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Simple but extremely effective autologous tumor vaccines

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A simple autologous tumor vaccine was created by mixing of autologous sublethally irradiated tumor cells with a non-specific immunomodulator MVE-2 (a polymer fraction of 1,2-co-polymer of divinyl ether and maleic anhydride). Two different tumor models, i.p. B-16 melanoma in C57Bl/6 mice and i.p. Sa-1 sarcoma in A/J mice, were used to assess the effectiveness of vaccine in the prevention of tumor development after challenge with viable tumor cells. Animals' survival was observed and the average day of death was calculated. With the prevaccination (7 days prior to tumor challenge) we managed to protect 60% of C57Bl/6 mice from tumor development - namely, they remained 100 days tumor free. The rest of the animals which ultimately developed i.p. melanomas survived statistically significantly longer than mock treated animals or animals receiving MVE-2 or non-replicating tumor cells alone. The results with sarcomas were less encouraging, since all prevaccinated animals finally developed i.p. sarcomas and died of it. Anyway, the survival of prevaccinated animals was significantly longer than the survival of mock treated animals, but a significant difference was obtained also when we compared animals treated with MVE-2 alone or irradiated tumor cells alone to mock treated animals. Interestingly, the animals that were treated with irradiated tumor cells alone had the longest survival. In conclusion, taking in account the results with genetically manipulated vaccines our own outcomes confirm that equally effective priming and triggering of the immune system could be obtained with a simple genetically unmanipulated and safe autologous tumor vaccine. The better results achieved with a less immunogenic B-16 melanoma remain unexplained so far.

Key words: biological therapy; vaccine therapy; neoplasms

Introduction

Due to the fact that nowaday methods of cancer treatment (chemotherapy, radiotherapy) are imperfect with regard to their toxicity and low specificity for tumor cells, tremendous efforts were put into the development of biological methods that would be more effective, more specific for tumor cells and less toxic for the treated organism. The commonly used biological therapies against cancer include different non specific immunomodulators (e.g. *Corynebacterium parvum, Bacillus Calmette-Guerin* - BCG, Muramyl dipeptide - MDP and its analogues), antitumor cytotoxic

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or immunomodulatory cytokines, growth factors, immunomodulatory monoclonal antibodies, host defense cells (i.e. tumor infiltrating lymphocytes, lymphokine activated killer cells), as well as tumor vaccines.[1-5] Among these methods, designing and application of tumor vaccines seemed to be the most attractive and promising ones, especially when the development of genetical engineering offered the possibility to create genetically modified vaccines.[3,6] However, the present-day knowledge still does not allow the preparation of absolutely controlled genetic constructs, which precludes their unlimited use in humans. The intention of this study was to design a potent autologous tumor vaccine, yet bypassing the dangers of low controllability of a genetically manipulated construct. Keeping this in mind, we designed a simple biphasic vaccine. We used unfractionated non-replicating (irradiated) autologous tumor cells as a source of tumor specific antigens that were intended to successfully and specifically prime the immune system. As the second component of the vaccine, used in order to enhance the cytotoxic macrophage activity, a non-specific immunomodulator MVE-2 was admixed to tumor cells.

Materials and methods

Tumor cells

Murine B-16 melanoma (clone F1) cells (American Type Culture Collection - ATCC, Rockville, Maryland) were grown in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal calf serum - FCS (Sigma, St Louis, MO), penicillin (100 units/ml, Pfizer, New York, NY), streptomycin (100 µg/ml, Pfizer) and gentamycin (11 µg/ml, Invenex, Chagrin Falls, OH). Fibrosarcoma Sa-1 tumour cells were grown *in vivo* as intraperitoneal tumours in A/J mice.

Animal tumor model

The experiments were performed on 8-10 week old syngeneic female C57Bl/6 or A/J mice (Institute Rudjer Bošković, Zagreb, Croatia). Animals were provided with food and water *ad libitum* and held at a constant room temperature (24°C) in a standard animal colony with the natural day/night cycle. Before the experiments, the animals were subjected to adaptation period of two to three weeks. At least 10 healthy animals, without signs of fungal or other infections, and with normal body weight, were included in each experimental group.

Intraperitoneal (i.p.) B-16 melanoma as well as i.p. Sa-1 sarcoma were employed as tumor models. Intraperitoneal B-16 melanoma tumors were induced by i.p. inoculation of a variable number of viable tumor cells: $2x10^5$, $5x10^5$ or $1x10^6$ in 0.2 ml EMEM supplemented with 2% FCS. Intraperitoneal Sa-1 tumors were induced by i.p. inoculation of $5x10^5$ viable tumor cells in 0.2 ml saline. The viability of tumour cells was determined by trypan blue dye exclusion test.

