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ORIGINAL ARTICLE

**Anne-Marie T. Ciupitu · Max Petersson · Koji Kono
Jehad Charo · Rolf Kiessling**

Immunization with heat shock protein 70 from methylcholanthrene-induced sarcomas induces tumor protection correlating with in vitro T cell responses

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Abstract The cytosolic members of the heat shock protein 70 (hsp70) family have in recent years been shown to elicit protective immunity mediated via binding to antigenic peptides in tumor and viral animal models. In this study we have used the methylcholanthrene (MC)-induced sarcomas MC57S and MC57X, previously shown to express individually distinct MHC-I associated peptides recognized by tumor necrosis factor-alpha (TNF-α) producing $CD8^+$ T cells. With hsp70 purified from tumor or liver tissue, we were able to confirm that tumor-derived hsp70 elicited in vivo protection against a challenge with the same tumor as that used for hsp70 isolation. We also observed that immunizing with hsp70 isolated from tumor tissue resulted in a significantly better protection than immunizing with hsp70 isolated from the liver tissue of healthy mice. In two out of three experiments however, immunization with liver-derived hsp70 as well as tumor-derived hsp70 resulted in a significantly delayed tumor outgrowth as compared to saline-injected controls. In vitro, T cell lines from mice immunized with tumor-derived hsp70 could recognize tumor cells from the same MC57 tumor as that used for the hsp70 purification, resulting in TNF-α production. In sera from hsp70-immunized mice we observed undetectable or only very low levels of anti-hsp70 antibodies, suggesting that it is possible to repeatedly immunize the mice with no significant interference from neutralizing antibodies. We therefore conclude that hsp70 immunization can lead to protection against the tumor it was purified from, with a low risk of eliciting neutralizing antibodies that could affect further immunizations.

A.-M. T. Ciupitu (✉) · M. Petersson · K. Kono
J. Charo · R. Kiessling
Department of Oncology-Pathology,
Karolinska Institute, R8:01,
171 76 Stockholm, Sweden
E-mail: ani.ciupitu@mtc.ki.se

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Abbreviations *hsp70* heat shock protein 70 · *MC* methylcholanthrene

Introduction

Much effort is invested in defining new tumor antigens and in efficient methods of delivering these as tumor vaccines to elicit anti-tumor responses in murine models and clinical trials [18, 20]. This work is ongoing both in murine models and in early clinical trials. Although many tumor antigens which are recognized by T cells in vitro have been found, their ability to elicit a response powerful enough to lead to rejection of the tumor in vivo remains low or unknown. The need to effectively induce immune responses to tumor antigens, which are often low affinity "self" antigens [2, 15, 19] has stimulated the search for new vectors and adjuvants to enhance their immunogenicity in vivo.

One promising vector candidate, recently shown to be able to efficiently activate a T cell or antibody response to covalently [16] or non-covalently [13, 17] bound epitopes, is heat shock protein 70 (hsp70). The role of the proteins of the hsp70 family is to chaperone proteins or peptides when these are being synthesized on the ribosome, prior to transport over membranes or when cellular proteins become incorrectly folded, for example after cellular stress [3, 6, 14]. The peptides bound to the hsp70 molecules will therefore reflect the proteins produced in that particular cell. The cytosolic members of the hsp70 family stirred the interest of tumor immunologists when they were shown to elicit tumor protection in vivo via the peptides to which they associated in tumor cells [17].

In this study we have used two methylcholanthrene (MC)-induced sarcomas, MC57S and MC57X [9, 10]. These tumors have previously been shown to express

individually distinct MHC-I associated peptides, recognized by tumor necrosis factor-alpha (TNF-α) producing $CD8^+$ T cells [11]. We investigated the ability of hsp70 to induce anti-tumor protective immunity in vivo, and the ability of T cells from hsp70-immunized mice to recognize in vitro the tumor from which this hsp70 originated. Our data confirm that hsp70 isolated from solid tumors can induce tumor protection in vivo.

Materials and methods

Mice

B6 (H-2^b) female mice were bred at the animal house of the Microbiology and Tumor Biology Center at the Karolinska Institute. All mice were immunized at 6 to 9 weeks of age.

Tumor cell lines

MC57X and MC57S (H-2^b) are two antigenically distinct MC-induced fibrosarcomas from C57BL/6 mice [9, 10]. RMA is a cell line derived from a Rauscher leukemia virus-induced T cell lymphoma, RBL-5, of B6 origin [8].

