Cryotreatment as a simple method for cell preparation in autologous tumor cell-based vaccination

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Abstract. Immunotherapies using autologous whole tumor cell vaccines have great potential in the treatment of cancer. Very few studies report the use of cryotreatment for the preparation of cells in cell-based vaccines. In this study, we demonstrated that a preparation containing cryotreated human breast cancer cells has the same capacity as a preparation containing irradiated human breast cancer cells to induce the activation of immune cells *in vivo*. The vaccine strategy proposed in this study may provide the experiment basis for the use of autologous or allogeneic breast cancer cells in the cell-based vaccine approach for the treatment of breast cancer and other types of cancer as well.

Introduction

Breast cancer is the most frequently occurring malignant disease in women and the second leading cause of cancer deaths in women in many of the regions of the world. According to the World Health Organization, >1.2 million people will be diagnosed with breast cancer this year worldwide. Although tumorectomy, radiotherapy, chemotherapy and hormone replacement therapy have been used successfully for the treatment of breast cancer, the limits of these existing treatment regimes for breast cancer are recognized. There are few effective therapeutic choices for patients with invasive and metastatic breast cancer (1,2). It is evident that novel therapeutic modalities for breast cancer need to be developed in order to eliminate residual circulating cancer cells and micrometastases.

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New therapies utilizing the immune system have proved effective in treating patients with advanced breast cancer (3-11). Cancer vaccine immunotherapy is certainly one of the most promising methods in cancer immunotherapy (8,12). Vaccination of cancer patients with autologous or allogeneic tumor cell-based vaccines has proved to be safe and elicit anticancer immune responses in clinical trials with patients affected by different types of malignancies (13-17). However some limitations have to be overcome before cell-based vaccine can be accepted as a new treatment for cancer. One of the limitations is the method used to prepare the cells. Irradiation is the common technique used to prepare cells in cancer cell-based vaccines, but its use necessitates expensive equipment and qualified personnel that are not always accessible. Therefore, the aim of this study was to investigate if a simpler, less expensive and faster method could replace irradiation. We investigated whether cells prepared by cryotreatment would be as efficient as cells prepared by the commonly used irradiation technique to induce an immune response in a nude mouse model.

Materials and methods

Cell lines. The human breast carcinoma cancer cell line MCF-7 was purchased from the American Type Culture Collection. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco-Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (BioMedia, Drummondville, Quebec, Canada), 50 μg/ml gentamycine (Gibco-Invitrogen) and 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO). The cultures were incubated at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air.

Mice. These experiments were approved by the University of Prince Edward Island and University of Moncton Animal Care and Use Committees and comply with Guidelines for the care and use of animals for research purpose. Female athymic nude mice CD1 nu/nu at 6-8 weeks of age were purchased from Charles River Laboratories (St. Constant, Quebec, Canada). Mice were housed in individual sterilized cages from the M.I.C.E.® Caging system (Animal Care Systems Inc., CO). Mice were given free access to sterile PICO Lab commercial food (Ren's Feed and Supplies, Quebec, Canada) and water.

Cell preparation for immunization. Cells were cultured in 75-cm^2 flasks. At 80% confluence, flasks were rinsed with phosphate-buffered-saline (PBS) (BD Biosciences, Mississauga, ON, Canada) and then filled up to the rim with PBS for irradiation treatment, or harvested for cryotreatment. For irradiation treatment, cells were irradiated at 10,000 cGy in a cesium irradiator (Dr. Georges-L.-Dumont Hospital, Moncton, NB, Canada). After irradiation, cells were harvested by scraping the culture flask with a cell scraper. Cells were then counted, centrifuged and resuspended in PBS at a concentration of 10^7 cells/ml. For cryotreatment, cells were centrifuged and resuspended in PBS at a concentration of 10^7 cells/ml. The cell suspension was then distributed in 1.5 ml cryotubes. Cells were then frozen by dipping in liquid nitrogen for 30 sec and slowly thawed at room temperature. This frozen-thaw process was repeated three times to increase the immunologic potency of the cells as observed in previous studies (18,19). After irradiation or cryotreatment, cell viability was determined by trypan blue exclusion assays. An equal volume of a solution of 0.4% of trypan blue (Sigma-Aldrich) diluted in PBS was added to the cell suspension, and viable and non-viable cells were counted on an hematocytometer plate under a microscope. The lack of cell growth was also confirmed by resuspending $1x10^5$ cells/ml in fresh culture medium. When the lack of cell growth was confirmed, the irradiated or cryotreated cells were then frozen at -80˚C until time of use.