Mice with i.p. tumors were monitored for the day of death, and the proportion of survivors (i.e. animals protected from tumor development) was notified. The average survival (AM) ± standard deviation (SD) ± standard error (SE) were calculated for animals that ultimately developed tumors and consequentially died of them. All given data are a summary of the results of at least two identical experiments.

MVE-2

A polymer fraction of 1,2-co-polymer of divinyl ether and maleic anhydride (MVE-2) (Hercules, Inc., Willington, Del.) was used as a non-specific immunomodulator in the experiments. It was chosen due to its potent macrophage-activating as well as other immunostimulatory properties. MVE-2 is astraight chain, C-C backbone, anionic polymer with molecular weight of about 15500.

Vaccine preparation and administration (vaccination)

In order to prepare a tumor vaccine, B-16 tumor cells were trypsinized (0.25% trypsin, Sigma) and washed three times in 10% serum containing medium. Sa-1 tumor cells were collected by peritoneal lavage with physiological saline and washed three times in saline. After that, the tumor cell pellets were resuspended in 2% serum containing EMEM (concentration $1x10^6$ cells/cm^2) and irradiated sublethally with 60 Gy on X-ray equipment Darpac 2000X (Gulmay Medical Ltd., Shepperton, UK). As sublethally irradiated were taken tumor cells which were neither clonogenic *in vitro*, nor tumorigenic *in vivo*. Irradiated tumor cells were counted in the counting chamber and the preferred number of cells (in 2% serum containing EMEM) was simply mixed with MVE-2. A standard tumor vaccine contained $1x10^6$ irradiated B-16 or Sa-1 tumor cells and 25 mg/kg of MVE-2. The mock treatment was performed with 0.2 ml of 2% serum containing EMEM.

In the protection experiments with i.p. tumor challenge, the standard tumor vaccine (as well as mock treatment, irradiated tumor cells alone or MVE-2 alone) was administered i.p. 7 days before injection of viable tumor cells. Volume per injection was 0.2 ml.

Statistical analysis

Survival curves were determined using the method of Kaplan and Meier, and Student-Newman-Keuls method (multiple comparison procedure) was used to calculate the significance. Analysis of statistical significance of the differences between study groups was made using the unpaired Student's *t*-test where P levels < 0.05 were taken as indicating significant differences.

Results

Intraperitoneal prevaccination with autologous Sa-1 tumor vaccine significantly increases the average survival of the mice challenged i.p. with viable Sa-1 tumor cells.

In order to determine the potency of autologous tumor vaccine to prevent i.p. Sa-1 tumor development, A/J mice were prevaccinated with autologous tumor vaccine (or with sole components of the vaccine) 7 days prior to the challenge with viable tumor cells. None of the experimental mice was fully protected from tumor development (no matter which pretreatment was applied), so eventually all mice developed i.p. tumors and consequentially died of them. The average survival ± standard deviation (AM±SD) among mock treated animals was 7.1±2.4 days (Figure 1). Next to this group were the mice preinjected with MVE-2 alone, with an average survival of 15.5±1.3 days. The difference in survival between the two groups was highly statisti-

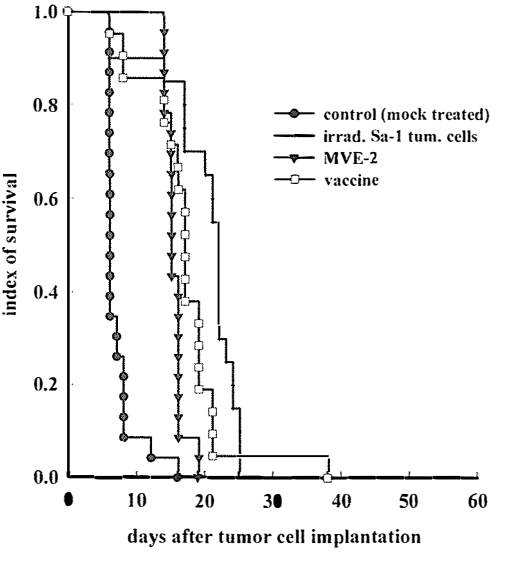

Figure 1. Survival data for vaccinated and control (mock treated, treated only with irradiated autologous Sa-1 tumor cells, or only with MVE-2) A/J mice challenged i.p. with $5x10^5$ viable Sa-1 tumor cells 7 days after the treatment.

cally significant ($p<0.0009$), thanks also to the extremely narrow time-range in which mock and MVE-2 treated mice died. The mice in the group preinjected with tumor vaccine survived significantly longer than the mock treated mice ($p<0.005$), but the average survival of 17.1±4.4 days did not significantly differ from the survival of the group preinjected with MVE-2 alone. Interestingly, the longest average survival was achieved in the mice preinjected only with irradiated Sa-1 tumor cells. It was 19.7±5.5 days, which is significantly longer than in the mock treated group ($p<0.000001$), but not significantly longer than in other groups.