Purification of hsp70 from MC57 tumor

In the work described here, we have purified the cytosolic members of the hsp70 family. After purification, presumably both the constitutive hsc73 and the inducible hsp72 are present. No discrimination between them has been made, as these two members of the hsp70 family are very similar in protein structure. For simplicity, the purified protein(s) will be referred to as hsp70 throughout the article.

Hsp70 was purified with only minor modifications, as previously described [13]: liver from C57BL/6 mice was homogenized in hypotonic buffer without detergent (10 mM Tris–Ac, 10 mM NaCl, 0.2 mM PMSF, pH 7.2) and centrifuged at 100,000 *g*. The supernatant was incubated with Active Blue 2 Sepharose beads (Sigma, St Louis, Mo.) for 30 min, after which the buffer was changed to buffer D (20 mM Tris–Ac, 20 mM NaCl, 15 mM *β*-mercaptoethanol, 3 mM $MgCl_2$, 0.5 mM PMSF, pH 7.5).

The sample was loaded on an ADP- or ATP–agarose column (Sigma, St. Louis, Mo.). After washing the column with salt buffer D (buffer D containing 0.5 M NaCl) and buffer D, the hsp70 was eluted with 3 mM ATP-containing buffer D. The buffer of the eluate was then changed to buffer A (20 mM sodium phosphate, 20 mM NaCl, pH 7.0), and the eluate was loaded on an DEAE–Sephacel column (Sigma, St. Louis, Mo.). After washing the column with buffer A, the hsp70 was eluted in 1.5-ml fractions with buffer A1 (150 mM NaCl in buffer A). Protein content in the different fractions was measured with the Bradford assay (Bio-Rad Laboratories, Hercules, Calif.) with bovine serum albumin (BSA) as standard. Protein purity was determined by running samples of the different fractions on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) 12.5% homogenous gel, after which the gel was silver-stained (see below).

At the beginning of the study, the hsp70 was purified by the following method. Minced tissue was homogenized in 2× M_1 buffer without detergent (20 mM Na_2HPO_4, 1 mM EDTA, 1 mM NaCl, 1 mM PMSF, pH 7.4) [5]. The lysed cells were centrifuged at 100,000 *g* for 90 min at 4°C. 100 mM 2-mercaptoethanol was added to the supernatant, and the filtrate was run on a Mono Q column (Econo-Pac Q, BioRad Laboratories, Hercules, Calif.). One-milliliter fractions were collected. The fractions containing the hsp70 were pooled and precipitated with 50% ammonium sulfate for 2 h at 4°C [17]. The supernatant was precipitated again with 70% ammonium sulfate for 2 h at 4°C, and the precipitate was saved.

SDS–PAGE and western blot

A sample of the purified hsp70 was run on SDS–PAGE 12.5% homogenous gels (Pharmacia Biotechnology, Uppsala, Sweden) using the PhastGel System (Pharmacia Biotechnology, Uppsala, Sweden). The gel was silver-stained or transferred to PVDF membrane (BioRad Laboratories, Hercules, Calif.). The membrane was blocked at 4°C with 5% bovine serum albumin (BSA; Sigma, St. Louis, Mo.) overnight and incubated for 1 h at room temperature with 1 μg/ml SPA-820 mouse anti-hsp/hsc70 antibody (StressGen Biotechnologies, Victoria, B.C., Canada) or with mouse sera. After washing for 3×5 min with 0.5% Tween-20 in phosphate-buffered saline (PBS), the membrane was incubated with 1 μg/ml peroxidase-conjugated rabbit anti-mouse antibody (Dakopatts, Denmark) for 1 h, washed as above, incubated again with 1 μg/ml peroxidase-conjugated swine anti-rabbit antibody (Dakopatts, Denmark) and washed again as described above. The blot was developed with 9 mg/ml 3,3'-diaminobenzidine (Sigma Chemical Co., St. Louis, Mo.) and 0.018% H_2O_2 in PBS for 10 to 50 s at room temperature, and then dried.

Immunizations and in vivo tumor outgrowth

Mice were immunized with either 10^6 irradiated (10^4 rad) MC57X or MC57S tumor cells, 10 μg hsp70 purified from one of the tumors or from the liver, or with 200 μl elution buffer s.c. once a week for 3 weeks. In some experiments mice were bled 4 to 5 days prior to beginning the first immunization, as well as 4 to 5 days after the last immunization to obtain sera for testing with enzyme-linked immunosorbent assay (ELISA). One week after the last immunization the mice were either challenged with 5×10^5 tumor cells, or they were killed and the lymph nodes were collected. Each group of mice consisted of 6 to 10 animals.

