Effectiveness of a Simply Designed Tumor Vaccine in Prevention of Malignant Melanoma Development

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We investigated the efficacy of a simple syngeneic tumor vaccine to induce specific antitumor immunity in female C57Bl/6 mice. Tumor vaccine was prepared by mixing irradiated B-16 melanoma tumor cells with the pleiotropic biological response modifier—maleic anhydride divinyl ether (MVE-2). Experimental animals were pretreated with the vaccine in order to prevent the development of intraperitoneal (i.p.) B-16 melanoma tumors after inoculation of viable tumor cells. More than 40% of prevaccinated animals challenged i.p. with 5×10^6 viable tumor cells were completely protected from tumor development and remained tumor-free 100 days after tumor cell inoculation. The percentage of tumor-free animals (survivors) rose to as much as 90% when the application of tumor vaccine was repeated two weeks after the first vaccination (i.e. one week after the inoculation of viable tumor cells). The induced antitumor response depended predominantly upon macrophage function, since vaccinated animals which were depleted of peritoneal macrophages died within the same time range as animals in the control group. Also, tumor-type specificity of the vaccine was confirmed by the fact that the animals vaccinated with B-16 melanoma vaccine were not protected from the development of another type of tumor. In conclusion, comparison of the experimental data with the data from the literature suggests that our simple tumor vaccine may be as effective as genetically engineered tumor vaccines. At the same time, this kind of vaccine is easier to control and thus safer to apply in humans when compared to genetically engineered vaccines.

Key words: Cancer — Prevention — Tumor vaccine — Melanoma

It has been widely recognized that current cancer therapies are not fully satisfactory mainly owing to insufficient effectiveness and severe toxic side effects. While surgery is the standard treatment for primary tumors, the treatment of metastatic disease is problematic. In the past few years we have witnessed a rapid development of different biological and genetic therapies created to fight malignant diseases.1–4 These therapies were designed to be more effective, more specific for tumor cells, and to cause no or negligible toxic side effects. The most attractive current biological approach seems to be the creation of specific tumor vaccines. Several distinct approaches to the design of tumor vaccines exist: 1) enhanced production of various cytokines that participate in immune processes (IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, tumor necrosis factor (TNF)-α); 2) expression of allogeneic HLA antigens; 3) replacement of defective p53 genes,12,13 or 4) introduction of “suicide genes” into target cells — genes that sensitize cells to drugs that are normally non-toxic to mammalian cells (e.g. herpes virus thymidine kinase—HSVTK gene). Many of these approaches include a genetic manipulation (that favors some characteristic or process able to induce the antitumor immune response) of autologous or homologous cells and usage of non-viral or viral vectors for gene transfer.16 However, the usage of genetically manipulated constructs carries certain risk owing to unexpected effects of the introduced genetic material in the recipient organism. So our idea was to prepare a vaccine without utilizing foreign genetic material. Our approach involves simple mixing of syngeneic sublethally irradiated tumor cells with a pleiotropic biological response modifier—MVE-2. The irradiated tumor cells are supposed to provide a sufficient quantity of tumor antigens, while the immunomodulator MVE-2 should at least multiply the number of cytotoxic macrophages that play a crucial role in the antitumor activity of the immune system, together with CTL.17–19

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).

Ehrlich-Lettre ascites carcinoma cells (ATCC) were grown in vivo as intraperitoneal (i.p.) tumors in C57Bl/6 mice.

Animal tumor model The experiments were performed...
on 8- to 10-week-old syngeneic female C57Bl/6 mice (Institute Rudjer Bosković, Zagreb, Croatia). Animals were held in a standard animal colony according to the “Principles of laboratory animal care.” 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 tumor and i.p. Ehrlich-Lettre ascites carcinoma were employed as tumor models. Intraperitoneal B-16 tumors were induced by i.p. inoculation with $5 \times 10^6$ viable B-16 tumor cells in 0.2 ml of EMEM supplemented with 2% FCS. Intraperitoneal Ehrlich-Lettre ascites carcinomas were induced by i.p. inoculation with $1.5 \times 10^6$ viable tumor cells in 0.2 ml of saline. The viability of tumor cells was determined by means of the 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 noted. The average survival (AM) ± SD ± SE was calculated for animals that ultimately developed a tumor and subsequently died of it.

