

Assessing the Effect of Freund Adjuvant on the 4T1 Breast Cancer Mice Model

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Abstract

Background and Objective: Cancer immunotherapy combined with other common treatments can be an effective way to overcome cancerous cells. The purpose of this study was to investigate the effect of Freund Adjuvant on breast cancer in the BALB/c model of mice.

Material and Methods: Twenty female inbred 6–7-week-old- BALB/c mice were randomly divided into two groups of Test and Control, each containing 10 mice. Breast cancer was induced by injecting 10^6 4T1 cells into the right flank region of mice. After the tumors were palpable; animals were immunized three times by intraperitoneal (IP) injection of Freund adjuvant in the test group and phosphate buffered saline (PBS) in the control group at same condition. During the study; tumor growth, body weight, and survival percentages in mice were measured by using the caliper method, and mortalities were recorded. Results were tabulated using Excel, and Graphpad Prism Version 8. Data were analyzed using One-Way ANOVA and T-test and the significance level for statistical tests was considered $p \leq 0.05$.

Results: The results showed that tumor mice given Freund Adjuvant had a significant reduction in tumor size compared to the control group ($P=0.01$) and no significant weight difference was observed between the two groups ($P=0.4$). Furthermore, Kaplan Meier showed that the survival of the mice in the Freund Adjuvant group was significantly increased compared to the control group ($P=0.009$).

Conclusion: This study showed that Freund Adjuvant may play an important role in improving the function of the immune system for cancer immunotherapy.

Keywords: Breast Neoplasms[MeSH], Immunotherapy[MeSH], Freund's adjuvant[MeSH], Animal model[MeSH]

Highlights

Immunotherapy is stimulating body's own immune system to work strongly or smartly to attack on cancer cells. Freund Adjuvant can be effective in immunotherapy for cancer by stimulating immune cells such as dendritic cells and T-cells and increasing the secretion of IFN- γ and other cytokines.

Introduction

Breast cancer is the most common malignancy in women all over the world, and the treatment is possible in 70-80 % of patients in the initial stage and Non-metastatic. Severe breast cancer is currently out of control with existing treatments (1-3). Conventional methods of cancer treatment are surgery, chemotherapy, hormone therapy, and radiotherapy. Conventional cancer treatments may reduce the size of the tumor, but it is transient and has no positive effect on patient survival, and also there is the risk of recurrence of the disease. Therefore, researchers today are looking for alternative therapies with low side effects (4). One of the new strategies in the treatment of cancer is immunotherapy, which is using the host immune system and strengthening specific immune responses against the tumor (5). The immune system can detect and respond to foreign markers, including tumor antigens. The goal of immunotherapy is to strengthen the host immune responses to the tumor cells, and it has no side effects for normal cells. Strengthening the body's immune system naturally in the defense against cancer and infections is the goal of complementary therapies (6-9).

Various immunity stimulants called adjuvants can affect the immune system. Adjuvants are compounds that can increase immunity response. Adjuvant leads to the increased Immunogenicity of weakened antigens and it

also plays a role in increasing the durability of the immune response and antigen presenting (10-12). Stimulating adjuvant and immune booster, categorized into the groups such as Mineral Compounds, Bacterial Products, Oily Emulsions, Immunological adjuvants, and Mucosa Adjuvants (13-14).

Some adjuvants can create an antigen depot effect, which can persist for weeks to months and allows antigen to be released slowly and continuously from the injection site (15-16). Some bacterial products are adjuvants, for example; Complete Freund Adjuvant (CFA) was developed by Jules Freund in the 1950s and consists of heat-killed mycobacteria, mineral oil, and surfactant with a high ability to stimulate the immune system (17-18). These types of adjuvants can induce their effect by influencing dendritic cells. The activation phase of dendritic cells is necessary to induce specific immune responses. Eventually, this issue can increase the ability of dendritic cells to induction T-Lymphocyte differentiation and leads to specific immune responses (19-21). Incomplete Freund Adjuvant (IFA), which contains the same water-in-oil emulsion with the mycobacteria omitted. This Adjuvant applies its effect by stimulating Th2 cells. This Adjuvant is used in clinical trials of vaccines that are a candidate for the treatment of diseases such as AIDS, Melanoma, kidney carcinoma, and Multiple Sclerosis (22). In this study, we investigated the in vivo effectiveness of Freund adjuvant for stimulating the immune system and quantified the efficiency of this therapeutic approach by measuring mice survival rates and rate of tumor shrinkage.

