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Antitumor Effect of Double Immunization of Mice with Mucin 1 and its Coding DNA

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Abstract. *Objective: To evaluate the antitumor effect of mouse immunization with human mucin 1 gene (muc 1) DNA plasmids combined with simultaneous injections of human mucin 1 (MUC1) protein. Materials and Methods: MUC1 DNA was cloned in pBK-CMV to prepare DNA plasmids and in pET22b(+) to produce proteins. Three strains of mice, immunized with DNA or DNA plus MUC1, were inoculated with tumor cells obtained from spontaneous tumors. IgG_{2a} production, MUC1-specific IFN-γ-producing $CD8^+$ T cells, tumor growth and mouse survival were monitored. Results: Only immunization with DNA plus proteins induced IgG_{2a} and intracellular IFN-γ production by $CD8^+$ T cells in the strains tested. DNA plus protein immunization induced a better mouse survival in comparison with the DNA groups. However, all immunized mice invariably developed tumors. Conclusion: Immunization with DNA plus proteins induced a better protection from tumor growth than immunization with naked DNA. However, the efficacy of immunization with MUC1-based antigens remains low.*

Although the immune system can clearly recognize cancer cells, there is little evidence that it does so to any effective consequence in patients with advanced breast cancer. Human mucin 1 (MUC1) is an epithelial mucin glycoprotein that is overexpressed in 90% of all adenocarcinomas including breast, lung, pancreas, prostate, stomach, colon and ovary, making MUC1 an attractive target for immune intervention. Low-level cellular and humoral immune responses to MUC1 have been observed in patients with solid adenocarcinomas, however, they are not sufficiently strong to eradicate the growing tumor. Different approaches have been suggested to improve the immunization efficacy of MUC1-based vaccines such as a vaccine formulation comprised of liposomal-MUC1 lipopeptide and human recombinant interleukin-2 (IL2); a novel breast cancer vaccine, Bacillus Calmette-Guerin (BCG)-hIL2MUC1; immunization with fused DC and MUC1-positive tumor cells (FC/MUC1); live recombinant vaccinia virus expressing the human *muc1* and *IL2* genes (TG1031) (1, 2). Most research demonstrated only a partial effect of MUC1 immunization in inducing cellular immunity. Another approach to immunotherapy for breast cancer is focused on the induction of antibodies to MUC1. It was shown that vaccination of breast cancer patients in remission with MUC1-keyhole limpet hemocyanin (KLH) conjugate induced high titers of IgM and IgG antibodies specific to MUC1 (3). However, there was no evidence of T cell activation in these patients. These results are also supported in mouse models. Mice immunized with liposomal-MUC1 lipopeptide and human recombinant IL2 developed T cells that expressed intracellular IFN-γ, and demonstrated a cytotoxic effect against MUC1-expressing tumor cells *in vitro*. However, the presence of MUC1-specific CTL did not translate into a clinical response as measured by time of tumor onset, tumor burden and survival (1).

In this work, we tried to induce an adaptive immune response to MUC1, using a protocol of immunization which ensured antigen presentation by two pathways associated with the activation of both humoral ($CD4^+$) and cellular ($CD8^+$) responses specific to MUC1.

Materials and Methods

Animals. Six-month-old BALB/cJCitMoise mice (hereafter, B/c), CBRB-Rb(8.17)1Iem (CBRB) and BYRB-Rb(8.17)1Iem (BYRB) mice bred at the Institute of Bioorganic Chemistry, Animal Department, Moscow, were used in this study (4). The protocol for the experimental animals was approved by the institutional committee.