**MVE-2** A 1,2-co-polymer of divinyl ether and maleic anhydride (MVE-2) (Hercules, Inc., Willington, DE) was used as a nonspecific immunomodulator in the experiments. It was chosen due to its potent macrophage-activating as well as other immunostimulatory properties. MVE-2 is a straight chain, C-C backbone, anionic polymer with a molecular weight of about 15,500.

**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. After that, the tumor cell pellets were resuspended in 2% serum containing EMEM (concentration $1 \times 10^6$ cells/cm²) and irradiated sublethally with 60 Gy on a Darpac 2000X X-ray unit (Gulmay Medical Ltd., Shepperton, UK). Sublethally irradiated tumor cells were neither clonogenic in vitro, nor tumorigenic in vivo. Irradiated tumor cells were counted and the preferred number of cells (in 2% serum containing EMEM) was simply mixed with MVE-2. Various modifications of the vaccine were done by changing the number of irradiated tumor cells ($5 \times 10^5$, $1 \times 10^5$, $5 \times 10^5$) or by changing the quantity of MVE-2 (10, 25, 40 mg/kg) added to the preparation.

The mock treatment was performed with 0.2 ml of 2% serum containing EMEM. A standard tumor vaccine contained $1 \times 10^6$ irradiated B-16 tumor cells and 25 mg/kg of MVE-2.

In the protection experiments with i.p. tumor challenge, the standard tumor vaccine was administered i.p. 30, 14, 10, 7, or 3 days before injection of viable tumor cells. In the experiments where animals with i.p. tumors were revaccinated, the standard tumor vaccine was delivered twice: firstly, 7 days before, and secondly, 7 days after injection of viable tumor cells. Volume per injection was 0.2 ml.

**Macrophage depletion tumor experiments** The depletion of activated peritoneal macrophages was performed as described by Belardelli et al. —a technique that had been shown to eliminate activated macrophages for a period of at least 4 days.28 Namely, mice (C57Bl/6) received i.p. injections of 25 mg of silica (Sigma) in 0.2 ml of EMEM. The silica particles ranged in size from 0.5 to 10 µm with approximately 80% of the particles between 1 to 5 µm. The silica was administered on days −7, −3, and 1, while the vaccine was administered on day −7 and viable tumor cells on day 0.

**Collection of peritoneal macrophages** To identify changes in immuno-competent cells in the peritoneal cavity of vaccinated animals we formed experimental groups according to the mode of i.p. treatment. Control groups consisted of (1) mock-treated animals without tumors (peritoneal lavage 7 days after treatment), (2) mock-treated animals exposed to i.p. tumor challenge (peritoneal lavage 17 days after treatment, i.e., 10 days after the inoculation of viable tumor cells), (3) MVE-2 treated animals without tumors (peritoneal lavage 7 days after treatment), (4) animals pretreated with MVE-2 and exposed to i.p. tumor challenge (peritoneal lavage 17 days after treatment, i.e., 10 days after the inoculation of viable tumor cells), (5) animals treated with irradiated tumor cells without tumors (peritoneal lavage 7 days after treatment), (6) animals pretreated with irradiated tumor cells and exposed to i.p. tumor challenge (peritoneal lavage 17 days after treatment, i.e., 10 days after the inoculation of viable tumor cells).

The mock treatment was performed with 0.2 ml of 2% serum containing EMEM, while for vaccination the standard vaccine was used. Complete pretreatment was executed i.p. 7 days before i.p. inoculation of viable tumor cells. The peritoneal cavity of experimental animals was rinsed three times with 3 ml of phosphate-buffered saline—PBS (pH 7.4 at 25°C; Sigma) and immediately cooled on ice.