The statistical significance for the in vivo data was calculated using a two-tailed *t*-test with two-sample unequal variance.

Generation of T cell lines

Cells from lymph nodes of immunized mice were cultured in complete media containing RPMI 1640 medium, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM/l 2-mercaptoethanol and 10% fetal calf serum (FCS; all from Life Technologies, Paisley, Scotland, U.K.). The cells were stimulated with irradiated autologous tumor on days 0 and 9, and 2 days after restimulation 50 U/ml interleukin-2 (IL-2) was added to the culture. The cells were cultured for 14 days and then tested for TNF-α release.

TNF-α release assay

10^5 T cells were plated in 100 μl culture media in a 96-well plate. 10^5 tumor cells were added in 100 μl culture media to each well and the mixture was incubated for 20 h at 37°C, 5% CO_2. Supernatants were collected and their TNF-α content was determined by testing their cytotoxic effect on WEHI 164 clone 13 cells in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay [7].

Anti Hsp70 ELISA

ELISA for measuring the presence of anti-hsp70 specific antibodies was set up using the following procedure: ELISA plates were coated with 2 μg/ml purified hsp70 (prepared by the ATP purification method mentioned above) in PBS overnight at 4°C. After washing three times in PBS containing 0.05% Tween-20, the plates were incubated for 10 min with 10% fetal calf serum (FCS) in PBS and washed as described above. The plates were then incubated for 3 h at 37°C with mouse sera diluted 10, 50, 100 or 1,000 times in PBS. After washing as above, the plates were incubated with

AP-conjugated antibody specific for mouse Ig, IgG, IgG1 or IgG2a (Southern Biotechnology Associates Inc., Birmingham, Ala.) for 1 h at 37°C. After a final washing step, the substrate *p*-nitrophenyl phosphate (Sigma, St. Louis, Mo.) diluted in DEA buffer (0.097% diethanolamine, 0.1 mg/ml $MgCl_2$, pH 9.8) was added, and the optical density (OD) was measured at 405 nm.

The standard used was the IgG2a anti-hsp/hsc70 antibody SPA-822 (StressGen Biotechnologies Corp., Victoria, B.C., Canada). For IgG1 no standard known concentration was available, which is why only the OD values were shown for this antibody.

Half of the sera was also tested by first coating the plates with either of the hsp70-specific capture antibodies DI73 (IgG2a) or DI69.7-1 (IgG1), kindly provided by Satish Jindal (Cambridge, Mass.) and run as above.

Results

Purification of hsp70

Hsp70 was purified from MC-induced sarcomas (MC57X or MC57S) induced in B6 mice [9, 10] or from the liver of healthy B6 mice using ADP affinity and ion exchange chromatography [13]. For some experiments where hsp70 without peptides was needed, ATP affinity purification was used instead of ADP since ATP leads to the release of peptide from the hsp70 complex [3, 6]. The purified hsp70 was tested for its level of purity by SDS–PAGE separation (Fig. 1; gel), and the identity of the hsp70 was further confirmed by western blot analysis (Fig. 1; blot). The purity of the hsp70 was >95%, as determined by densitometry.

At the beginning of the study, hsp70 was purified by ammonium sulfate precipitation [5, 17]. Since this method resulted in an hsp70 purity which did not exceed 65% of the final protein content in the sample (data not shown), this approach was later replaced with the more recent ADP affinity chromatography method.

Tumor-derived hsp70 protects against tumor outgrowth in vivo

We wanted to analyze the capacity of hsp70 isolated from B6-derived MC tumors to induce tumor protection in vivo. For this purpose, groups of mice were immunized with tumor-derived hsp70 purified by ADP affinity chromatography, a method which preserves peptides bound to hsp70 [13]. As control, mice were immunized with irradiated MC tumors of the same type (MC57X or S) as that from which the hsp70 was derived, or with buffer only. After three immunizations 1 week apart, the mice were challenged with live tumor and tumor outgrowth was monitored for at least 50 days. Pooled data from the five experiments performed are summarized in Table 1 and two representative experiments with the MC57X tumor are shown in Figs. 2 and 3. We were able to confirm [13, 17] that tumor-derived hsp70 elicited in vivo protection. Thus, 64% of the mice immunized with hsp70 survived challenge with the same tumor as that from which the hsp70 was purified (Table 1). This resulted in a significant protection ($P = 0.0003$) compared to that observed in the PBS-immunized mice, where only 27% of the mice survived. A comparable protection was induced using hsp70 purified by either the ADP affinity method or the ammonium sulfate method (Fig. 2).