Experimental design. To compare the two different methods of cell preparation, 15 female nude mice were divided into three groups of five. Each mouse received a subcutaneous injection on the right flank region using a 21-gauge needle at day 0 and a second at day 8. The first group received $2x10^6$ irradiated tumor cells suspended in 200 μl of PBS, the second group received $2x10^6$ cryotreated tumor cells suspended in 200 μl of PBS, and the third group received 200 μl of PBS. At day 23, mice were anesthetized with xylazin/ketamin (4 and 10 mg/kg, respectively) and blood was collected by cardiac puncture with a 1-cc syringe and a 5/8" 21 gauge needle.

To determine the best vaccination strategy with cryotreated cells, two additional sets of experiments were performed. In the short-term experiment, two groups of five female nude mice were used. At day 0, mice were injected subcutaneously in the back of the neck using a 21 gauge needle with 10^6 cryotreated MCF-7 cells suspended in 100 μl of PBS. Control mice received 100 μl of PBS. At day 14, cardiac puncture was performed on each anesthetized mouse. In the long-term experiment, at day 0, mice were injected subcutaneously in the back of the neck with $2x10^6$ cryotreated tumor MCF-7 cells suspended in 200 μl of PBS. Control mice received 200 μl of PBS. Seven days after the first injection (day 7), mice received a second injection in the right flank. At day 28, all mice were anesthetized in order to collect blood samples by cardiac puncture. In all experiments, mice were monitored daily for gross anatomical changes and body weights were determined twice weekly.

Blood samples. Blood samples were collected from anesthetized mice by cardiac puncture in EDTA tubes (BD Biosciences, MA, USA). EDTA tubes containing blood were kept on ice. Blood samples were then centrifuged for 10 min at 2,000 rpm and blood cells were analyzed the same day. An erythrolysis solution was made by dissolving 8.29 g NH_4Cl, 1 g $KHCO_3$, and 0.372 g EDTA in 1 l of distilled water. The pH of the solution was adjusted to 7.2-7.4 then the solution was sterilized by filtering through a 0.2-μm filter and stored at 4˚C. Blood cells were then placed in a 50-ml centrifuge tube and 30 ml of erythrolysis solution was added for each ml of blood. Each tube was then slowly mixed for 10 min, centrifuged and washed with a solution of PBS containing 0.5% (w/v) bovine serum albumin and 0.1% sodium azide (PAB) three times. Cells were then stored at 4˚C to preserve them until flow cytometry analysis.

Flow cytometry analysis. Following erythrolysis, immune cells were transferred into cytometer tubes with three quarters of the tubes volume in PAB. Tubes were then centrifuged for 1 min at 3,500 rpm and supernatant was removed by decantation. Antibodies were then added: 5 μl of APC-labeled goat anti-mouse CD19 (BD Biosciences) and 10 μl of FITC-labeled goat anti-mouse I-A(d) (BD Biosciences). Cells were then incubated at 4˚C for 20 min. Cells were washed with PAB and resuspended in 250 μl of paraformaldehyde. Cells were counted (minimum 5,000 events) with a fluorescence activated cell sorter (FACS, Becton-Dickinson) and analyzed by using the CellQuest software. The percentage of cells expressing I-A(d) and CD19 was calculated.