**Determination of peritoneal lymphocyte proportions in vaccinated animals** Samples containing peritoneal macrophages were centrifuged (1000 rpm), cells were washed twice with PBS, counted in a counting chamber and prepared for flow cytometry. Flow cytometric analysis was performed using a fluorescence-activated cell sorter (FACSort; Becton Dickinson, Mountain View, CA). A three-parameter analysis was performed to determine the expression of CD25 molecules by CD3+, CD4+ and CD8+ cells.
Cells were labeled with monoclonal antibodies and analyzed. Isotype controls (Becton Dickinson) and a control of viable cells (LIVE/DEAD kit; Molecular Probes, Eugene, OR) were included in the analysis. Monoclonal antibodies conjugated with fluorochromes: CD3 (PerCP), CD4 (PerCP), CD8 (PerCP), CD25 (FITC) were purchased from Becton Dickinson.

Statistical analysis Survival curves were determined using the method of Kaplan and Meier, and the 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, and P levels <0.05 were taken as indicating significant differences.

RESULTS

Optimal effect in the prevention of i.p. B-16 tumor development is achieved with tumor vaccine composed of 1×10^6 irradiated tumor cells and 25 mg/kg MVE-2 applied 7 days before i.p. B-16 tumor challenge. The two most important questions were: (1) whether the irradiated syngeneic B-16 tumor cells together with MVE-2 given i.p. as a vaccine could prevent the development of i.p. B-16 tumors, and, if they could, (2) to determine the optimal timing for administration. To answer these questions, experimental mice were injected i.p. at different times (prior to challenge with viable tumor cells) with vaccines that were composed of variable numbers of irradiated tumor cells (at a constant quantity of 25 mg/kg of MVE-2), or variable quantities of MVE-2 (at a constant number of 1×10^6 irradiated tumor cells).

Among the animals vaccinated with vaccine containing 5×10^5 irradiated tumor cells and 25 mg/kg of MVE-2, 40% survived more than 100 days after tumor challenge, and these were considered as fully protected. The remaining 60% died between days 14 and 66 following tumor challenge (with an AM±SD of 43.2±22.0 days), although this was significantly later than control animals (P<0.001) (Fig. 1, panel A). Almost equally effective antitumor protection was achieved with the vaccine containing 1×10^6 irradiated tumor cells and 25 mg/kg of MVE-2: 41.4% of vaccinated animals remained free of tumors, while the rest of them died between days 16 and 54 following tumor challenge. The average day of death in this group was 32.4±12.8 days, showing a highly significant difference in survival compared to controls (P<0.000009) (Fig. 1, panel A). The highest chosen number of irradiated tumor cells (5×10^6) did not augment the effectiveness of the tumor vaccine. Namely, in the group vaccinated with this vaccine, only 30% of animals were fully protected (i.e., free of tumors). Survival among the animals that ultimately developed tumors was similar to that with the previously described vaccines: animals died between days 15 and 54.

Fig. 1. Survival data for vaccinated and control (mock-treated, treated only with irradiated syngeneic B-16 tumor cells, or only with MVE-2) C57Bl/6 mice challenged i.p. with 5×10^5 viable B-16 tumor cells 7 days after the treatment. The influence of different concentrations of irradiated B-16 tumor cells (panel A), and different quantities of MVE-2 (panel B) on the effectiveness of tumor vaccine in prevention of i.p. tumor development and consequently on survival of experimental animals. The experimental groups consisted of 10 to 33 animals. A, ○ control (mock-treated), ■ 5×10^5 irradiated tumor cells, ▲ 1×10^6 irradiated tumor cells, ◆ 5×10^6 irradiated tumor cells, □ 5×10^5 irradiated tumor cells+25 mg/kg MVE-2, △ 1×10^6 irradiated tumor cells+25 mg/kg MVE-2, ◆ 5×10^6 irradiated tumor cells+25 mg/kg MVE-2. B, ○ control (mock-treated), ■ 10 mg/kg MVE-2, ▲ 25 mg/kg MVE-2, △ 40 mg/kg MVE-2, □ 10 mg/kg MVE-2+1×10^6 irradiated tumor cells, ◆ 25 mg/kg MVE-2+1×10^6 irradiated tumor cells, □ 40 mg/kg MVE-2+1×10^6 irradiated tumor cells.
AM±SD was 37.3±14.1, and a significant difference in survival was observed as compared to control groups (P<0.007) (Fig. 1, panel A).