Although hsp70 immunization resulted in a clear tumor protection, the protection observed after immunization with irradiated tumor cells was significantly stronger ($P < 0.001$), resulting in a 98% survival rate of mice (Fig. 2 and Table 1).

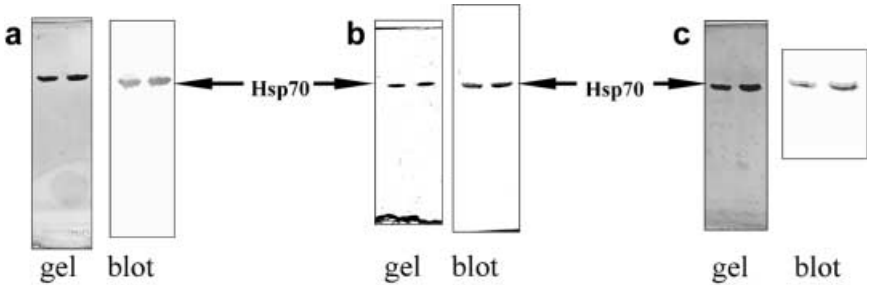
Table 1 Protection of mice from tumor outgrowth

Immunization[a]	Irradiated tumor	Hsp70 from tumor	Hsp70 from liver tissue	Buffer
Total no. of mice	42	42	22	45
No. of surviving mice	41	27	9	12
% surviving mice	98	63	41	27
Significance[b]	$P < 0.001$	$P < 0.001$	$P = 0.27$	–

[a] Mice were immunized with irradiated tumor cells (MC57X or MC57S), hsp70 purified from the respective tumor, hsp70 purified from B6 liver or with buffer as described in Materials and methods, and challenged with live 5×10^5 tumor cells of the same type as that used for the immunization. Data are pooled from five experiments. The liver hsp70 group was included in three of the experiments

[b] Significance was calculated by comparing the mice in each group with those in the buffer group, using a two-tailed Student's *t*-test with two-sample unequal variance

Fig. 1 Silver-stained SDS–PAGE and western blot of purified hsp70. The hsp70 was purified either from tumor by ADP (**A**), from liver by ADP (**B**) or from liver by ATP (**C**) affinity chromatography and ion exchange chromatography. Samples were run on SDS–PAGE and were silver-stained or western-blotted followed by immunodetection using hsp70-specific antibody as described in Materials and methods