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Antigens. MUC1 coding DNA and protein fragments corresponding to exons 234 and 456 of the *muc1* gene were used in this work. Total RNA was isolated from B/c homogenized mammary carcinoma using a standard method (5). Coding DNA was used as a template in polymerase chain reaction with a set of *muc1*-specific primers designed to include BamHI and XhoI sites. Both fragments were cloned into BamHI and XhoI sites of the pET22b(+) (Novagen, San Diego, USA) vector, according to the manufacturer's instructions. Plasmids used for immunization of mice were prepared using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, USA). The bacterial expression of the *muc1* gene fragments MUC234 and MUC456 was fulfilled in *E. coli* BL-21 strain, using a standard method (6). Recombinant MUC1 proteins were isolated by Ni-chromatography (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Animal experiments. B/c, CBRB and BYRB females were used as mammary carcinoma donors, while males of the same background were used as tumor recipients. Intact male mice from group 1 (3 per group of each strain) were immunized twice, with a one-week interval, by subcutaneous (*s.c.*) injection at the base of the tail with 5 μg of each: plasmids *muc234*, *muc456*, proteins MUC234 and MUC456 in 100 μl of sterile water. The second group (3 per group of each strain) was immunized with the same dose of DNA (*muc234* and *muc456*) only. The antigens were dissolved in sterile water and gently admixed with an equal volume of Gerbu LQ adjuvant (Gerbu Biochemicals, Gaiberg, Germany). Control groups (4 mice per group of each strain) were treated with the adjuvant dissolved in water. The experiments with the B/c mice were repeated three times, with CBRB mice twice and with BYRB mice only once. The number of mice per group was always 3-5. A comparison of the effect of immunization between different strains of mice was made. Tumor suspensions were prepared as described earlier (7). One million tumor cells were inoculated into the right upper flank of mice in 200 μl of phosphate-buffered saline (PBS). The size of the tumors was measured in three dimensions by calipers and a mean diameter was calculated, as described earlier (7). The mouse survival was monitored twice a week.

ELISA. Antigen-specific IgG_1 and IgG_{2a} were determined, according to the manufacturer's instructions (Pharmingen) using biotinylated isotype-specific anti-mouse antibody and ExtrAvidin-conjugated horseradish peroxidase (HRP) (Sigma, Moscow, Russian Federation).

Intracellular cytokine staining. Spleen cells depleted from erythrocytes were cultured at 37˚C and 5% CO_2 overnight in complete RPMI-1640 medium (Sigma) with 10% fetal calf serum (BioClot Ltd., Germany), L-glutamine and antibiotics (Sigma), in the presence of a mixture of MUC234 and MUC456 at 5 μg/ml of each. Brefeldin A (Sigma) (50 μg/ml) was then added and the cells were incubated for a further 6 h. The cells were stained with rat anti-mouse CD4 or CD8 Mab (Caltag, San Francisco, CA, USA) in PBS with 1% bovine serum albumin and 0.05% NaN_3 (PBAN) for 1 h, then washed in PBAN and blocked for 30 min with 10% of normal mouse serum in PBAN to prevent FcR binding. Afterwards, the cells were fixed in 1% paraformaldehyde prepared in PBAN for 1 h at room temperature. Permeabilization was fulfilled by freshly prepared 0.2% saponin (Sigma) in PBAN (saponin buffer). After washing, the cells were incubated with anti-IFN-γ-phycoerythrin (PE) mouse antibodies in saponin buffer for 2 h at 4˚C. Isotype controls included a mixture of mouse IgG_1 and IgG_{2a} labelled with FITC and PE. Afterwards, the cells were washed in PBS with 1 mM EDTA and transferred into FACS tubes.

Flow cytometry. To identify $CD4^+$- and $CD8^+$-producing IFN-γ splenocytes, cell surface molecules were labelled with antibodies and analyzed by flow cytometry using FACScan (Becton Dickinson, Franklin Lakes, NJ, USA) and CELLQuest software. Live lymphocytes were gated using a two-dimensional display of a mixture of anti-CD3-fluorescein isothiocyanate (FITC) and anti-B220-FITC antibody fluorescence *vs.* side scatter. The following Mabs were used for flow cytometry: anti-CD4-FITC, anti-CD8-FITC, anti-IFN-γ-phycoerythrin (PE) and isotype control Abs (Caltag).

Statistics. The Student's *t*-test was used to determine the difference between Abs levels, the number of IFN-γ-producing T cells and tumor size. Mouse survival was compared by the nonparametric Mann-Whitney test, because of the low number of mice per group.

Results

Induction of humoral response to MUC1. Mice were immunized either with plasmids only (DNA groups) or by a combination of DNA (*muc234* and *muc456*) and protein antigens (MUC234 and MUC456). Immunization with DNA induced a Th1/Th2 response associated with IgG_1 and IgG_{2a} production in B/c mice only (Figure 1). On the contrary, immunization with DNA plus proteins induced a well-shaped Th1 response in CBRB and BYRB mice visualized only by IgG_{2a} production, while Th2-associated IgG_1, as well as IgG_{2a}, were produced by B/c mice.