When the quantity of MVE-2 was modulated at a constant concentration of irradiated tumor cells (1×10⁶) the best protection of vaccinated animals was achieved with vaccine containing 25 mg/kg (Fig. 1, panel B). Vaccine created with 10 mg/kg of MVE-2 induced full antitumor protection in only 20% of vaccinated animals. Animals with tumors started to die by day 18 and the last died on day 48. The average day of death was 39.0±10.1 days, which significantly differed from that of the control groups (P<0.008). When the vaccine was created with 40 mg/kg of MVE-2, full protection of vaccinated animals was achieved in 20% of them. The rest died between days 25 and 85 after tumor challenge, with an average survival of 43.3±22.9 days (statistically significantly different in comparison with control groups, P<0.03).

The best prevention of tumor development was observed when animals were prevaccinated 7 days prior to i.p. tumor challenge. Namely, more than 40% (45.5%) of these animals were fully protected and survived for more than 100 days without any signs of tumor development. In other experimental groups the proportion of survivors varied between 12.5% (prevaccination 30 days before tumor challenge) and 30% (prevaccination 10 days before tumor challenge) (Fig. 2).

On the basis of the above described results, we prepared the standard vaccine for other experiments with 1×10⁶ irradiated tumor cells and 25 mg/kg MVE-2 per injection volume of 0.2 ml.

Repeated vaccination improves the protection of experimental animals from the development of i.p. B-16 tumors The question was whether the tumor vaccine applied repeatedly could extend or/and enhance the priming of the immune system and consequently offer better tumor prevention. To address this question, we vaccinated one group of animals with a standard vaccine once, 7 days before tumor challenge, and another group twice, 7 days before and 7 days after challenge with viable tumor cells. This time we gained 41% of fully protected animals after single injection of tumor vaccine, and an encouraging 90% after a repeated application of vaccine (Fig. 3).

The survivors are partially protected (without revaccination) from B-16 melanoma tumor development after an i.p. rechallenge with viable B-16 tumor cells In order to determine whether the vaccination (one application, 7 days before first tumor challenge) in survivors (animals that remained without any sign of tumor development 100 days after the first tumor challenge) induced a long-lasting protective immunity, we rechallenged these animals with 5×10⁵ viable B-16 tumor cells without previous revaccination. Again, 20% of rechallenged survivors remained tumor-free for more than 100 days after inoculation of viable tumor cells. The remaining
80% of rechallenged survivors died between days 20 and 33 (AM ± SD was 28.5 ± 4.3 days) following (the second) inoculation of viable tumor cells, but still survived significantly longer than the control animals that died in 18.5 ± 4.0 days on average (P < 0.01) (Fig. 4).

Tumor vaccine created with irradiated B-16 melanoma cells is tumor-type specific

Experimental animals were prevaccinated with the standard vaccine and 7 days later challenged i.p. with syngeneic Ehrlich-Lettre ascites carcinoma cells in order to define whether the tumor vaccine is tumor-type specific or not. The most important fact is that none of the applied treatments significantly increased the survival of animals, compared to mock-pretreated animals (AM ± SD of the day of death in this group was 20.9 ± 5.1 days). The mice preinjected with MVE-2 alone unexpectedly died first in a short period of time (between days 16 and 20 following tumor challenge), having an average day of death of only 18.0 ± 1.5 days (the average day of death of animals preinjected with irradiated B-16 cells alone was 24.7 ± 2.4 days and the average day of death of animals treated with the vaccine was 22.0 ± 2.8 days) (Fig. 5).

Peritoneal macrophages play a crucial role in the mechanism by which tumor vaccine induces protection against tumor development

To determine the role of peritoneal macrophages in generating an antitumor immune response following the vaccination, we compared the survival rate of prevaccinated animals depleted of peritoneal macrophages with the survival of normal prevaccinated mice. Therefore, some of the experimental animals were injected 3 times with silica particles that had been shown to eliminate activated macrophages for a period of at least 4 days. From Fig. 6 it is clear that silica-treated prevaccinated animals (i.e., vaccinated animals depleted of peritoneal macrophages) died of i.p. B-16 tumors significantly earlier (P < 0.0003) than animals receiving i.p. tumor vaccine and having no silica injections (40% of them were fully protected from tumor development). Actually, the depletion of macrophages completely abolished the protective immunity induced by the vaccine. No silica toxicity-related deaths were noted among the healthy mice injected with silica according to the same schedule as tumor-bearing animals.