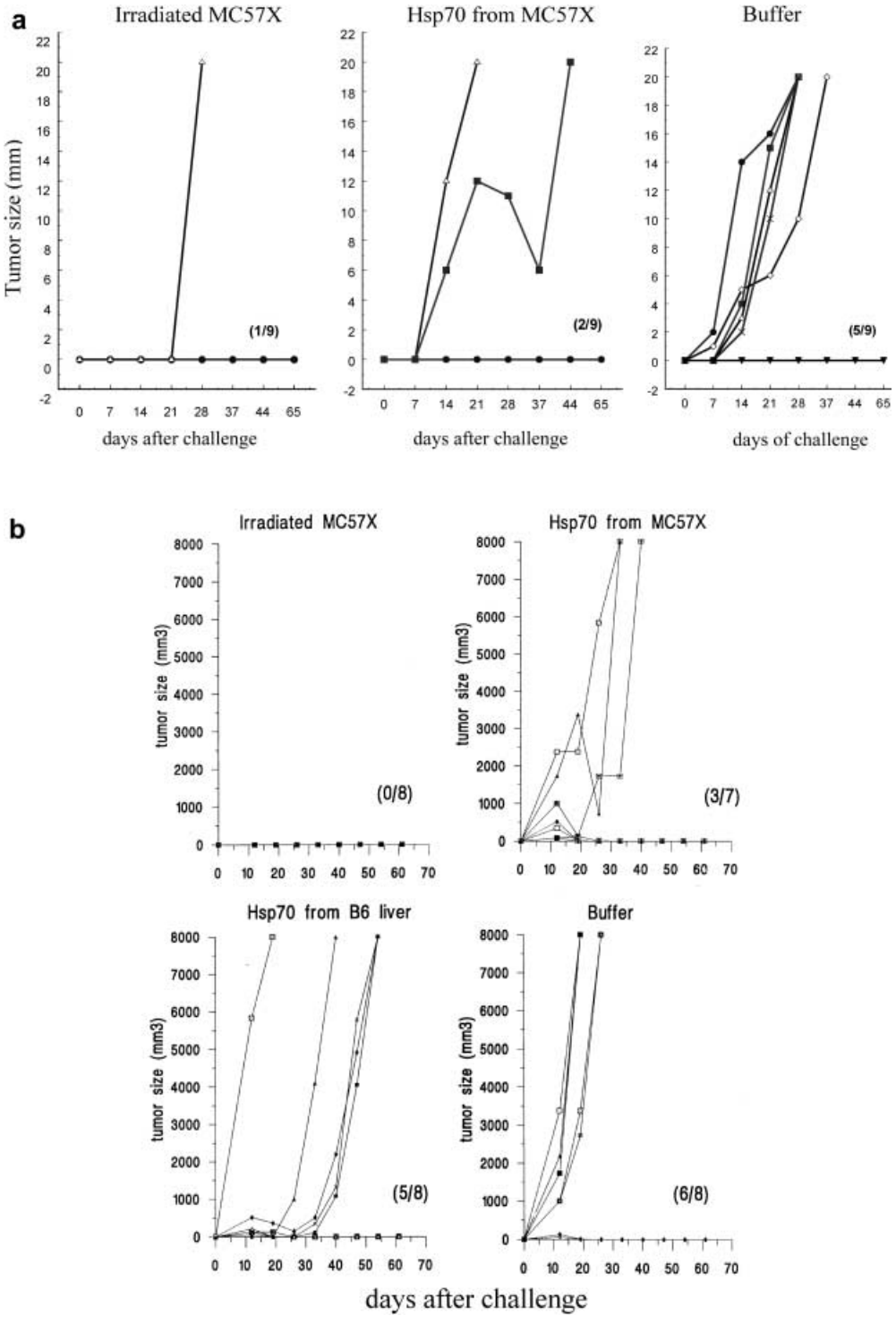


Fig. 2 Tumor rejection in vivo. Mice were immunized with irradiated MC57X cells, hsp70 from MC57X, hsp70 from liver or buffer as described in Materials and methods, and challenged with 5×10^5 live MC57X cells. Each line represents the tumor growth in one mouse. The numbers in brackets indicate the number of mice with growing tumor/number of total mice in the group. **A** The hsp70 was purified by precipitation. **B** The hsp70 was purified by affinity chromatography. Each line in the graphs represents one mouse, except the line for no outgrowth, as several lines can be overlaid at 0 outgrowth

Liver-derived hsp70 delays tumor outgrowth

We also analyzed the data in Table 1 to determine whether immunizing with hsp70 from tumor tissue would result in a significantly better protection than by immunizing with hsp70 from liver tissue derived from healthy mice. We observed that 41% of the mice immunized with hsp70 from liver tissue survived the challenge with live tumor. This protection was significantly lower than in the 63% of mice that survived the tumor challenge after immunization with tumor hsp70 ($P < 0.05$).

Immunization with the liver-derived hsp70 resulted in a somewhat higher number of protected mice compared to buffer-injected control mice (41% compared to 27% surviving mice, respectively), although this difference was not statistically significant (Table 1). In two out of the three experiments in which liver hsp70 was included, liver hsp70 immunization resulted in a higher

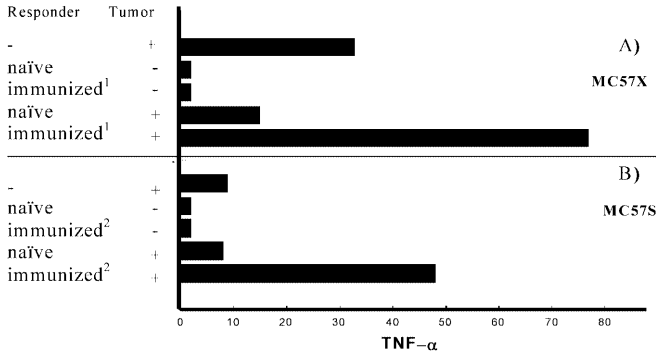


Fig. 3 T cell lines from mice immunized with hsp70 from MC57 tumors produce TNF-α in vitro when stimulated with tumor cells. Lymph node cultures from naive mice or from mice immunized with either hsp70 from MC57X (**A**) or hsp70 from MC57 S (**B**) were stimulated with the same tumor as that from which the hsp70 was purified, as described in Materials and methods. The supernatants were tested for TNF-α after 18 h of incubation. One experiment out of three is shown. A The T cell responder was isolated from the lymph node of a mouse immunized with hsp70 purified from MC57X. B The T cell responder was isolated from the lymph node of a mouse immunized with hsp70 purified from MC57S