Materials and Methods**• Cell Culture**

This study is an experimental-laboratory type. Breast cancer cells of 4T1, a mouse breast cancer cell line (ATCC CRL-2539), were

purchased from the Pasture Institute, Tehran, Iran (National Cell Bank of Iran). Cells in the medium containing RPMI (Gibco, Germany) enriched with 10% FBS (Gibco, Germany), 100 U/mL penicillin, 100 µg/mL streptomycin (Sigma, Germany) were cultured in an incubator at 37 °C and 5% CO₂. 4T1 cells are sticky, so in order to subculture, when the cells reached at least 70% growth, first the cells were separated from the bottom of the flask by 500 µL of 0.25% trypsin-EDTA (Gibco, Germany), and 1×10⁶ cell were seeded into new flasks.

• **Animals Study**

For this study, 20 BALB/c female mice with an age range of 6-7 weeks and weighing approximately 19 to 20 g were purchased from the Pasture Institute of Tehran and kept in an animal house under controlled and standard conditions at temperature 22 ± 2 °C, humidity 55 ± 2% with a 12-hour cycle of light /dark and standard feeding. All animal experiments were conducted under the guidelines of the Shahid Beheshti University, Tehran, Iran, for the care and use of animals.

• **Tumor Induction**

From 4T1 breast cancer cell line (Pasture Institute, Iran) used for the creation of mouse breast cancer model. Breast cancer cells were cultivated in the environment RPMI with 10% FBS in a flask T75. Then RPMI-containing 4T1 cells at a concentration of 1×10⁶ cells in 100 µL were injected subcutaneously into the right flank of BALB/c mice and the day of cell injection was considered day 0. Tumors appeared and were palpable on the tenth day (after injection). At this level, mice were divided randomly into two animal groups of 10.

• **Treatment Groups and Injection Process**

In this study, two experimental groups were used, in which 10 mice were randomly

selected in each group. The first group to evaluate the effect of Freund adjuvant on the process of growth of tumors and strengthen the immune system and the second group as a control group received respectively Freund adjuvant and PBS at equivalent volumes. The prescription procedure was performed in three doses in a volume of 50 µl intraperitoneal (IP) with an insulin syringe for 3 consecutive weeks.

• **Measuring the Tumor Growth**

Once the tumors were palpable, tumor width (W) and length (L) were measured using a digital caliper with an accuracy of 0.01 mm, regularly until the end of the work. Using the following formula, tumor volume was calculated:

$$\text{Tumor volume (mm}^3\text{)} = A \times B^2 \times 0.5$$

In this formula **A** indicates the length of the tumor and **B** Indicates the width of the tumor.

• **Measuring the Body Weight of Mice**

The weight of mice was monitored regularly to the end of the healing process, by digital scales with an accuracy of 0.001g.

• **Study of the longevity of Mice**

To evaluate the efficacy of immunization, five mice from each group were kept in standard conditions until the normal death coming. After the last death in both groups, data were analyzed by a Kaplan-Meier test.

• **Statistical Analysis Method**

In this study to evaluate and compare data used from the software Graphpad Prism Version 8, one-way statistical test of variance (One-Way ANOVA) and T-test and the significance level for statistical tests were considered $P \leq 0.05$.

Result

• Measurement of Tumor Volume

The results of tumorizing mice and the treatment process are shown in [Figure 1](#). Approximately 10 days after the injection of cancer cells 4T1, tumor growth was clearly visible, and as soon as the tumors became palpable, measured by caliper and the healing process began. The results of tumor growth in the control group and the treatment group are presented in [Figure 2](#). The results of this study show, that there is a significant difference in tumor size between the control group and the treatment group, and tumor growth in the control group compared to the treatment group shows a significant increase ($P=0.01$).

• Body Weight Results

Mice weight measuring to the end of the healing process regularly, by digital scales.