An increase in proportion of peritoneal T cells (CD3+) as well as of peritoneal CD8+ T cells was noted in vaccinated animals

In order to define the role of certain subpopulations of immuno-competent cells in the prevention of tumor development, we analyzed peritoneal exudates of experimental animals with a fluorescence-activated cell sorter. An increased proportion of peritoneal T cells (CD3+) was found in animals receiving the standard tumor vaccine (AM ± SD was 20.8 ± 1.9) compared to control animals (16.4 ± 2.7; P = 0.018). Such an increase was not observed in animals receiving MVE-2 alone or irradiated tumor cells alone. Also, an increased proportion of CD8+ T cells (compared to control) was detected in vaccinated animals as well as in animals receiving MVE-2 (P < 0.04). The AM ± SD of proportion of CD8+ T cells in control...
The proportion of CD4+ T lymphocytes did not change significantly in any group.

DISCUSSION

Biological therapy is a key component of any clinical cancer research effort. The commonly used biological therapies against cancer include non specific immunomodulators (e.g., Corynebacterium parvum, Bacillus Calmette-Guerin—BCG, Muramyl dipeptide—MDP and its analogues), antitumor cytotoxic or immunomodulatory cytokines, growth factors, immunomodulatory monoclonal antibodies, and host defense cells (i.e., tumor-infiltrating lymphocytes, lymphokine-activated killer cells), as well as tumor vaccines. Current gene therapy procedures include genetically modified tumor cells as vaccines, adoptive cellular therapy with genetically modified effector cells, tumor cell modification with prodrug-activating enzyme genes, introduction of multidrug resistance genes into bone marrow or stem cells, or introduction of wild-type tumor suppressor genes into tumors with mutated (non-functional) or lost tumor suppressor genes.

Nevertheless, the preparation of a potent anti-tumor vaccine does not necessarily demand a genetic change of tumor cells. Hock et al. noted that sublethally irradiated tumor cells admixed with C. parvum have an immunogenic activity comparable to that of genetically transformed cells. Also, Allione et al. have demonstrated that irradiated tumor cells admixed with C. parvum activate the immune system more effectively than vaccines comprising non-replicating (irradiated) tumor cells engineered to produce certain cytokines or growth factors.

So our intention was to create a tumor vaccine as simple and at the same time as potent as possible, that could be used in humans repeatedly without undesirable side effects. To achieve a substantial immunological antitumor effect it is necessary at least (1) to provide the immunocompetent cells with a sufficient amount of major histocompatibility complex (MHC) and other tumor-specific antigens as well as (2) to trigger cytotoxic T lymphocyte (CTL). Keeping in mind that a wide repertoire of tumor antigens is present on tumor cells themselves, we chose syngeneic tumor cells as a source of tumor-specific antigens. In this way, we believe, the risk of escape mutants is minimized. At the same time, when using the unfractionated tumor material, the identity of the effective tumor antigens need not be known, which expands the list of cancer types that can be treated.

Another component of our tumor vaccine was supposed to be an immunostimulator that would function as a triggering agent for immuno-competent cells. As the main candidates, we considered non-specific immunostimulators: BCG, MDP, and some of its analogues, as well as MVE-2. In the preliminary experiments (data not shown), the most promising results were obtained with MVE-2, which is not surprising, since MVE-2 is known to have a broad spectrum of activities ranging from antitumor to antibacterial. The implementation of these diverse activities is through immunomodulatory effects: enhancement of NK and cytotoxic macrophage activity, induction of IFN synthesis, stimulation of polymorphonuclear cell production and differentiation, as well as potentiation of antibody-independent microbicidal activity of phagocytes.

On the other hand, MVE-2 exhibits very few toxic side effects in the treated organisms. Moreover, it has also been utilized in bioconjugates, e.g. with TNF-α or neocarzinostatin, in order to reduce the toxicity of these antitumor substances.