proportion of mice with tumors appearing late after injection as compared to the buffer-injected mice, in which the majority of tumors appeared early ($P = 0.006$; Table 2). This delay in tumor outgrowth was seen to the same extent in the mice immunized with tumor-derived hsp70.

In these two experiments, tumor outgrowth was fast, with lethal tumor burden within 3 weeks for most of the buffer-immunized mice. In the third experiment the tumors grew significantly more slowly, and reached large sizes only after 5 weeks in the buffer-immunized mice. In this experiment, no difference was observed in the rate of tumor outgrowth between the buffer control and the hsp70-immunized groups (data not shown).

Table 2 Immunization with hsp70 from liver or MC57 tumor delays tumor outgrowth

Immunization[a]	Hsp70 from tumor	Hsp70 from liver tissue	Buffer
No. of mice with late tumor outgrowth[b]	4	7	1
No. of mice with early tumor outgrowth[c]	2	3	12
Significance[d]	$P = 0.017$	$P < 0.006$	–
Total no. of mice with tumor outgrowth	6	10	13
No. of mice with no tumor outgrowth	9	6	5
Total no. of mice	15	16	18

[a]Mice were immunized with hsp70 purified from MC57X tumor, hsp70 purified from B6 liver or with buffer, as described in Materials and methods, and challenged with live MC57X tumor cells. Data are pooled from two experiments

[b]Tumor growth was defined as early if the tumor size reached a diameter of 12 mm (or 1,800 mm^3) within 3 weeks. Recovery of the mouse after the tumor had reached this size was never observed. Tumor growth was defined as late if the tumor size reached a diameter of 12 mm after 3 weeks

[c]If the tumor was smaller than 12 mm (or 1,800 mm^3) 3 weeks after challenge, tumor growth was considered to be late

[d]The significance was calculated using Fisher's exact test. The proportion of early and late tumor outgrowth in each hsp70 group was compared to that in the buffer group

Immunization with hsp70 induces T cells that can respond by tumor-specific production of TNF-α in vitro

To investigate whether immunization with hsp70 purified from tumors could elicit tumor-specific responses in vitro, mice were immunized with irradiated tumor cells, with hsp70 purified from tumor tissue, or with buffer. Lymph node cells from the immunized mice were cultured in vitro with irradiated tumor cells from the same tumor as that used for immunization. After 14 days, the cultures were tested for TNF-α production when stimulated with the immunizing tumor. We found that T cells from mice immunized with hsp70 purified from MC57S did indeed produce TNF-α when mixed with MC57S tumor cells, while T cells from naive mice did not (Fig. 3). In another experiment the T cells were also stimulated with MC57X or RMA T cell lymphoma, which did not result in any TNF-α production (data not shown). The same pattern of TNF-α production was observed when using hsp70 purified from the MC57X tumor (Fig. 3).

Repeated hsp70 immunization does not elicit an hsp70-specific antibody response

One of the important parameters for a vector of antigenic epitopes to be effective is that it should not itself be immunogenic, since this could potentially result in its rapid elimination e.g. by neutralizing antibodies [12]. We therefore measured the production of anti-hsp70-specific antibodies in the sera of immunized mice before the first and after the last immunization (Fig. 4).

In one experiment, the anti-hsp70 antibody levels remained low in all hsp70-immunized groups, although interestingly mice in the tumor immunized group showed some anti-hsp70 antibody response (Fig. 4a). In a second experiment, three of the immunized groups showed increased levels of hsp70-specific antibodies: two groups immunized with irradiated tumor, and the group immunized with hsp70 purified from MC57X (Fig. 4b). This increase was observed in the total Ig, IgG and IgG1 subclasses, but not in IgG2a. However, when the sera were diluted 1,000 times the levels of antibody were no longer higher in the sera from the immunized mice compared to those before immunization or to the other groups. The titers were not high, and did not amount to more than 10 times the naturally existing levels of the anti-hsp70-specific antibody.