Induction of cellular response to MUC1. The cellular response, induced by MUC1 immunization, was estimated by IFN-γ intracellular expression in $CD4^+$ and $CD8^+$ T cells. Induction of MUC1-specific IFN-γ production was fulfilled by *in vitro* stimulation of splenocytes from immunized mice with MUC1 proteins. The results presented are calculated from 3 separate experiments (total 9 to 12 animals per group) as they were highly reproducible. The highest level of IFN-γ expression was found in mice immunized with DNA and MUC1 fragments (Figure 2). Among $CD8^+$ T cells, from 4.3 to 4.8% increased IFN-γ production when stimulated *in vitro* with MUC1 (Table I). Among the total number of cells, from 0.8 (CBRB) to 1.3% (B/c) of $CD8^+$ T cells were MUC1-specific (data for B/c are shown in Figure 2). Spontaneous IFN-γ production was higher both in the DNA- and DNA plus proteins-immunized groups than in the control groups (Table I). An increased number of $CD4^+$ T cells in B/c, but not CBRB, mice also expressed IFN-γ when stimulated with MUC1 *in vitro* (Figure 2).

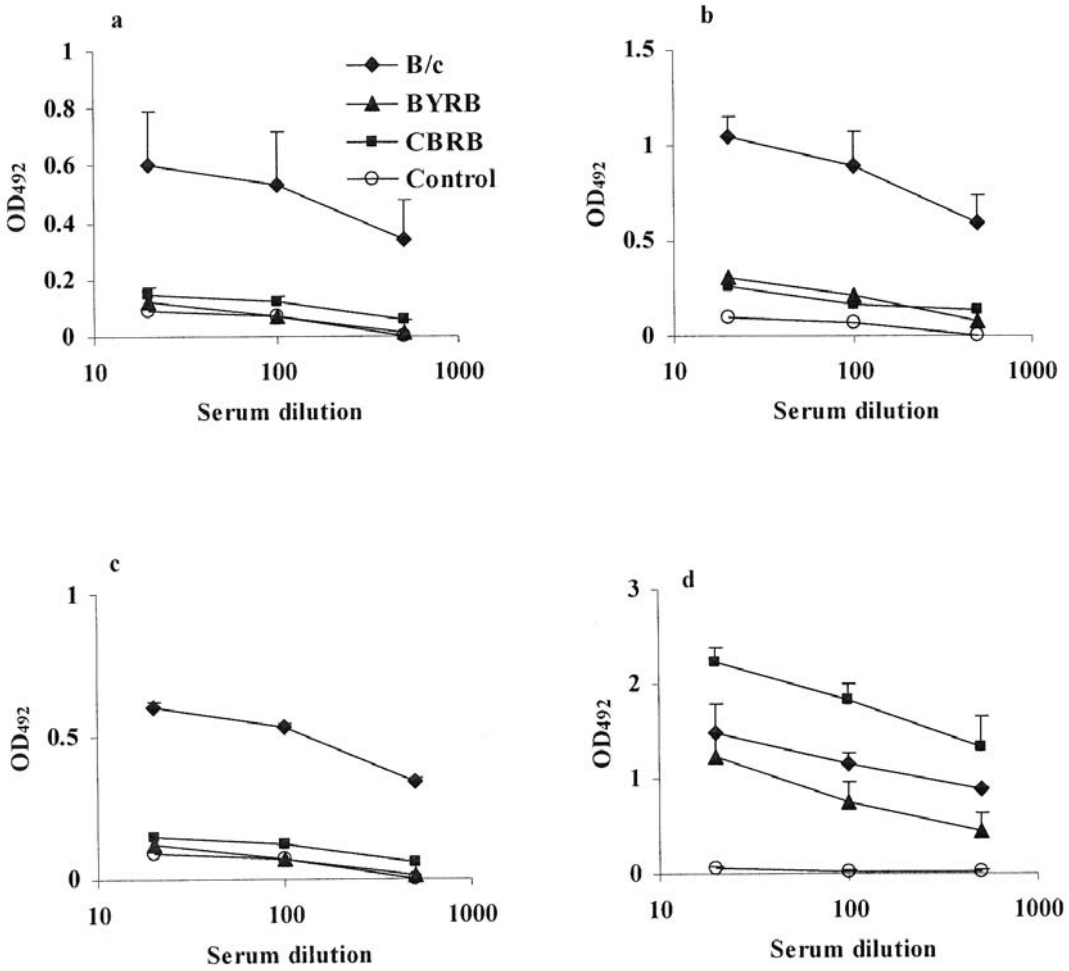

Figure 1. *IgG_1 (a and c) or IgG_{2a} (b and d) antibody production in B/c, BYRB and CBRB mice immunized either with plasmids (a and b) or plasmids plus proteins (c and d). Sera were collected a week after the second immunization.*