Since many studies indicated that the main value of tumor vaccines is their usage in the prevention of disease recurrence, as well as in prevention of metastatic spread after surgical excision of primary tumors, we designed experiments where the protective effectiveness of tumor vaccine was examined. Our simple vaccine was comparable in effectiveness to the most promising current genetically modified vaccines. Namely, more than 40% of
animals were fully protected (against highly aggressive B-16 tumor cells) after a single application of the vaccine. Using similar tumor models, other authors have reported protection of between 30% and 60% of tumor-challenged animals (100% protection was obtained in a single case) when they applied genetically modified tumor vaccines.\(^5, 31, 32\)

In our study, the best antitumor protection was achieved when animals were prevaccinated 7 days prior to tumor challenge, which is in agreement with the results of other authors.\(^31, 32\) When the vaccine was applied twice, surprisingly good results were obtained, since the percentage of survivors rose to as much as 90%. Moreover, in some animals we managed to induce a long-lasting antitumor immunity after a single vaccination only. These results are promising because they confirm that the immune system can be triggered against a certain type of tumor for a long period of time, which is one of the prerequisites to apply tumor vaccines in humans as an adjuvant therapy. The observation that a tumor vaccine is tumor type-specific indicates that syngeneic (or autologous) tumor cells are nearly mandatory for the preparation of an optimal tumor vaccine and that tumor-specific antigens have an important role in triggering the antitumor immunity.

It is generally believed that the development of a CD8+ CTL response is important for the generation of antitumor immunity\(^33-35\) and that the antigen-presenting cells play the main role in activating CTL. Considering that, we investigated the mechanisms by which the tumor vaccine develops antitumor protection. We examined the changes in the proportions of CD3+, CD8+, CD4+ and CD25+ (as a fraction of CD3+ cells) in ascitic fluid of vaccinated animals, as well as the effectiveness of tumor vaccine after the elimination of activated macrophages. There was an increase in the proportion of intraperitoneal CD8+ T cells, mostly on account of an increase in the number of CD8+ cells (the proportion of CD4+ remained unchanged). However, the precise mechanisms via which the tumor vaccine triggered this part of the immune system remain to be established. Nevertheless, our results may be consistent with the findings of authors reporting that CD8+ cells are necessary for effector responses against tumors.\(^6, 36\)

On the other hand, following the elimination of activated peritoneal macrophages, mice vaccinated with the standard vaccine died within the same time range as controls, confirming that macrophages and other antigen-presenting cells have a crucial role in the mechanisms via which the tumor vaccine induces the antitumor immunity. This observation was not unexpected, since macrophages have diverse effects both on tumor biology (e.g., tumor growth rate, neovascularization and formation of the tumor stroma) and on the establishment of antitumor immunity.\(^37-39\) We believe that i.p. administration of our vaccine resulted predominantly in the triggering of immunologic antitumor defense mechanisms—especially the mechanisms by which macrophages participate in antigen presentation activities, direct cellular cytotoxicity and the release of cytokines important for stimulation of other effector cells (T and B lymphocytes, NK cells).

In conclusion, we created a syngeneic tumor vaccine with a potent preventive antitumor activity and with no apparent toxic side-effects. None of the vaccine components when applied alone could significantly prolong the survival of treated animals. The simplicity of the vaccine enables its rapid and uncomplicated production, without extensive preparation of primary cell cultures, and without expensive and time-consuming genetic manipulation of tumor cells. The tumor cells, once removed on surgery, can be stored and used for several preparations, with an enhanced effect after repeated application. Such a tumor vaccine is tumor type-specific and does not include any foreign genes or viral components that could potentially escape the control of the vaccinated organism. The major mechanism through which our vaccine induces antitumor immunity involves activation of tumor-associated macrophages and their direct cytotoxic action on tumor cells. Most probably other macrophage functions and effects (including the antigen-specific activation of CD8+ T lymphocytes) are also involved in the antitumor response. We suggest that the presented vaccine could have potential advantages as an adjuvant agent after the excision of primary tumors in humans to prevent metastases or recurrence of the disease.

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