The increase in hsp70-specific Ig was not seen in the group of mice immunized with hsp70 purified from the liver in either experiment (Fig. 4a and b), regardless of

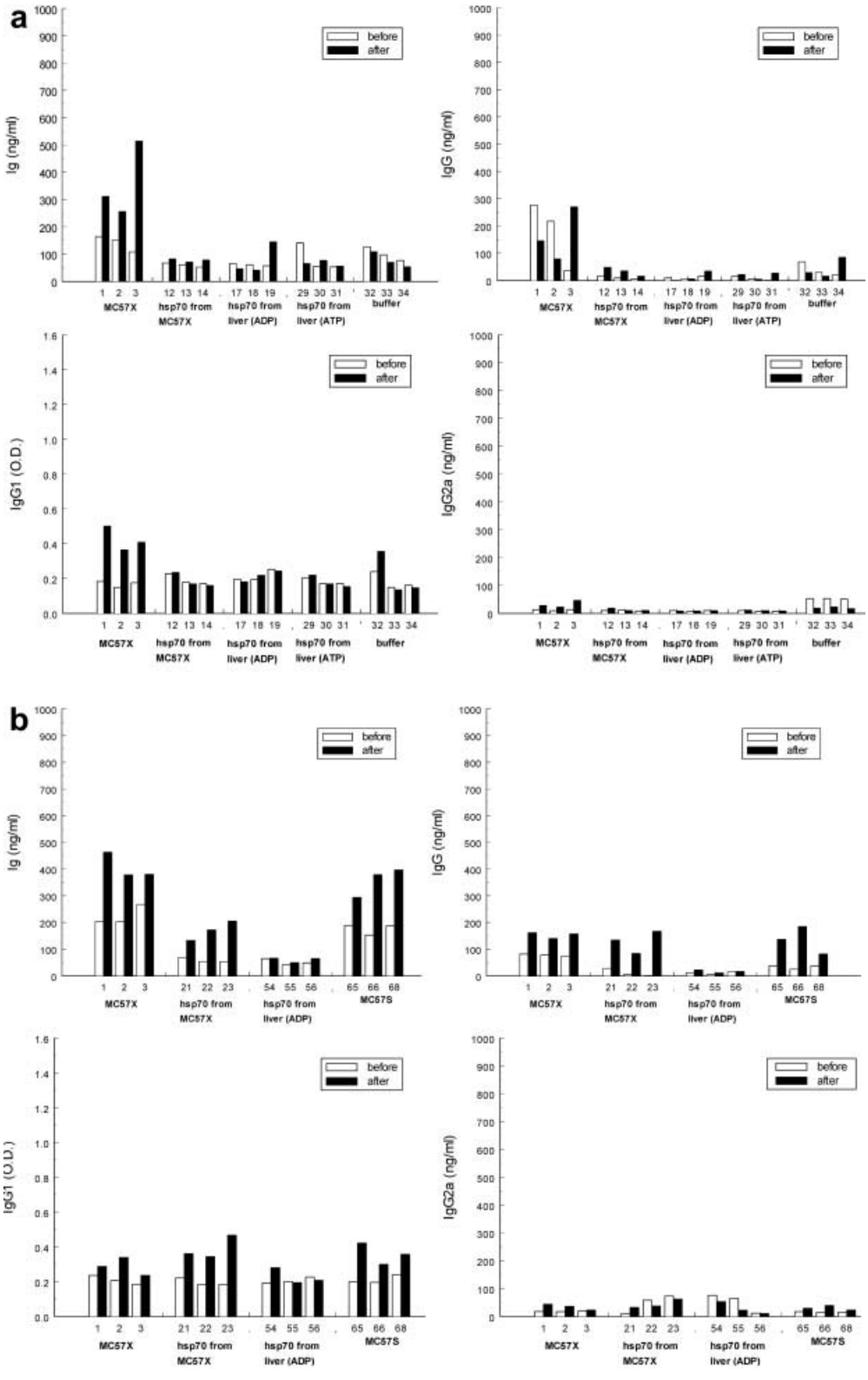


Fig. 4 Titers of anti-hsp70-specific antibodies in sera from mice immunized with MC57 tumor, hsp70 or buffer. Mice were immunized with the indicated tumor or protein, as described in Materials and methods. Sera were collected 4 to 5 days before the first immunization and 4 to 5 days after the last immunization from eight mice per group (from which three representative sera are shown here). The sera were diluted 10 times and tested by ELISA for total Ig, IgG, IgG1 and IgG2a antibodies specific for hsp70. The data are expressed as relative amount of antibody compared to the standard antibody IgG2a anti-hsp/hsc70 antibody SPA-822 (StressGen Biotechnologies Corp., Victoria, B.C., Canada). No standard was available for IgG1,, which is why only the OD values are shown for this antibody. **A** Experiment 1; **B** Experiment 2

whether the liver hsp70 was purified with ADP (still binding liver peptides) or ATP (no bound peptides). We therefore concluded that the hsp70 per se does not elicit significant antibody responses using our immunization protocol.

