A apoptotic killing of B-chronic lymphocytic leukemia tumor cells by allicin generated in situ using a rituximaballiinase conjugate

Fabian D. Arditti, Aharon Rabinkov, Talia Miron, Yair Reisner, Alain Berrebi, Meir Wilchek, and David Mirelman

Introduction

Allicin, a highly active component from freshly crushed garlic, is produced upon the reaction of the small molecular weight molecule alliiin, with the enzyme alliinase (EC 4.4.1.4). Because alliiin was shown to be toxic to various mammalian cells in vitro, we devised a novel approach for the therapy of B-cell malignancies based on site-directed generation of alliiin. Alliiinase was conjugated to the monoclonal antibody rituximab, which recognizes the CD20 antigen, and the resulting conjugate was targeted to CD20+ B chronic lymphocytic leukemia (B-CLL) and other B-cell lymphomas. Upon addition of alliiin, alliiin was formed in situ, killing the CD20+ tumor B cells via apoptosis. Following a 72-hour treatment, an 85% and 96% reduction was observed in the number of viable B-CLL and EBV-transformed B cells, respectively. Using the human/mouse radiation chimera for the evaluation of allicin, we showed a significant reduction in the number of recovered B-CLL, mantle cell lymphoma, or EBV-transformed B cells. We conclude that our system offers a new powerful and less toxic therapy for B-CLL and other B-cell malignancies. Furthermore, combining alliiinase with the appropriate monoclonal antibody may extend the application of this approach to other conditions in which the elimination of a specific cell population is desired. [Mol Cancer Ther 2005;4(2):325–31]

Materials and Methods

Chemicals

3-(2-Pyridyldithio) propionic acid N-hydroxysuccinimide ester (SPDP), DTT, and pyridoxal 5'-phosphate were purchased from Sigma (St. Louis, MO). Alliiin was synthesized as described (16). Allicin was prepared by applying synthetic alliiin onto an immobilized alliinase (EC 4.4.1.4) with its substrate, alliiin (S-allyl-L-cysteine sulfoxide; ref. 1), has become in recent years an object of interest in light of its antiproliferative and cytotoxic effect (2–5). Alliiinase and alliiin are enclosed in different compartments within the garlic clove, so that intact garlic cloves do not contain alliiin but rather its precursor, alliiin. Following crushing of the garlic clove, alliiin is exposed to alliiinase, which results in the generation of allicin. Allicin is an unstable, short-lived hydrophobic molecule that penetrates biological membranes with ease and reacts rapidly with free thiol groups (6–8). Therefore, it disappears from the circulation within a few minutes after injection (9). This explains why the versatile and valuable attributes of allicin, including its potent antibiotic and cytotoxic effects, were shown thus far mostly in vitro (3, 4, 6). Nevertheless, we have recently shown that allicin can kill tumor cells if it is generated on the surface of target cells. This was achieved using as a delivery system monoclonal antibodies which have emerged as important therapeutic agents against a number of malignancies (10–14). In our previous study, we conjugated the enzyme alliinase to a monoclonal antibody (mAb) directed against ErbB2, which is expressed on the surface of several tumor cells (15). After the mAb-alliiinase conjugate was bound to target tumor cells, the substrate, alliiin, was added. In the presence of alliiin, the tumor-anchored alliiinase produced allicin, which killed tumor cells expressing ErbB2 whereas normal tissues were unharmed. In the present study, we show that the same principle can also be applied to kill malignant B cells expressing either low or high levels of CD20 [human B chronic lymphocytic leukemia (B-CLL) cells or B-cell lymphomas, respectively]. Targeting of alliiinase to CD20+ tumor cells was achieved by its conjugation to the humanized anti-CD20 mAb rituximab (Rituxan Mabthera), which is extensively in use in the clinic, mainly for non-Hodgkin lymphomas. Our results show that alliiin generated in situ by the targeted rituximab-alliiinase conjugate following the addition of alliiin was able to kill CD20+ B-CLL cells as well as two other human B-cell lymphoma lines, both in vitro and in vivo.
Biochemical Analysis
Protein concentration was measured at 280 nm, using \( E_{280} = 77,000 \text{ mol/L} \cdot \text{cm}^{-1} \) for alliinase and 210,000 mol/L \cdot \text{cm}^{-1} \) for purified mAb \((E_{280} = 1.54)\). The number of SPDP residues on the modified proteins was determined according to ref. 20. Purification of alliinase was done as described (21). Quantitative determination of alliin and allicin was done by high-performance liquid chromatography as well as by reaction with 2-nitro-5-thiobenzoate (18, 22).

