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13. ABSTRACT *(Maximum 200 Words)*

Several studies have shown that certain stress proteins can function as potent vaccines against a specific cancer when purified from the same tumor. We have hypothesized in our application that hsp110 may be an excellent candidate for cancer vaccines. Here, we describe the studies of the vaccine potential of hsp110 and its ER homologue grp170, two long recognized but unstudied stress proteins. Vaccination with these two HSPs purified from Meth A fibrosarcoma caused the complete tumor regression. A significant growth inhibition of Colon 26 tumor was also seen in the immunized animals. HSP vaccination significantly extended the life span of tumor-bearing mice when applied after tumor transplantation and decreased experimental lung metastasis in a melanoma tumor model. A tumor specific CTL response developed in the mice immunized with tumor derived hsp110 or grp170. Antibody depletion assay demonstrated that NK cells in conjunction with CD4+ and CD8+ T cells were involved in the protective immunity elicited by HSP vaccination. Furthermore, treatments of the mice with bone marrow derived dendritic cells pulsed with hsp110 or grp170 from tumor also elicited a strong anti-tumor response indicating that dendritic cells can be used to efficiently mediate this therapeutic approach. Additionally, we showed that mild, fever-like hyperthermic conditions, which have been shown to stimulate other immunological functions, also stimulate the vaccine efficiency of hsp110 as well as hsc70, but not grp170. Peptides profile stripped from tumor-derived hsp110 was also analyzed. We also extend our studies to the development of hsp110 vaccines targeting breast tumor antigen. Immunization with ICD (intracellular domain of Her2/neu) complexed with hsp110 elicited antigen specific immune response. These studies indicate that these large stress proteins can be used in heat shock protein-based cancer immunotherapy.

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INTRODUCTION

Tumor derived heat shock protein (hsp)-peptide complexes (particularly hsp70 and grp94/gp96) have been demonstrated to serve as effective anti-tumor vaccines (1, 2). This approach takes advantage of the peptide binding properties of stress proteins which are responsible for their functions as molecular chaperones in numerous processes such as protein folding, transport, assembly, and peptide trafficking in antigen presentation (3, 4). Purification of specific heat shock proteins from tumor also provides its associated immunogenic peptides. In addition, vaccination with hsp/grp-peptide complexes derived from tumors circumvents the need to identify a large number of CTL epitopes of a cancer and the technical limitations associated with that approach.

Heat shock proteins (HSPs) are highly conserved and abundant proteins in both eukaryotes and prokaryotes. The heat shock proteins of mammalian cells can be classified into several families based on their size and structure (hsp25, hsp70, hsp90, and hsp110). However, in addition to hsps, a second set of stress proteins has been long observed which are localized in the endoplasmic reticulum (ER). These stress proteins have been referred to as glucose regulated proteins (grps) (such as grp78, grp94, and grp170), which are regulated by stresses which disrupt the function of the ER (5). While individual stress proteins have been studied for several years, the largest of the above hsp and grp groups, hsp110 and grp170, have been almost entirely ignored. These two stress proteins have only been cloned within the last few years and their characterization remains at a very preliminary level (6-12). In our laboratory, analysis of secondary structure indicated that, while exhibiting similarities to hsp70, hsp110 and grp170 appear to exhibit a peptide-binding cleft with significantly enlarged "lid" domain (13). This suggests that hsp110/grp170 binding affinities and/or capacities differ from hsp70. Our studies have confirmed that hsp110 exhibit a different peptide-binding capacity (9, 12) and grp170 is involved in immunoglobulin chain binding (14). Most notably, Grp170 may be the ATPase responsible for peptide import into the ER from TAP (Transporter associated with Antigen Processing) (15-17). Based on the previous studies on these two stress proteins in our laboratory and the previously demonstrated effectiveness of a few other stress proteins as vaccines, we hypothesized in our application that hsp110 may be an excellent candidate as anti-tumor vaccine. Here, we described the analysis of the vaccine potential of hsp110 purified from tumor using conventional approach. Additionally, we also explored the possibility of using hsp110 complexed with her2/neu as vaccines targeting breast cancer. Furthermore, we extended our investigation to another high molecule weight heat shock protein grp170, which is an ER homologue of hsp110.

BODY

Purification of hsp110, grp170 from tumor and normal liver tissue

In order to evaluate vaccine potential of hsp110, we first optimized the purification protocols for hsp110 using syngeneic transplantable mouse tumor CT26 tumor tissue (Objective 1, task A), Typical biochemical methods were used and this approach consists essentially of preparing cell lysates with hypotonic buffer, clarification of lysate by ultracentrifugation, removal of glycoproteins with Con A-Sepharose, ion-exchange chromatography (FPLC) using Mono Q column and salt gradient to elute hsp110. Further clean-up of preparation can be achieved with size-exclusion column superose 12. In addition, we also perfected the condition for isolating grp170 (ER homologue of hsp110). Grp170 was recovered using Con A-sepharose column after incubation with buffer containing 15% α-D-methylmannoside (see detail in Appendices). Hsp110 and grp170 were purified simultaneously from tumor and liver. The purity of the proteins was assessed by SDS-PAGE and silver staining as shown in figure 1. Approximately 20-50 μg hsp110 and 10-40 μg grp170 were obtained from each gram-wet weight of tumor or tissue. The yield of grp170 from tumor is usually higher than that from normal tissue as a result of a higher level of grp170 expression in the tumor, possibly due to a hypoxic tumor fraction.