Protective effect of MUC1 immunization. There was no significant difference in the tumor sizes in the CBRB and BYRB models, while the tumors grew a little quicker in the B/c mice immunized groups (data not shown). However, the mice immunized with DNA plus proteins showed a tendency to a better survival in comparison with the control groups in all strains studied (Figure 3). Unexpectedly, the B/c and CBRB mice immunized with DNA died earlier than the controls, while no difference was found in the BYRB strain. The difference in both the decreased survival in the DNA-immunized group and improved survival in the DNA plus proteins group in comparison with controls was not statistically significant for individual strains, possibly due to the low number of mice per group. However, when mice from different strains were pooled, the results demonstrated a statistically significant difference estimated by Mann-Whitney statistics: $p=0.048$ for DNA *versus* control group and $p=0.045$ for DNA plus proteins *versus* controls (Figure 4). The difference between the DNA- and DNA plus proteins-immunized groups was also significant by *t*-test ($p=0.037$).

Discussion

This induction of an adaptive immune response targeting tumor cells involves intensive immunization by tumor-associated antigens. The role of B cells and antibody responses in antitumor defense is questionable in experimental models, in spite of their usage in clinics (8). On the contrary, the induction of cellular adaptive immunity looks more promising for the production of anticancer vaccines. Indeed, many papers demonstrate a better effect of DNA-based approaches for tumor therapy (9). However, their clinical relevance is not much higher than for antibody-inducing protocols (3).

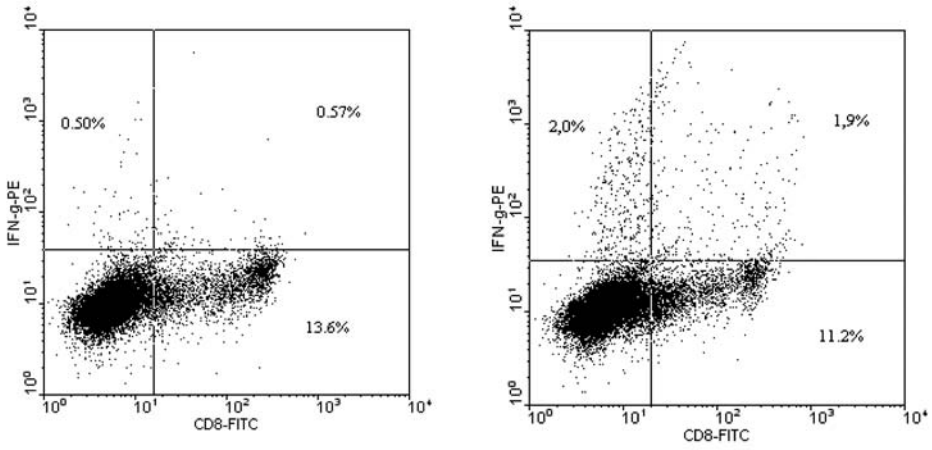

Figure 2. *Flow cytometry analysis of intracellular MUC1-specific IFN-γ production by CD8+ T cells from B/c mice immunized with plasmids and proteins stimulated (right side) or not (left side) in vitro overnight with 10 μg of a 1:1 mixture of MUC234 and MUC456. Live lymphocytes were gated using a two-dimensional display of a mixture of anti-CD3-fluorescein isothiocyanate (FITC) and anti-B220-FITC antibody fluorescence vs. side scatter. The cut-off level was selected using the isotype control Ab as equal to zero. The percentage of positive cells among all live cells is shown in the quadrants.*

Table I. *Spontaneous and antigen-induced intracellular expression of IFN-γ by CD4+ and CD8+ T cells from spleens of B/c and CBRB mice immunized with DNA (muc234 and muc 456) or DNA and proteins (MUC234 and MUC456).*