Enzymatic Activity
Aliinase activity was determined by the 2-nitro-5-thiobenzoate method as described (18). The resulting enzymatic activity of the Rituxan-Alliinase conjugate was 104 units/mg protein. A unit of activity was defined as the amount of enzyme required to release 1 \( \times 10^4 \) units/mg protein. A unit of activity was defined as the amount of enzyme required to release 1 \( \mu \text{mol} \) of pyruvate per minute. An ELISA system using 96-well plates was developed to assay the activity of the enzyme conjugates in a cell-free system. Wells were coated with protein A (5 \( \mu \text{g/mL} \)) and quenched with bovine serum albumin (1% in PBS). Binding of PBS-diluted mAb-alliinase conjugates to the wells was done at 24°C for 2 hours. After removal of unbound conjugates, the activity of adsorbed conjugated alliinase was determined by using 2-nitro-5-thiobenzoate (3 \( \times 10^{-4} \) mol/L) containing alliin (0.6 \( \times 10^{-3} \) mol/L) in 50 mmol/L phosphate buffer, and 2 mmol/L EDTA (pH 7.2). The decrease in \( A_{412} \) nm was determined after 60 minutes. Wells to which no enzyme was added served as control.

Protein Modifications
SPDP modification of proteins was done as described (15). The modified alliinase was stored in 50% glycerol at -20°C. Modification of rituximab (Hofmann-La Roche, Basel, Switzerland) was done with a 10 molar excess of SPDP over the mAb. The degree of modification was 2.5 to 2.9 SPDP residues/mAb molecule. The modified rituximab was stored in PBS at 4°C. Preparation of rituximab-alliinase conjugate was done as described (15). Conjugates were stored at -20°C in 50% glycerol.

Preparation of Human/Mouse Chimera
Chimeric mice for in vivo experiments were prepared as described (23). Briefly, BALB/c mice were exposed to split lethal total body irradiation. One day later, irradiated mice were injected with bone marrow cells from non-obese diabetic/severe combined immunodeficient mouse donors through the tail vein. All strains of mice were from the Weizmann Institute Animal Breeding Center (Rehovot, Israel). All the animal studies and protocols were approved by the Weizmann Institutional Animal Care and Use Committee.

Tumor Cells and Cultures
B-CLL peripheral blood mononuclear cells (PBMC) were obtained from heparinized whole blood drawn from five patients at Rai stage IV with their informed consent. Blood cells were subjected to Ficoll density gradient centrifugation and the mononuclear cells were diluted to the desired concentration before their injection into mice (100 \( \times 10^6 \) cells/mouse, by an i.p. injection) or in vitro (10^6 cells/mL/well, in 24-well plates) treatment. CD20+ mantle cell lymphoma (MCL) line was kindly provided by G. Inghirami (NYU, New York, NY). A CD20+ EBV immortalized B-cell line is routinely grown in our lab. Because both of these cell lines induce rapid proliferating tumors, cells were injected i.p. at 4 \( \times 10^5 \) cells/mouse or cultured in 24-well plates at 2 \( \times 10^5 \) to 3 \( \times 10^5 \) cells/mL/well. Cells were recovered by peritoneal lavages or sampled from the culture well and analyzed for the presence of the below-mentioned surface markers by fluorescence-activated cell sorting (FACS) analysis.

Fluorescence-Activated Cell Sorting Analysis
The viability of cells isolated from peritoneal washes of engrafted mice was determined 7 to 10 days after the inoculation of human cells by trypan blue dye exclusion or following incubation with a mixture of monoclonal antibodies labeled with different fluorochromes (see below) by FACS analysis. Incubation with the antibodies was for 20 minutes at 4°C. After washing off the antibodies, two or three color analyses were done using a FACSscan analyzer (Becton Dickinson, Franklin Lakes, NJ). The following labeled anti-human antibodies were used to recognize specific surface molecules: CD19-APC (Pan B cells/B-CLL), CD3-PercP (Pan T cell), and CD45-PercP (pan-leukocytes) were purchased from Becton Dickinson; CD20-FITC (B cells, some B-CLL) and CD5-PE (T cells, B-CLL tumor B cells) were purchased from DAKO, Glostrup, Denmark.