As shown in [Figure 3](#). There was no significant difference in mice body weight between the control group and the treatment group ($P=0.4$).

• Survival Rate

One week after the last dose of the drug, five mice in each group were sacrificed for studying further results, and the remaining mice were kept for studying longevity until normal death coming. The results of studying mice longevity in the control group and treatment group are presented in [Figure 4](#). Results show mice survival in the treatment group is higher than the control group ($P=0.009$). This increase in survival in the treatment group seems very promising in using Freund Adjuvant as cancer immunotherapy.

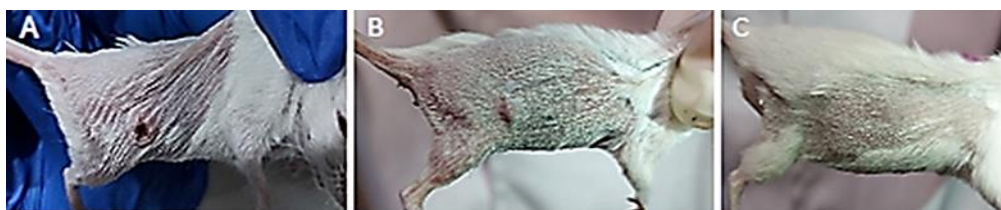


Figure 1. BALB/c mice with breast cancer tumors

A) Tumor status of mice from control group B) Tumor status of mice receiving the drug in the first injection C) Status of mice in the treatment group at the end of treatment

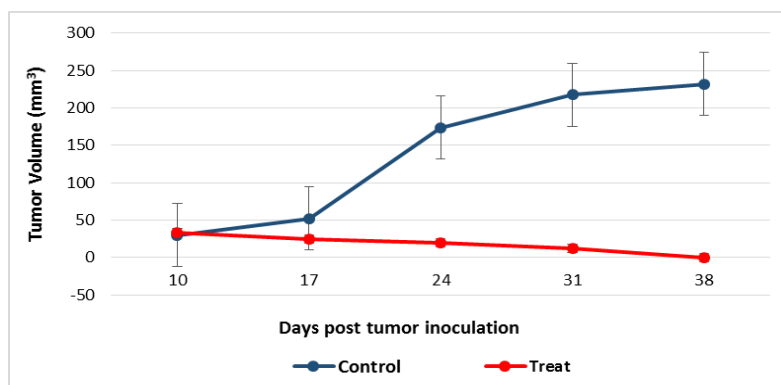


Figure 2. The tumor growth process in control and treatment groups

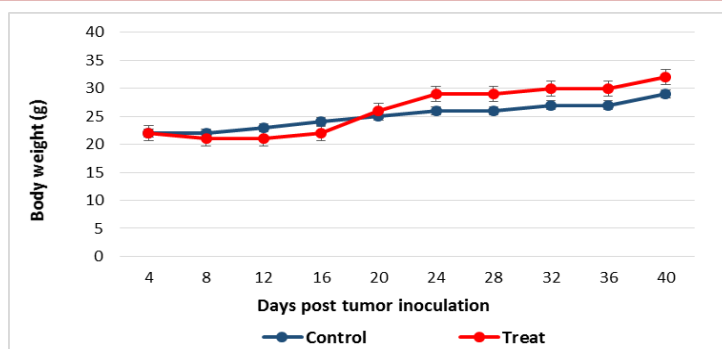


Figure 3. Results of the final weight of mice in control and treatment groups

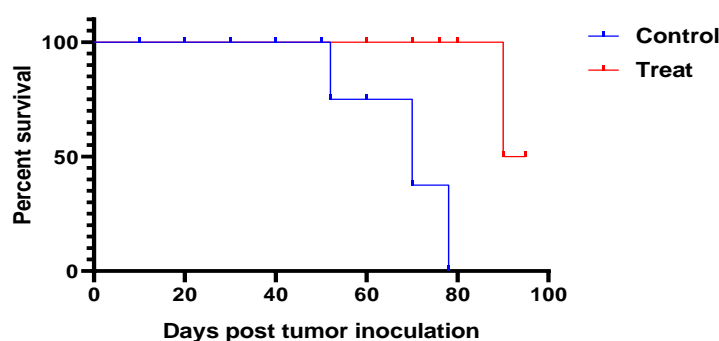


Figure 4. Survival curve in control and treatment groups

Discussion

Cancer immunotherapy, although being established over a century ago, is an emerging field in cancer treatment. The immune system contains multiple immune cell types which have been demonstrated to play complex roles in cancer development, progression, and elimination of tumor masses (23).