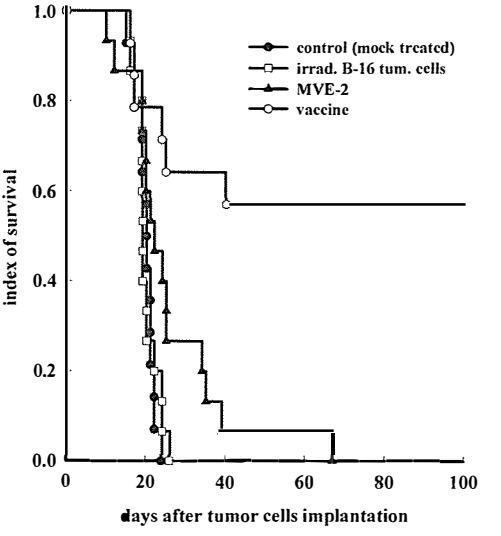

Figure 2. Survival data for vaccinated and control (mock treated, treated only with irradiated autologous B-16 tumor cells, or only with MVE-2) C57Bl/6 mice challenged i.p. with $5x10^5$ viable B-16 tumor cells 7 days after the treatment.

Intraperitoneal prevaccination with autologous B-16 tumor vaccine prevented tumor development in 60% of prevaccinated mice challenged i.p. with viable B-16 tumor cells.

Encouraging results with i.p. Sa-1 tumor model motivated us to investigate the same system of autologous tumor vaccination on poorly immunogenic i.p. B-16 malignant melanoma. Like in previous experiments, mice were pretreated with autologous vaccine or with an individual component of the vaccine, and their survival after challenge with viable B-16 tumor cells (7 days after the treatments) was compared to the survival of mock treated mice. The results obtained were even better than in Sa-1 tumor model, since 60% of prevaccinated mice remained tumor free more than 100 days after the challenge with viable highly tumorigenic B-16 tumor cells (Figure 2). The remaining 40% of prevaccinated animals eventually developed tumors, surviving on average 23.2±9.1 days. Among the mice pretreated with a single component of the vaccine (MVE-2 or irradiated B-16 cells alone) no survivors were found. Average survival of mock treated mice was 19.8±2.3 days, whereas of the mice preinjected only with irradiated B-16 tumor cells or of the mice preinjected only with MVE-2, the average survivals were 20.0±2.8 and 26.1±13.8 days, respectively. Neither the survival of animals preinjected with MVE-2 alone, nor the survival of prevaccinated animals (the 40% that died of tumors) was statistically significantly different in comparison with the survival of mock treated animals, particularly due to the wide scatter of the days of death in the groups.

Number of viable tumor cells used for tumor challenge is not critically important for survival of prevaccinated mice.

To answer the question how strong the primed antitumor immunity was and whether a greater tumor challenge avoided more easily such immune surveillance, we designed the experiments where the mice injected with mock treatment, vaccine or irradiated tumor cells alone were 7 days later challenged with a different number of viable B-16 tumor cells: $2x10^5$, $5x10^5$ or $1x10^6$. It was amazing to see, in repeated experiments, that

the highest number of survivors (i.e. against tumor development fully protected mice) was just among the prevaccinated mice challenged with the highest concentration of viable tumor cells (Figure 3). In this group 77.8% survived, while in the group challenged with $5x10^5$ viable tumor cells, 60% of mice survived and in the group challenged with $2x10^5$ viable tumor cells, 55.5 % of prevaccinated mice survived. The animals in prevaccinated groups that ultimately developed tumors, after the challenge with $2x10^5$ viable tumor cells, $5x10^5$ viable tumor cells, or $1x10^6$ viable tumor cells survived for 36.4±25.0 days, 32.4±12.9 days, and 27.0±14.4 days on average. No survivors were observed in the mock treated groups as well as in groups treated with irradiated tumor cells alone. Mock treated animals, challenged 7 days later with $2x10^5$ viable tumor cells, $5x10^5$ viable tumor cells, or $1x10^6$ viable tumor cells had an average survival of 20.4±5.8 days, 18.5±4.1 days, and 19.6±5.7 days, respectively. When the animals were preinjected with irradiated tumor cells ($5x10^5$) and seven days later challenged with $2x10^5$ viable tumor cells, they survived on average 21.7±4.3 days. When challenged with $5x10^5$ viable tumor cells, the animals survived 23.2±9.1 days, and when challenged with $1x10^6$ viable tumor cells 23.5±6.5 days.