Detection of Apoptosis by Annexin Staining
Samples from in vitro cultures as well as cell samples from peritoneal washes were incubated with a mixture of selected monoclonal antibodies as described above. After washing off the unbound free antibodies, samples were incubated with 5 \( \mu \text{L} \) of Annexin-APC (Bender MedSystems, Vienna, Austria) or Annexin-CY5 (PharMingen, San Diego, CA) in annexin buffer for 10 minutes at room temperature. Subsequently, unbound annexin was washed out and samples were analyzed by FACS as described above.

Competitive Inhibition of Conjugate Binding by Rituximab
To examine the CD20 antigen specificity of allicin killing by the rituximab-alliinase conjugate, target cells were preincubated for 30 minutes at 4°C with rituximab alone in excess (20 \( \mu \text{g/mL} \) cells). Following preincubation, unconjugated rituximab was washed off and the cells were incubated with the rituximab-alliinase conjugate at different concentrations under the same conditions as with rituximab alone. After washing off unbound conjugate, the cells were transferred to 6- or 24-well plates and incubated in the presence or absence of the substrate alliin. Cells were assayed for annexin staining and for surface markers by FACS analysis at different time intervals.

Statistical Analysis
Statistical significance was established by the Student’s \( t \) test. In vitro experiments were repeated three to five times and in vivo experiments were done thrice. Results are shown as mean ± SD.
Results

Allicin Induces In vitro Apoptosis in a Dose-Dependent Manner

In order to examine whether allicin could override the antiapoptotic barriers in tumor B-CLL cells, we cultured B-CLL PBMC from B-CLL patients at stage IV in the presence of increasing concentrations of pure allicin. As shown in Fig. 1, a dose-dependent apoptotic killing (Annexin-V+) of B-CLL tumor cells was induced by allicin following 48 hours in culture. The percentage of apoptotic B-CLL cells increased from background levels (6.6 ± 0.6%, mean ± SD.) in the absence of allicin to reach a plateau of around 95% at allicin concentrations of 6.5 to 8.1 μg/mL.

Killing of CD20+ Tumor B Cells In vitro by Allicin Generated In situ by Rituximab-Alliinase Conjugate

In contrast to most B-cell malignancies, the CD20 antigen is expressed at low levels on B-CLL cell surface. This fact could explain the failure of rituximab alone to induce a considerable response in patients with this malignancy (11, 24–26). Therefore, we used rituximab as a targeting device to direct the enzyme alliinase to the surface of CD20+ B-CLL cells, where it can generate cytotoxic allicin upon addition of the substrate, alliin. PBMC (10^6 cells/well) from B-CLL patients at Rai stage IV (n = 5) were incubated with or without (untreated) 4 μg/mL rituximab-alliinase conjugate. Following removal of the unbound conjugate molecules, alliin (100 μg/mL) was added to part of the cells previously incubated with the conjugate (Rtx-all + alliin). As seen in Table 1, allicin generated in situ by the conjugate induced a substantial reduction in the number of living cells (CD19+ Annexin- ) following 48 hours from the addition of alliin. This result was highly reproducible with the cells of all five patients tested.

To further validate these results, we evaluated the dependence of this effect on different concentrations of conjugate. Following removal of unbound conjugate from B-CLL cell cultures incubated with different concentrations of the conjugate, tumor cells were incubated in the presence or absence of alliin. After 48 hours in culture, cells from different treatment wells were counted and tested for the detection of apoptotic tumor B cells by anti CD19-FITC as well as by Annexin-Cy5 using FACS. As shown in Fig. 2A, at 4 μg/mL conjugate and 100 μg/well alliin, a significant (60 ± 10%) decrease in the amount of viable (annexin-negative) cells was observed (from 16.9 × 10^3 ± 1.7 × 10^3 cells to 7.7 × 10^3 ± 0.8 × 10^3 cells, mean ± SD). Additionally, we tested the effect of increasing doses of allicin generated by increasing amounts of conjugate on B-cell lymphomas expressing high levels of CD20. As found for B-CLL, allicin generated in situ induced a strong dose-dependent killing of CD20+ EBV immortalized cells as well as of MCL cells (Fig. 2A).

