab immunotherapy. This effect, presented in Table 1, has been observed in both hematologic malignancies [7,8,32] and solid tumors [8,9,33–36]. The overall response and complete response rates of rituximab

alone are 76 and 71%, respectively. Outcome data of published series are heterogeneous and four patients among the 22 patients who achieved response relapsed after a median interval of 7 months [8]. These results clearly demonstrate the efficacy of rituximab immunotherapy in the treatment of PTLD, but without any evidence of prolonged and durable remission. It therefore appears necessary to combine other treatments with immunotherapy to significantly improve the outcome of PTLD patients. Few clinical reports have evaluated the combination of rituximab with other treatments (Table 2), namely rapamycin [37], a combination of immunoglobulin, prednisone and interferon [38], reduction of immunosuppression [39], chemotherapy [15,40,41], radiotherapy [40] or surgery [40]. These results concern a small number of patients that do not allow any definitive conclusions on the efficacy of these combinations.

Various reports have also evaluated the role of gemcitabine in malignant lymphoma, as shown in Table 2. Gemcitabine is a pyrimidine antimetabolite characterized by a unique mechanism of action, which includes cytotoxic self-potentiation, masked DNA chain termination and potent inhibition of DNA repair [42]. Gemcitabine has shown a peculiar efficacy in relapsed or refractory T-cell non-Hodgkin's lymphoma (NHL) with a mean response rate and complete response rate of 68 and 13%, respectively [43,44]. In contrast, intermediate results were obtained in Hodgkin's disease with a mean response rate of 32% [45–47], and a poorer response was observed in intermediate/high-grade lymphomas and low-grade lymphomas with a mean response rate of 23% [48,49] and 14% [50–52], respectively. Gemcitabine was also combined in chemotherapy regimens, giving better responses in NHL. A study of a small series of elderly patients with relapsed diffuse large B-cell lymphoma treated with rituximab and gemcitabine showed a response rate of 71% with 29% of complete responses and a median time to progression of 12 months [53]. This observation is concordant with the one showing that pretreatment of NHL tumor cells with rituximab sensitizes drug-resistant cells to drug-mediated cytotoxicity, such as that induced by gemcitabine [54]. In our

Table 1 Rituximab therapy in posttransplant lymphoproliferative disorders

References	Risk factor	*N*	Treatment	% RR (CR)	Outcome
Faye *et al.* [7]	HM	1	rituximab	100 (100)	6 months CR
Cook *et al.* [33]	lung transplant	3	rituximab	67 (67)	6 and 8 months CR
Milpied *et al.* [8]	SOT (26) + HM (6)	32	rituximab	69 (62)	OS_1 73% 4 relapses/22
Zompi *et al.* [39]	liver transplant	3	rituximab + reduction of immunosuppression	67 (67)	–
Dotti *et al.* [40]	SOT	5	rituximab after CT or RT or surgery	100 (60)	–
Faye *et al.* [32]	HM	12	rituximab ± DLI	67 (67)	0 relapse/8
Skoda-Smith *et al.* [41]	HM	1	rituximab after CT and ASCT	100 (100)	10 months CR
Jenkins *et al.* [38]	HM	1	rituximab + IVIG + ganciclovir + IFN + prednisone	100 (100)	14 months relapse
Ganne *et al.* [9]	SOT	8	rituximab	87 (87)	No relapse/7
Garcia *et al.* [37]	Renal transplant	2	rituximab + rapamycin	100 (100)	–
Orjuela *et al.* [15]	SOT	6	rituximab + cyclophosphamide + prednisone	100 (83)	1 year median duration of response
Yedibela *et al.* [34]	Liver transplant	2	rituximab	100 (50)	2 and 10 months deaths
Ghobrial *et al.* [35]	SOT	15	rituximab	73 (–)	median OS 13 months
Savoldo *et al.* [36]	Liver transplant	6	rituximab	100 (100)	3 relapses/6

RR, response rate; CR, complete response; HM, hematologic malignancies; SOT, solid organ transplantation; OS_1, 1-yearoverall survival; CT, chemotherapy; RT, radiotherapy; DLI, donor lymphocyte infusion; ASCT, autologous stem cell transplantation; IVIG, intravenous immunoglobulin.

Table 2 Gemcitabine in relapsed or refractory malignant lymphomas

References	Type of lymphoma	*N*	Treatment	% RR (CR)	Outcome
Fossa *et al.* [48]	DLBCL	30	G	20 (0)	6 months MTP
Santoro *et al.* [45]	HD	22	G	39 (9)	6 months MTP
Savage *et al.* [49]	DLBCL and MCL	13	G	31 (8)	2-20 months MTP
Zinzani *et al.* [46]	HD	14	G	43 (14)	–[a]
Zinzani *et al.* [43]	cutaneous T-cell NHL	44	G	70 (11)	10–15 months MTP
Dumontet *et al.* [50]	low-grade NHL	36	G	25 (6)	5 months MTP
Sallah *et al.* [44]	T-cell NHL	10	G	60 (20)	13.5 months MTP
Venkatesh *et al.* [47]	HD	29	G	22 (0)	6 months MTP
Ganjoo *et al.* [51]	FL and SLL	13	G	8 (0)	2 months MTP
Larson *et al.* [52]	low-grade NHL	20	G	0 (0)	–
Wenger *et al.* [53]	DLBCL	7	G–rituximab	71 (29)	12 months MTP

RR, response rate; CR, complete response; MTP, median time to progression; G, gemcitabine; HD, Hodgkin's disease; FL, follicular lymphoma; SLL, small lymphocytic lymphoma; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma.
[a]Three patients in partial response were treated by involved-field irradiation or autologous bone marrow transplantation. The two patients in CR are still in remission after 12 and 15 months.

report, we observed an impressive efficacy of combined rituximab and gemcitabine in EBV-associated lymphoma. Despite the fact that no data are currently available on the efficacy of gemcitabine alone on posttransplant lymphoproliferations, our results and those reported by Wenger *et al.* suggest that combined rituximab and gemcitabine could be an effective treatment for PTLD.

In conclusion, our study characterized the histopathologic, cytogenetic and genetic features of a new EBV-associated human B-cell lymphoma obtained after Hodgkin's xenograft in immunodeficient mice. This model resembles posttransplant lymphoproliferative disease. We evaluated the therapeutic potential of a combination of rituximab and gemcitabine in xenografted tumors in SCID mice, and observed a high level of efficacy of this treatment. This observation therefore suggests that combined rituximab and gemcitabine could be an effective treatment of PTLD.

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