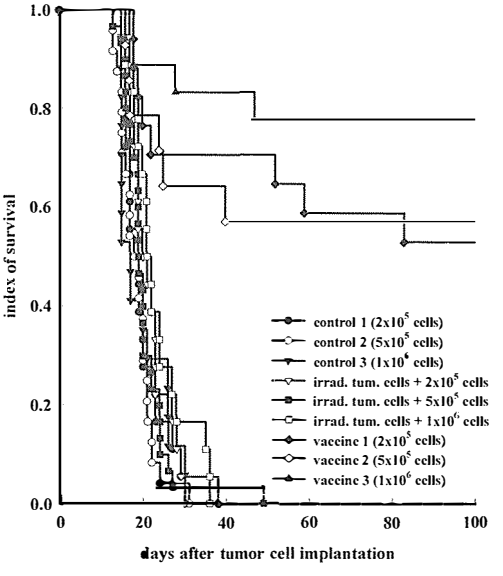

Figure 3. Survival of vaccinated and control i.p. B-16 tumor-bearing animals challenged with different concentrations of viable B-16 tumor cells 7 days after the treatment. The results of controls preinjected with MVE-2 are not plotted since they do not differ from the results in other control groups.

Discussion

The experiments reported here were motivated by a large number of recent studies showing that a host response to tumor challenge could be influenced by inoculation of tumor cells genetically engineered to express particular cytokines, MHC antigens, products of tumor suppressor genes, or B7 activation antigen.[6,7] At the same time, it seemed that the leading idea for the development of these vaccines was not always only their effectiveness for triggering host specific antitumor immunity, yet to design as complicated as possible constructs containing some foreign genes. Contrary to this concept, our efforts were put in the development of a simple tumor vaccine that would be easy to produce, and safe for application in cancer patients.

Therefore, we elaborated the autologous tumor vaccine where non-replicating (irradiated) tumor cells were employed as a source of the specific antigens, and the non-specific immunomodulator MVE-2 as an agent for the stimulation of cytotoxic macrophage activity. Keeping in mind that the most important factor for achieving specific antitumor immunity is natural immunogenity of tumor cells, we tested our vaccines on two different tumor models. It was encouraging to see that autologous Sa-1 tumor vaccines were capable of inducing a protective effect in A/J mice (even though there were no survivors), but it was most unexpected when in accordance with

the results of Dranoff *et al.* we could confirm that moderately immunogenic non-replicating tumor cells were comparable in their efficacy of stimulating the immune system to genetically modified irradiated tumor cells.[8] Namely, in our experiments the longest survival after the challenge with viable Sa-1 tumor cells was achieved by pretreatment with irradiated Sa-1 tumor cells alone.

The results in poorly immunogenic B-16 melanoma tumor model additionally verified the hypothesis that, for the development of an efficient tumor vaccine, it is not always obligatory to manipulate the tumor cells genetically. Specifically, the percentage of animals fully protected against tumor development in our experiments (60%) was comparable to the results of the authors who employed genetically modified tumor cells as tumor vaccine.[8-10] An even better evidence of the effectiveness of genetically non-manipulated vaccines provided Allione at al. who compared the efficacy of the vaccine containing irradiated autologous tumor cells admixed with *C.parvum* to the vaccines containing replicating or non-replicating tumor cells engineered to produce cytokines. Only the replicating tumor cells which were transfected with GM-CSF gene (Granulocyte Macrophage Colony Stimulating Factor) were more efficient than the above described simply created vaccine.[11]

Astonishing and interesting results were obtained when the antitumor protection was assessed after the challenge with different concentrations of viable B-16 tumor cells. The best protection was achieved in the prevaccinated animals that were inoculated with the largest tested number of viable tumor cells though, according to our expectations these mice were to die first. So far, there is no material evidence to explain this, yet we can speculate that this fact speaks for a critical number of tumor cells that are essential for the triggering of immune system. Such a speculation is supported by the results of the authors who confirm that the most potent tumor vaccines are designed when replicating tumor cells are transfected with a gene of interest.[11]

In conclusion, we demonstrated a brand new approach in tumor vaccine preparation, which resulted in a tumor vaccine, simple but effective enough to merit further investigation in animal tumor models as well as in humans. The main advantages of this kind of vaccine, besides its efficacy, are facile preparation, high controllability, and absence of toxic side effects or immunological deposits.

Acknowledgment

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