Discussion

In this study we investigated the capacity of hsp70 isolated from MC-induced sarcomas MC57S and MC57X

to induce tumor protection in vivo and stimulate T cells to respond in vitro. The reason for choosing these MC tumors for this study was that they had been previously demonstrated to possess strong tumor-associated transplantation antigens in vivo [9; 10] and to express individually distinct MHC-I associated peptides, recognized by TNF-α producing $CD8^+$ T cells in vitro [11]. We here observe that hsp70 purified from the same MC57 tumors can "mimic" the immunogenic properties of the intact irradiated tumor cells, eliciting tumor protection in vivo and priming T cells to recognize tumor cells in vitro.

Although the protection with tumor-derived hsp70 was not as strong as that induced by immunization with irradiated tumor cells, it was still significant and was observed in all five experiments. There was also a significant tumor protection when comparing the tumor-derived hsp70 with the liver-derived hsp70, strongly implicating the contribution of tumor-derived hsp70 bound peptides. These results therefore confirm previous reports that hsp70 has a protective action against the outgrowth of experimental sarcomas [13, 17]. The protection seen with the hsp70 immunization was specific to the tumor from which the hsp70 had been purified, as hsp70 from MC57X could not protect the mice from MC57S (data not shown).

Our in vivo results were consistent with our in vitro T cell data, in which T cell cultures from hsp70-immunized mice were tested for TNF-α production when mixed with the same tumor as that from which the hsp70 originated. The T cell cultures from mice immunized with tumor-derived hsp70, but not the cultures from naive mice, produced TNF-α when stimulated with the cognate tumor. We have previously reported that $CD8^+$ as well as $CD4^+$ T cells producing TNF-α in vitro in response to stimulation with the specific MC57 tumor are present in the lymph nodes of mice immunized with irradiated tumor cells [11]. The subtype of the hsp70-induced T cells in the present system is currently being analyzed, although immunization with hsp70 has previously been shown to activate $CD8^+$ T cells [1, 4].

Interestingly, there was also a small increase in survival of the mice immunized with liver-derived hsp70 when compared to the buffer-injected group (41% compared to 27%, respectively), although this difference was not statistically significant ($P = 0.27$). Since in two out of the three experiments involving liver-isolated hsp70 we observed a delay in tumor appearance compared to the saline-injected group (Fig. 2b and Table 2), we concluded that liver-derived hsp70 can temporarily increase tumor resistance. In the experiment where immunization with liver-derived hsp70 did not delay the appearance of tumors, tumor outgrowth was also very slow in the control group, indicating that the non-specific effect of liver-derived hsp70 may be short-lived. It is possible that hsp70 might cause a non-specific and short-lived activation of the immune system, unrelated to the role of hsp70 as a provider and protector of antigenic tumor-specific peptides. As the liver hsp70 was purified from healthy mice, the difference in outgrowth cannot be explained by metastasis of the tumor to the liver and thereby tumor peptides in lower concentrations in the liver hsp70.

To analyze whether repeated immunizations with hsp70 would elicit hsp70 specific antibodies, pre- and post-immunization sera were tested for hsp70-specific antibodies. Interestingly, antibodies specific for hsp70 were only consistently detected in the groups immunized with irradiated tumor cells (MC57X or S), while immunization with tumor-derived or liver-derived hsp70 induced no or in one out of two experiments only low levels of anti-hsp70 antibodies. It seems therefore that hsp70 itself does not elicit significant antibody responses. This should also be considered in approaches where hsp70 mixed in vitro with known antigen peptides can elicit protective responses in vivo that correlate with CTL induction in vitro [1, 4]. Moreover, the antibody data together with the apparently healthy condition of the mice after more than 4 months of repeated hsp70 immunizations suggest that the risk of inducing autoimmunity is low. This argues that hsp70 is a safe vector to use also for multiple immunizations as a vaccine against cancer and infectious agents.

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