Detection of serum tumor-specific antibodies. MCF-7 were placed in a 96-well plate at a concentration of 10^5 cells per well. The plate was then incubated overnight or until 80% confluence. Wells were then washed twice with 100 μl of PBS with calcium and magnesium (BD Biosciences). Pre-cooled methanol was added to each well and plates were placed at -20˚C for 10 min in order to fix the cells to the plates. Plates were then washed twice with PBS. Wells were then filled with 5% PBS/Triton (v/v) for 5 min and then washed twice with PBS. This step was repeated with PBS/gelatin (0.2 g/100 ml) and the wells were then blocked by 1% (1 g/100 ml) BSA in PBS. Previously made dilutions of mice serum (1:20) were added to the wells and the plates were incubated at 37˚C in a humidified atmosphere of 5% CO_2 for 2 h. Every mouse serum sample was tested in triplicate. Wells were washed and the PBS/Triton and PBS/gelatin steps were repeated. Then, 100 μl of goat anti-mouse IgG-HRP (Santa Cruz Bio Technology, Santa Cruz, CA, USA) diluted to 1/1,000 in PBS/gelatin was added to the wells and the plates were incubated for 2 h at 37˚C in a humidified atmosphere of 5% CO_2. After being washed, 100 μl of HRP substrate was added to each well and the plates were kept in dark for 20 min. The reaction was stopped by adding 100 μl of 4 M H_2SO_4 and the absorbance was read with a spectrometer (λ = 415 nm) (Spectromax Plus, Molecular Devices).

Statistical analysis. Results are expressed as the mean ± SD. Statistical significance of differences between treatment groups was evaluated by Student's t-test for unpaired observations using the Analysis Toolpak of Microsoft Excel. In all analyses, differences with a $p<0.05$ were considered significant. All probability values are two-tailed.