Immunization	**CD4+ T cells**		**CD8+ T cells**	
	Spontaneous	MUC1[d]	Spontaneous	MUC1
B/c	% among CD4+ T cells		% among CD8+ T cells	
Control	2.81±0.80[c]	1.62±0.17	3.80±1.31	3.47±1.02
DNA[a]	1.73±0.15	2.56±0.82 **(+0.8)[e]**	5.91±2.84	7.73±2.37 **(+1.8)**
DNA+ proteins[b]	3.05±1.25	4.62±1.15 **(+1.6)**	10.61±2.51	14.92±3.35 **(+4.3)**
CBRB				
Control	2.73±1.15	2.97±0.95	4.43±2.19	4.80±2.85
DNA	10.91±3.66	5.67±2.57	11.60±4.15	12.27±3.74
DNA+ proteins	7.72±2.25	6.63±3.15	9.32±3.33	14.19±4.15 **(+4.8)**

[a]Mice were immunized with 5 μg of each *muc234* and *muc 456* plasmids in Gerbu adjuvant twice with a one-week interval. Tumor cells were inoculated a week after the last immunization. IFN-γ production was analyzed a month after tumor inoculation.

[b]Mice were immunized by a combination of 5 μg of each DNA and 5 μg of each protein MUC234 and MUC456.

[c]Average and standard deviation represent percentage of PE-positive cells among either CD4 or CD8 cells. The cut-off level was estimated using isotype control Abs. The data are calculated for 3 experiments conducted for B/c mice (in total 10 mice per group) and 2 experiments conducted for CBRB mice (in total 7 mice per group). The data for 12 and 8 mice are shown for corresponding control groups.

[d]Splenocytes from immunized or control mice were stimulated *in vitro* with 5 μg of each MUC234 and MUC456 overnight.

[e]Statistically relevant ($p<0.05$) increase in stimulated cultures is shown in bold in brackets.

To study the effect of anticancer vaccines and drugs, different *in vivo* and *in vitro* models are used. The results obtained in these models can not always be transferred into clinical practice for different reasons. For example, mouse models of breast cancer only partially resemble human pathology, although many features of breast carcinogenesis in humans can also be found in mice (7). The advantage of our approach was the simultaneous use of different strains of mice. In our collection, there are several strains with either a high incidence of spontaneous mammary carcinomas (CBRB, BLRB, BYRB) or strains where carcinomas can be easily obtained by *in vivo* passage (B/c, A/Sn). The results obtained in genetically different mice permit the separate analysis of the effects of vaccination and genetic variability.

We demonstrated that immunization of mice of three different strains with both MUC1 and its coding DNA against mammary carcinoma does induce a slightly better survival, especially when compared with DNA-immunized groups. However, all immunized mice invariably developed tumors with the same kinetics as the control animals. These results are in agreement with results obtained by Mukherjee *et al*. (1), where a MUC1-specific cellular response was induced. However, the presence of MUC1-specific CTL did not translate into a clinical response as measured by time of tumor onset, tumor burden and survival. On the other hand, our results are not in agreement with another publication by Johnen *et al*. (9), where MUC1 immunization was highly protective. The difference between the results is related to the mouse model in which the effect of MUC1 immunization was studied. Both Mukherjee *et al.* and our group used mouse breast cancer models that demonstrate peripheral and central tolerance to MUC1 and develop spontaneous tumors of the mammary gland. On the contrary, the tumor cell line MC38, expressing human