In a separate experiment, we evaluated the response of tumor cells to increasing amounts of allicin generated by increasing concentrations of alliin using a constant amount of conjugate (4 μg/well). We found that the number of apoptotic B-CLL cells increased with the concentration of added alliin. Thus, after the addition of 40, 80, and 160 μg/well of alliin, the number of apoptotic cells increased from a background level of 20% to 61%, 81.7%, and 93.7%, respectively (Fig. 2B). We also examined the progression of the effect mediated by allicin on tumor B-cell surface over time. As seen in Fig. 3, the number of apoptotic (CD19+ Annexin-V+) tumor B cells, exposed to allicin generated in situ, increased over time, with a highest number of apoptotic cells at the latest time point tested (72 hours). In contrast, when a fixed amount of pure allicin (not generated in situ) was added to the cells, there was no increase in cell death with time. Furthermore, cell death was not observed when conjugate treatment was not followed by the addition of the substrate alliin. In addition, cells that do not contain the CD20 antigen, such as the neuroblastoma cell line LAN-1 or T cells, were not targeted nor damaged by the rituximab-alliinase conjugate (data not shown).

Alliinase Activity Is Specifically Delivered to CD20+ Cells by Rituximab

The specificity of the rituximab-alliinase conjugate was investigated by preincubation of the target cells with excess of free rituximab to block the binding sites of the subsequently added mAb-conjugated alliinase. As shown in Fig. 4, preincubation of B-CLL or EBV immortalized cells with free rituximab blocked subsequent binding of the conjugate to tumor B cells and prevented their killing.

Tumoricidal Effect of Targeted Allicin In vivo

In order to assess the in vivo antitumor potential of rituximab-alliinase targeted allicin in a preclinical animal model, we used the human/mouse radiation chimera, which allows engraftment of human tissues, including hematopoietic tumors, in mice (11, 23, 24, 27–29). Chimeric
mice were prepared as described (28, 30). Seven to ten days after i.p. infusion of either 100 × 10^6 fresh B-CLL PBMC, 4 × 10^6 MCL cells, or 4 × 10^6 EBV immortalized B cells, mice were divided into three treatment groups, each consisting of six mice treated as follows: control (only PBS), conjugate only (80 µg/mouse), and conjugate (80 µg/mouse) followed 1 day later by alliin (3 mg/mouse, twice a day, during 3 days). As shown in Fig. 5, a single injection of the conjugate followed by repeated administration of alliin induced a significant reduction in the amount of recovered B-CLL from 63 × 10^5 ± 12.5 × 10^4 to 16.5 × 10^3 ± 3.5 × 10^4 (n = 3, P < 0.03), MCL from 5.3 × 10^4 ± 9.6 × 10^4 to 12 × 10^4 ± 3.2 × 10^3 (n = 3, P < 0.03), and EBV immortalized B cells from 75.4 × 10^4 ± 8.7 × 10^4 to 15.6 × 10^3 ± 2.6 × 10^3 (n = 3, P < 0.01). These effects were observed in peritoneal cell samples taken 24 hours after the last alliin injection.

**Discussion**

We have recently reported the specific and efficient killing of ErbB2+ tumor cells by allicin molecules that were generated in situ from alliin by a conjugate consisting of the enzyme alliinase and a monoclonal antibody specific against ErbB2 (15). In the present study, we show that generation of allicin molecules in situ by a rituximab-alliinase conjugate can be used to kill leukemia/lymphoma cells expressing the CD20 cell surface molecule. Generation of allicin at the target tumor cell surface is catalyzed from the substrate Alliin by the enzyme alliinase, which was chemically ligated to the commercially available humanized anti-CD20 mAb, rituximab. Our in vitro and in vivo results show that following the addition of alliin, allicin was generated in situ by the rituximab-alliinase conjugate and this killed B-CLL tumor cells, which express low levels of CD20, as well as two B-cell lines (MCL and EBV transformed B-cell line), which express higher levels of this surface antigen. In contrast, CD20-negative cells like the neuroblastoma LAN-1 or T cells were not affected by the rituximab-alliinase conjugate.

Considering that purified B-CLL cells die shortly in culture if separated from their feeder cells (31–33), we used in our in vitro assays fresh unseparated PBMCs from five B-CLL patients, which contained >90% double positive CD19+CD5+ pathologic cells. These cells survive in culture in the absence of any manipulation for a period of at least 1 to 2 weeks (30). The humanized mAb rituximab is, at present, in extensive clinical use for the treatment of non-Hodgkin lymphoma, which express high levels of CD20 on the cell surface (34–36). In contrast to non-Hodgkin lymphoma and most tumor cells, B-CLL consists of nondividing cells that express low levels of CD20. Nevertheless, several studies have been conducted to analyze the efficacy of standard, escalated, or fractionated doses of rituximab in B-CLL. Overall responses were moderate and complete remission was only occasionally achieved (11, 24–26).