This study describes the assessment of novel potential cancer treatment, Complete Freund's Adjuvant (CFA), which is injected intraperitoneally in an attempt to induce an anti-tumor immune response, leading to tumor regression. CFA has been used in research for decades, primarily to produce research antibodies. CFA is composed of heat-killed mycobacteria in mineral oil and surfactant, which when emulsified with an aqueous solution creates a thick water-in-oil emulsion, which persists at the injection site.

These results indicate that Freund adjuvant can be used as an effective means in preventing the metastasis of cancer cells in mice breast cancer metastatic model. These effects may be due to the stimulation effect of Freund adjuvant on the cellular immune response.

This project was based on the initial hypothesis that when injected intraperitoneal, the mycobacterial PAMPs in CFA would activate tumor-infiltrating APCs, including dendritic cells, already carrying tumor antigens, which would then migrate to the draining lymph nodes and prime tumor-specific T cells.

Freund adjuvant as a potent immune stimulant would induce the recruitment of inflammatory cells to the tumor site, and local pro-inflammatory cytokine secretion would overcome the immunosuppressive milieu

within the tumor to allow anti-tumor responses against the tumor (24).

Judith et al. (25) demonstrated that immunization with an antigen and Complete Freund's Adjuvant induced interferon- γ -secreting and antigen-specific T cells. IFN- γ is known to exhibit anti-proliferative effects on tumors and to induce apoptosis (26). This process depends on the activation of the STAT1 (signal transducers and activators of transcription) signal transduction pathway.

Moreover, IFN- γ can control the angiogenesis process by inducing the production of IP10 (Interferon inducible protein) and MIG (Monokine induced by interferon γ) that inhibit angiogenesis and tumor progression (26, 27).

Production of IFN- γ not only increases the proliferation of lymphocytes but also increases the secretion of IFN- γ and changes other cytokine levels (27).

Garcia et al (28) evaluated the effect of IFN- γ and IFN- γ receptor on breast cancer cells and showed that IFN- γ could inhibit cell growth in breast cancer. Therefore, Freund adjuvant by increasing IFN- γ can reduce the size of the tumor.

Karlynn et al. (29) have shown that high rates of CD8+ and CD4+ T-cell responses to peptide vaccines for cancer are administered in Incomplete Freund's Adjuvant (IFA) emulsions (30, 31). High rates of tumor regression have also been observed after vaccination with peptide vaccine using IFA as an immunological adjuvant (32).

Melssen et al. (33) reported that vaccines using peptides emulsified in IFA can induce CD8 T-cell responses in 70–80% of patients, and can also induce CD4 T cell responses in most patients.

Activation of CD8+ cytotoxic T cells regarded as a major anti-tumor mechanism of

the immune system. Also, CD4+ T cells are required for the generation and maintenance of effective CD8+ cytotoxic and memory T cells. CD4+ T cells play an important role in the development of effective anti-tumor immunity (34, 35).

These studies showed that there was a significant association between the stimulation effect of Freund adjuvant and immune system response, which not only increased the proliferation of lymphocytes but also increased the secretion of IFN- γ and other cytokines. As the initial hypothesis suggested that T cells and IFN- γ are potentially mediated candidates for tumor regression, thus CFA would be useful as an adjuvant increased immune response for cancer immune therapy.

Conclusion

According to studies that show the ability of Freund adjuvant to strengthen the immune system; in the present study, we showed that Freund adjuvant increases survival by improving the function of host immune responses in cancer immunotherapy. All the results reported above show that this compound after three doses of injection could to significantly reduce the size and growth of the tumor compared to the control group and also no weight loss was observed in the treatment group. In order to perform better in this area including; combining other drugs with Freund Adjuvant requires further studies, and we hope to find a wider range of Freund Adjuvant application in cancer immunotherapy in the future.

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Conflict of Interest

The authors declare no conflict of interest.

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