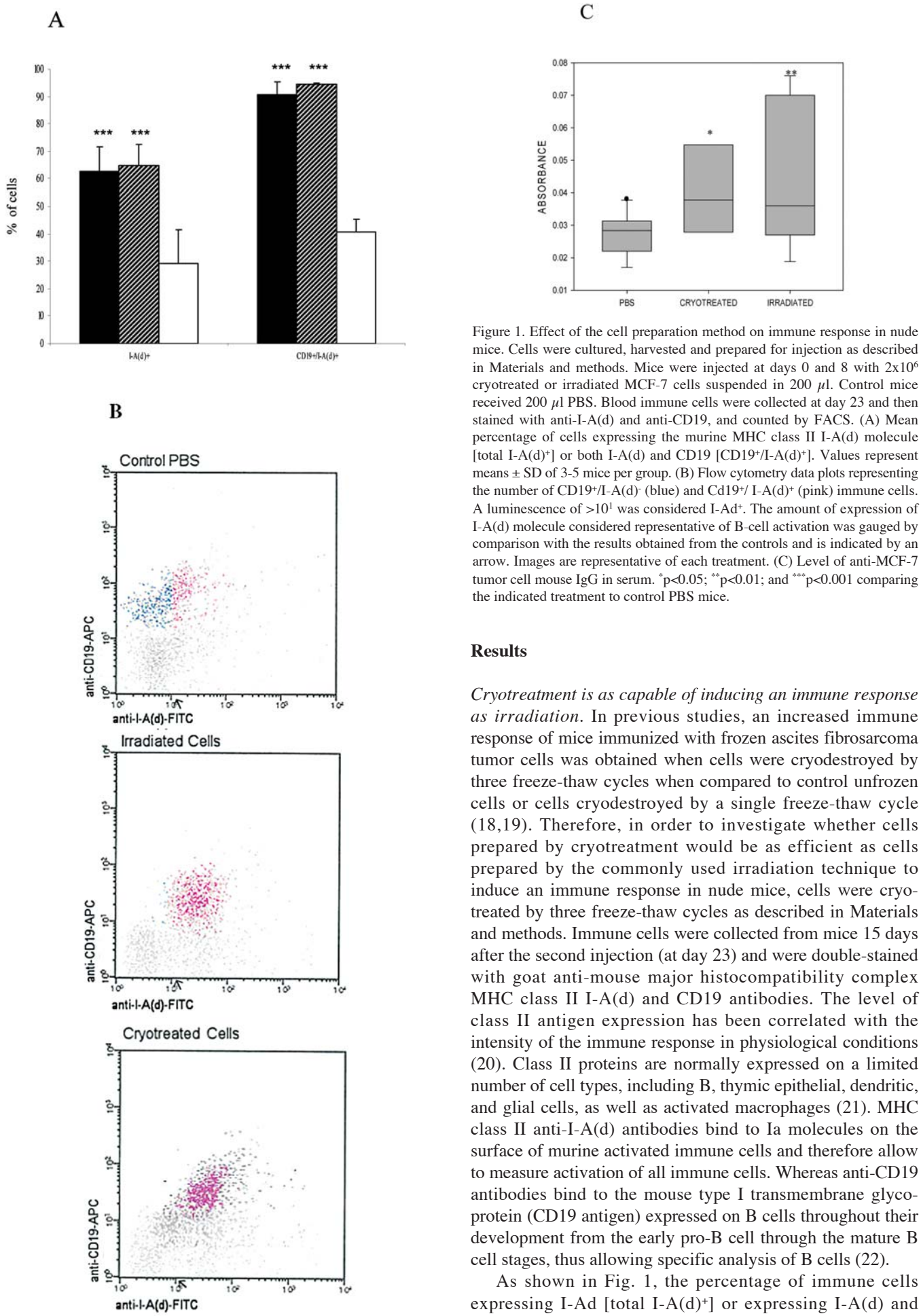

Figure 1. Effect of the cell preparation method on immune response in nude mice. Cells were cultured, harvested and prepared for injection as described in Materials and methods. Mice were injected at days 0 and 8 with $2x10^6$ cryotreated or irradiated MCF-7 cells suspended in 200 μl. Control mice received 200 μl PBS. Blood immune cells were collected at day 23 and then stained with anti-I-A(d) and anti-CD19, and counted by FACS. (A) Mean percentage of cells expressing the murine MHC class II I-A(d) molecule [total I-A(d)$^+$] or both I-A(d) and CD19 [CD19$^+$/I-A(d)$^+$]. Values represent means ± SD of 3-5 mice per group. (B) Flow cytometry data plots representing the number of CD19$^+$/I-A(d)$^-$ (blue) and Cd19$^+$/ I-A(d)$^+$ (pink) immune cells. A luminescence of $>10^1$ was considered I-Ad$^+$. The amount of expression of I-A(d) molecule considered representative of B-cell activation was gauged by comparison with the results obtained from the controls and is indicated by an arrow. Images are representative of each treatment. (C) Level of anti-MCF-7 tumor cell mouse IgG in serum. $^*p<0.05$; $^{**}p<0.01$; and $^{***}p<0.001$ comparing the indicated treatment to control PBS mice.

Results

Cryotreatment is as capable of inducing an immune response as irradiation. In previous studies, an increased immune response of mice immunized with frozen ascites fibrosarcoma tumor cells was obtained when cells were cryodestroyed by three freeze-thaw cycles when compared to control unfrozen cells or cells cryodestroyed by a single freeze-thaw cycle (18,19). Therefore, in order to investigate whether cells prepared by cryotreatment would be as efficient as cells prepared by the commonly used irradiation technique to induce an immune response in nude mice, cells were cryotreated by three freeze-thaw cycles as described in Materials and methods. Immune cells were collected from mice 15 days after the second injection (at day 23) and were double-stained with goat anti-mouse major histocompatibility complex MHC class II I-A(d) and CD19 antibodies. The level of class II antigen expression has been correlated with the intensity of the immune response in physiological conditions (20). Class II proteins are normally expressed on a limited number of cell types, including B, thymic epithelial, dendritic, and glial cells, as well as activated macrophages (21). MHC class II anti-I-A(d) antibodies bind to Ia molecules on the surface of murine activated immune cells and therefore allow to measure activation of all immune cells. Whereas anti-CD19 antibodies bind to the mouse type I transmembrane glycoprotein (CD19 antigen) expressed on B cells throughout their development from the early pro-B cell through the mature B cell stages, thus allowing specific analysis of B cells (22).