Although a number of studies reported the ability of rituximab to induce apoptosis by itself, the bulk of its cytotoxic activity is known to be mediated by the opsonization of the target cell, leading to antibody-dependent cell cytotoxicity and complement fixation. However, the immune status of the patient is crucial to allow an effective immune response and, very often,

**Table 1. Antitumor activity of allicin generated in situ by the rituximab-alliinase conjugate and alliin**

<table>
<thead>
<tr>
<th>B-CLL patients*</th>
<th>Untreated</th>
<th>Rtx-all</th>
<th>Alliin</th>
<th>Rtx-all+alliin</th>
<th>% Tumor eradication†</th>
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<tr>
<td>#1</td>
<td>11,547</td>
<td>11,267</td>
<td>11,547</td>
<td>4,817</td>
<td>58</td>
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<tr>
<td>#2</td>
<td>11,280</td>
<td>9,131</td>
<td>10,604</td>
<td>3,454</td>
<td>69</td>
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<tr>
<td>#3</td>
<td>11,079</td>
<td>11,331</td>
<td>N/T</td>
<td>4,727</td>
<td>57</td>
</tr>
<tr>
<td>#4</td>
<td>10,800</td>
<td>12,000</td>
<td>11,625</td>
<td>2,745</td>
<td>75</td>
</tr>
<tr>
<td>#5</td>
<td>12,000</td>
<td>12,000</td>
<td>14,800</td>
<td>1,600</td>
<td>87</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>11,341 ± 410</td>
<td>11,145 ± 1,055</td>
<td>12,143 ± 1,585</td>
<td>3,468 ± 1,218</td>
<td>69 ± 11</td>
</tr>
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</table>

**SS lymphoma‡**

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<tr>
<td>SS1</td>
<td>20,100</td>
<td>21,000</td>
<td>20,400</td>
<td>1,800</td>
<td>91</td>
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<tr>
<td>SS2</td>
<td>21,452</td>
<td>20,319</td>
<td>18,540</td>
<td>4,215</td>
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<td>SS3</td>
<td>24,300</td>
<td>24,300</td>
<td>20,700</td>
<td>1,440</td>
<td>94</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>21,950 ± 2,144</td>
<td>21,872 ± 2,129</td>
<td>1,988 ± 11,700</td>
<td>2,484 ± 1,509</td>
<td>88 ± 7</td>
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</table>

**EBV lymphoma§**

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<tbody>
<tr>
<td>MCL1</td>
<td>16,935</td>
<td>17,072</td>
<td>13,683</td>
<td>2,755</td>
<td>84</td>
</tr>
<tr>
<td>MCL2</td>
<td>25,500</td>
<td>25,500</td>
<td>26,500</td>
<td>5,500</td>
<td>78</td>
</tr>
<tr>
<td>MCL3</td>
<td>16,800</td>
<td>15,600</td>
<td>18,800</td>
<td>3,600</td>
<td>79</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>19,744 ± 4,985</td>
<td>19,390 ± 5,342</td>
<td>19,661 ± 6,452</td>
<td>3,951 ± 1,406</td>
<td>80 ± 3</td>
</tr>
</tbody>
</table>

* B-CLL obtained from five patients in stage IV with their informed consent.
† This value was calculated as follows: [(untreated – (Rtx-All-alliin)) / untreated] × 100.
‡ SS lymphoma is an EBV immortalized human B cell line grown routinely in our laboratory and MCL is a cell line (Grantas) derived from human Mantle cell lymphoma.
patients are immunocompromised as a result of previous cycles of chemotherapy. Moreover, large amounts of the therapeutic antibody are required to counteract complement inhibitory molecules (i.e., CD55 and CD59), which are present on the surface of malignant B cells (37, 38).

In our study, we enhance the capacity of rituximab to cause tumor cell death by using it as a targeting element. The specificity of rituximab enabled us to anchor the enzyme alliinase to the cell surface of CD20+ tumor cells. Because CD20 is not internalized following its binding to rituximab (24, 35), the prolonged presence of the conjugate on the cell surface and repeated administration of high doses of the substrate allin enabled the continuous in situ enzymatic generation of cytotoxic allicin molecules. Allicin, being a hydrophobic molecule easily, penetrates through membranes and reacts with thiols immediately afterwards (7, 8), thus targeting its effect only in the cells on which it is produced.