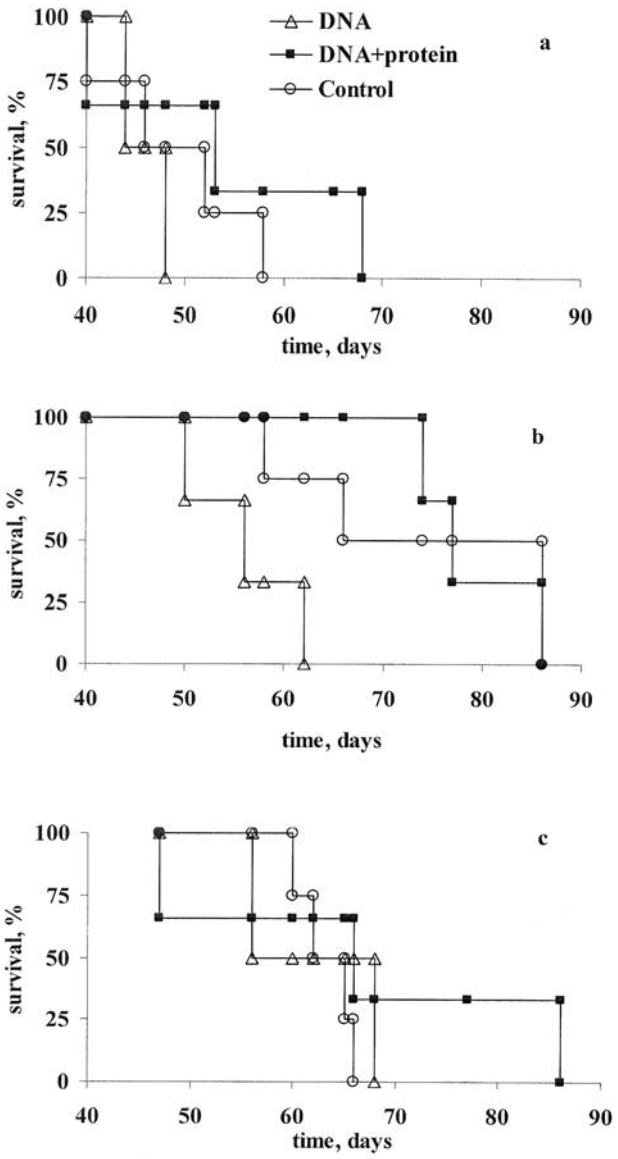

Figure 3. *Survival of B/c (a), CBRB (b) and BYRB (c) mice, immunized either with muc1 DNA or DNA and MUC1 proteins (DNA+protein), after tumor inoculation. No statistically significant difference was found between groups, probably due to the low number of animals per group (3-4).*

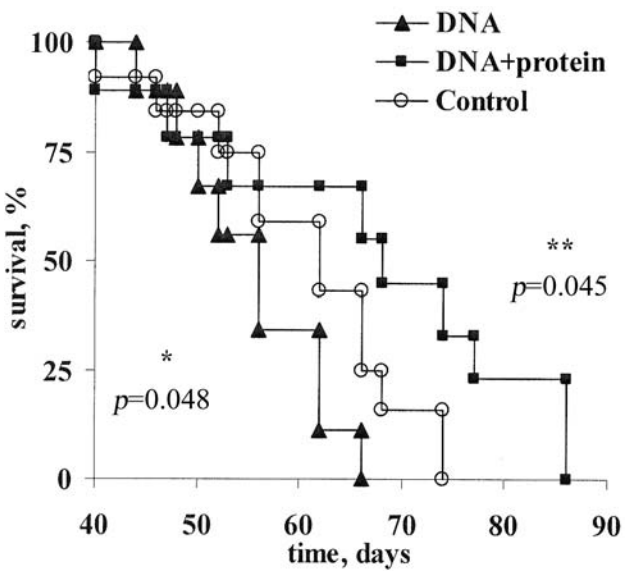

Figure 5. *Survival of pooled groups of mice (B/c, CBRB and BYRB), immunized with DNA or DNA+proteins. Due to the increased number of mice per group (9 in immune groups and 12 in a control group), a statistical difference was found using nonparametric methods between the immunized and control groups. The probability of coincidence between the DNA-immunized group and control is shown with one asterisk and between the DNA+protein group and control, with two asterisks. The difference between the DNA- and DNA+proteins-immunized groups was statistically significant using the t-test (p=0.037).*

MUC1, was used in the work published by Johnen *et al*. Even minor genetic differences can help the immune system to mount an antitumor response. That is why care in the choice of the model should be taken when different protocols or drugs are tested.

The low antitumor effect of the MUC1 immunization could be a result of the inability of effector cells to migrate to the initial site of tumor growth due to the lack of danger signals, such as the chemokine gradient, or adhesion molecules, such as selectins, ICAM and VCAM. Another reason for the low efficacy of immunization could be connected to MHC class I loss by the breast carcinoma cells in our models. Finally, effector cells, stimulated without sufficient signals from APC, can quickly become anergic (10). Taken together, it can be concluded that the induction of even both humoral and cellular adaptive immune responses to a single tumor-associated protein antigen (mucin 1) only slightly improves the survival of mice. Some other immune mechanisms should be activated to protect mice from carcinoma growth from the very early stages.

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