As shown in Fig. 1, the percentage of immune cells expressing I-Ad [total I-A(d)$^+$] or expressing I-A(d) and

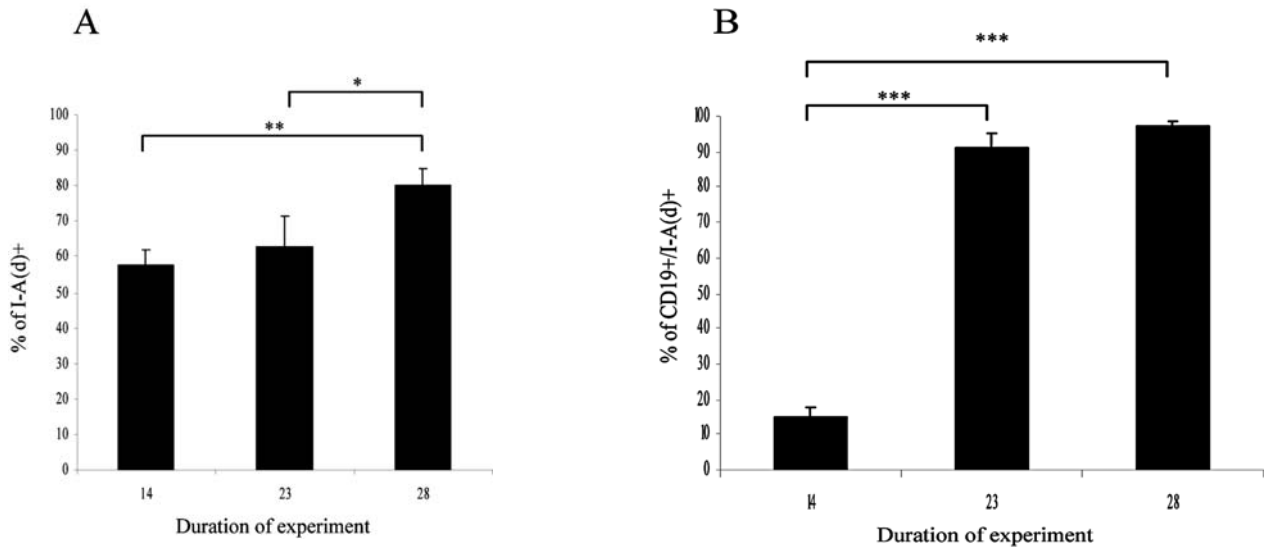

Figure 2. Comparison of different regimens. Cells were cultured, harvested and prepared for injection as described in Materials and methods. In the short-term experiment (14 days), mice were injected once at day 0 with cryotreated MCF-7 cells and blood samples were collected 14 days post-injection. In the long-term experiments, mice were injected at day 0 and received a boost one week later. Blood samples were collected 15 (23-day experiment) or 21 (28-day experiment) days after the boost injection. (A) Values represent mean percentage of cells expressing the murine MHC class II I-A(d) molecule [total I-A(d)$^+$] ± SD of 3 mice per group. (B) Values represent mean percentage of cells expressing both the murine MHC class II I-A(d) molecule and CD19 molecules [CD19$^+$/I-A(d)$^+$] ± SD of 3 mice per group. *p<0.05; **p<0.01; and ***p<0.001.

CD19 [CD19$^+$/I-A(d)$^+$] after injection of cryotreated or irradiated tumor MCF-7 cells was significantly higher than in the control groups (p<0.001). However, no statistical difference could be observed between the two types of cell preparation. Then we compared the production of human cancer cell specific antibodies. In mice that were injected with either cryotreated or irradiated cells, human cancer cell-specific antibodies production was significant higher than in mice injected with PBS (Fig. 1C). However, no significant difference could be observed between cryotreatment and irradiation. These data demonstrate that cryotreatment is as effective as irradiation in the preparation of cells.

Comparison of different vaccination regimens. To determine the best strategy for immune activation *in vivo*, we compared the immune activation in mice grafted with cryotreated cells in three different regimens. In the short-term vaccination regimen, immune activation was monitored for 14 days after a single injection of cryotreated cells. Whereas in the long-term vaccination approaches, mice received a boost injection one week after the first one and immune activation was monitored either 15 or 21 days after the boost injection. As shown in Fig. 2A, a significant higher level of total immune activation was obtained in the 28-day experiment when compared to the short-term experiment and the 23-day long-term experiment. No significant difference was observed between the short-term and the 23-day experiments. However, when we look at only B cell activation (Fig. 2B), significant higher percentages of activated B cells were found in both long-term experiments compared to the short-term experiment. Next, we measured serum tumor-specific mouse IgG antibodies by using a tumor cell-based assay. High level of tumor-specific antibodies were detected in sera from mice that received a boost injection 15 days after the first injection (23-day experiment). Similar levels of IgG were found in the short-term (14-day) and the long-term (28-day) experiments when compared to the serum of the mice that received PBS (Fig. 3).