The in situ generation of allicin on the targeted cells overcomes the known problem of the short-lived allicin molecules in the circulation. Clinical trials will be needed, however, to verify the lack of toxicity of allicin in humans following its generation in situ. These trials should test also the immunogenicity of the conjugate as a new entity, although the humanized structure of rituximab and the fact that alliinase is a dietary protein that is "known" to the human immune system are expected to minimize unwanted reactivities.

Allicin killed tumor B cells by apoptosis, which could be detected by Annexin-V in in vitro assays. However, owing to annexin being known to occasionally bind nonspecifically to viable B cells in vivo (39), we evaluated our in vivo assays also by determining the amounts of CD19+CD45+ double positive cells recovered from the peritoneum of engrafted mice as well as by counting trypan-blue-positive versus -negative cells.
The combination of FACS analysis of peritoneal washes of mice from different treatment groups and trypan blue dye exclusion method allowed the quantitative analysis of the antitumor effect mediated by allicin generated in situ.

The main advantages of the mAb-alliinase conjugate over previously devised antibody-directed enzyme prodrug therapies, immunotoxins, or drug-conjugated antibodies are (a) the very low concentrations of alliinase-mAb conjugate needed for injection because the cytotoxic allicin molecules are continuously generated on the surface of the target cell following the repeated administrations of alliin; (b) the possibility to administer large amounts of the inert prodrug alliin, which is a natural ingredient of garlic; and (c) the selectivity of the allicin killing mechanism.

In order to exert their effect, conjugates of antibodies with toxins or cytotoxic drugs have to penetrate the target cell membrane either by internalization via the antigen receptor or by endocytosis. High concentrations of conjugates are needed because each antibody molecule carries only one or two toxin or drug molecules, and although they are capable of killing the targeted cells, they can also affect normal cells and cause them damage as well. The mode of action of allicin is entirely different: rather than interacting with DNA, allicin chemically modifies free SH (thiol) groups that are usually present in peptides or proteins. Its action with thiol containing proteins. Biochim Biophys Acta 1998;1379: 330–44.

In cancer cells which have a reduced level of reduced glutathione due to long-lasting oxidative stress, the cytotoxic effect of allicin could be even more pronounced.

**Figure 4.** The specificity of the conjugate rituximab-alliinase. Fresh B-CLL PBMC or EBV immortalized B cells (1 × 10⁶/well) were preincubated with (*) or without (●) 20 μg free rituximab for 30 min at 4 °C. Following removal of unbound rituximab, the cells were incubated with either 1 or 4 μg of conjugate under the same conditions as in preincubation. Following removal of excess conjugate, cells were plated and cultured in the presence of alliin (100 μg). Cells were alternatively treated with alliin alone as a negative control. Cell survival was calculated after 72 h in culture by the percentage of CD19−Annexin-V negative cells from total CD19+ cells. Columns, mean (B-CLL, n = 3 for B-CLL samples from three different patients; EBV immortalized B-cell line, n = 3 for three independent experiments); bars, SD.

**Figure 5.** Killing of tumor B cells by rituximab-alliinase targeted allicin in vivo. Chimeric mice (4–6 per treatment group) were engrafted i.p. with fresh B-CLL PBMC (100 × 10⁶ cells/mouse), or MCL or EBV immortalized B cells (4 × 10⁶ cell/mouse). Two weeks later, mice were injected i.p. with conjugate in 2 mL PBS or with PBS alone. Starting the day later, part of the mice that received the conjugate were injected i.p. with 3 mg alliin, twice a day, for 3 days. One day later, tumor cells were recovered by peritoneal lavages with PBS, counted, and stained for CD19-FITC and CD45-PercP. PBS-treated mice (●); mice treated with conjugate alone (■); mice treated with conjugate + alliin ( []). Columns, mean (B-CLL, n = 3 for B-CLL from three different patients; MCL and EBV immortalized B-cell line, n = 3 for three independent experiments); bars, SD.

In conclusion, the in situ generated allicin technique could offer a more powerful and less toxic therapy than conventional approaches. In addition, by using an appropriate antibody or ligand, the principle of this novel approach shown here for B-cell malignancies could be extended to other tumors as well as to other conditions in which the elimination of a specific population of cells is desired, such as autoimmunity, graft rejection, or graft versus host disease.

**References**

Molecular Cancer Therapeutics

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