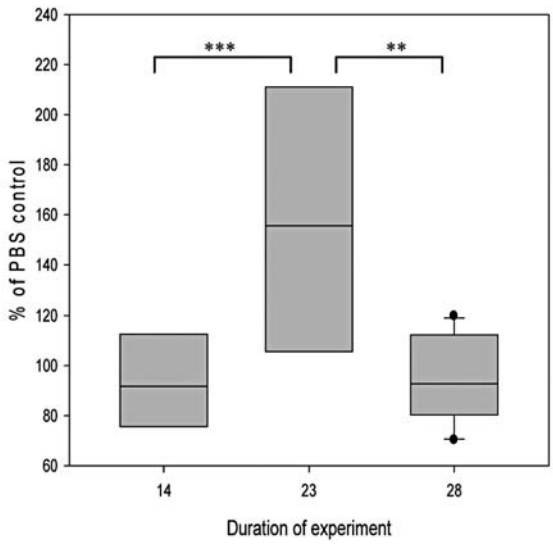

Figure 3. Production of tumor-specific antibodies. Mice were injected once (14-day experiment) or received a boost a week after (23- and 28-day experiments) with cryotreated MCF-7 cells. Blood samples were collected at the end of the experiment and levels of tumor-specific IgG antibodies were measured by an adapted ELISA cell-based assay performed on serum samples collected on 5 mice per group. Values represent means of absorbance ± SD of all 5 mice (1-2 samples per mice). Data are expressed as percentage of PBS control group. *p<0.05; **p<0.01; and ***p<0.001.

Discussion

In the present study, we have investigated the efficacy of cryotreatment as a method for cell preparation to induce immune activation for the improvement of tumor cell-based vaccines. The immunodeficient murine model used in these

experiments was chosen for its capacity to accept xenografts to allow us to evaluate a new cell-based vaccine strategy using human breast cancer cells. This study was not aimed and designed to study and investigate the host immune response in details. Nude mice are known to possess a normal functional number of B cells, mast cells, macrophages, LAKs and natural killer cells (NKs) (23-27). Even though this animal model does not permit the evaluation of a complete host immune response, we believe it is appropriate for the purpose of this study.

In this study we wanted to investigate if a simpler, less expensive and faster method such as cryotreatment could replace irradiation for the preparation of the cells. We observed that submitting human breast cancer cells to three successive freeze-thaw cycles was as potent as irradiation in inducing an immune response in nude mice. Our observations are consistent with other previous studies that report effective induction of immune responses in mice immunized with cryotreated tumor cells (18,19,28,29). Very few studies report the use of cryotreatment for the preparation of cells in cell-based vaccines and the present study might help in the development of this promising new therapeutic approach for treatment of cancer.

In addition, we determined that high level of immune activation can be achieved using free cryotreated cells. In this study we observed a better immune response when a boost injection was administered to the mice and the immune activation lasted up to 28 days. Additional experiments would be needed in order to determine the frequency of boost injections to maintain an immune response more than a month. However, based on the production of tumor-specific antibodies, the best strategy would probably be a boost injection every 2-3 weeks. Among solutions that have been proposed to reduce the number of injections in vaccine strategy is the utilization of polymers. Thus, a possible improvement of the vaccine strategy presented in this study could be the slow release of tumor cells or tumor specific antigens in mice immunized with cryotreated cells incorporated into a polymer-based vehicle. Many polymers have been studied for their immunization capabilities and researchers have shown that continuous release of antigen was as effective as multiple injections (30-32). Such a strategy could help reducing the number of injections needed for immunization, therefore improving patient compliance and cost-effectiveness as previously shown with Tetanus Toxoid vaccine delivered by PLGA microspheres (33).

Overall, these results are encouraging and show that cryotreated tumor cells could be an effective way to deliver tumor cell vaccines *in vivo*. Experiments aiming to verify whether vaccination with cryotreated tumor cells will protect the immunized mice when challenged with viable tumor cells would be needed to better evaluate the potential of therapeutic applications of the vaccine strategy described in this study.

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