While working out the purification protocol, we observed that hsp110 exists in a large complex of 400 kDa to700 kDa. Another two heat shock proteins hsc70 and hsp25 were found to directly interact with hsp110. Furthermore, it was observed that luciferase migrate into this chaperone complex following heat shock, when it was added to this in vitro system, suggesting that these heat shock protein might form a chaperoning machine in vivo. The chaperoning functions of this complex are being examined and a study of the vaccine potential of the complete complex is under consideration Deletion mutant analysis demonstrates that peptide-binding domain is required for interaction with hsp25, but not with hsc70 (For further detail, see reprint attached in appendices).

Vaccine studies of hsp110 and its ER homologue grp170

As outlined in Objective 2 of application, we then investigated whether immunization with purified hsp110 and grp170 could protect mice against tumor challenge. For this purpose, the methylcholanthrene-induced fibrosarcoma (Meth A) tumor model was initially employed. We immunized mice twice with 40 μg (dose based on preliminary data) hsp110 or grp170 and then challenged them with Meth A cells by intradermal injection (Figure 2). It is seen mice immunized with hsp110 and grp170 were protected from the Meth A tumor challenge. Interestingly, and similar to studies of others, most hsp110/grp170 vaccinated animals transiently developed tumors which then regressed and disappeared. However, in the mice which were immunized with grp170, two of five mice failed to develop any measurable tumor mass.

To test the generality of these observations on the vaccine activity of hsp110 and grp170 in the Meth A sarcoma tumor system, we next chose the Colon 26 tumor model. This model was chosen since it has proven to be less immunogenic than the Meth A and to be generally more resistant to various therapies. Groups of mice (five mice per group) were immunized with PBS or with varying quantities of tumor-derived hsp110 or grp170. Hsp110 or grp170 were also isolated from the livers of the same animals and this or PBS was used as control. As seen in

figure 3, all mice that were immunized with PBS or liver derived hsp110 or grp170 developed rapidly growing tumors. In contrast, mice immunized with hsp110 and grp70 from CT26 tumor showed a significant tumor growth delay, in general agreement with the above Meth A results. The inhibitory of hsp110 or grp170 vaccination on tumor growth was dependent on the dose of HSP used for immunization. While mice immunized with 20 μg (per injection) of HSP only slightly slowed tumor growth, those immunized with 40 or 60 μg of hsp110 or grp170 showed increasingly significant tumor growth delays. On each day examined (e.g., 15, 21, 27 days after challenge), the mean volumes of the tumors that developed in mice immunized with hsp110 or grp170 at doses of 40 and 60 μg were significantly smaller than those of control mice ($p < 0.01$, student's t test). However, the differences in the mean volumes of the groups injected with PBS or liver derived hsp110/grp170 preparations were not significant. Lastly, mice immunized with Meth A derived hsp110 or grp170 were not resistant to challenge with CT 26 tumor cells. Although tumor growth was not preventable in this highly aggressive and rapidly growing tumor system, this data demonstrates that hsp110 and grp170 have specific anti-tumor effects.

In considering the clinical application of a tumor vaccination strategy, it is more realistic to treat animals with tumor present at the time of vaccination. Thus, the aggressive CT26 tumor was again examined in using a therapy approach. Tumor cells were first established in the flank of mice (10 mice each group). When tumors were readily palpable after inoculation, animals were treated with liver or CT 26 derived hsp110 or grp170 on a weekly basis. It was found that tumor bearing mice treated with autologous tumor HSP showed significantly longer survival times compared to the untreated mice or mice immunized with liver derived hsp110 or grp170 (Figure 4), all control mice died within 30 days, but approximately half of each group survived to 40 days and 20% of grp170 treated mice lived beyond 60 days, clearly demonstrating a beneficial anti-tumor effect. In parallel with previous data, grp170 appears to be more efficient than hsp110 on an equal mass basis.

Since cellular immunity appeared to be critical in mediating the observed antitumor effects (18-20), we analyzed the ability of tumor-derived hsp110 and grp170 preparations to elicit a tumor specific $CD8^+$ T cell response. Splenocytes generated from immunized mice were used as effector cells in CTL assay (Figure 5). Splenocytes from mice immunized with CT26 cell derived hsp110 or grp170 preparations showed specific lysis for CT26 tumor cells only, but not Meth A tumor cells; conversely, splenocytes from animals immunized with Meth A tumor cells were only effective against Meth A cells and not CT 26 cells. This again demonstrates that vaccination with hsp110 or grp170 elicits a tumor specific CTL response.

Dendritic cells (DCs) have been known to be highly specialized antigen-presenting cells and to be the principal activators of naïve T cells in vitro and in vivo (21). In order to investigate whether antigen presenting cells could be involved in the anti-tumor response elicited by hsp110 or grp170 immunization, we tested the ability of DCs to acquire an anti-tumor activity, presumably by presentation of hsp110 or grp170 chaperoned peptides. DCs were prepared from mouse bone marrow and then pulsed with grp170 or hsp110 purified from the CT26 tumors. Cells were injected intravenously back to the mice. Ten days after the second immunization, mice were challenged with CT 26 tumor (Figure 6). It was observed that tumors grew rapidly in the mice that received PBS or (non-pulsed) DCs alone. However, tumor growth was significantly

delayed in mice immunized with DCs pulsed with hsp110 or grp170. Moreover, based on the immunization effects in the mice which received 10^6 DCs pulsed with 20 μg protein and those that received two 40 μg protein by subcutaneous injections, it is found that hsp110/grp170 pulsed DC based immunotherapy was both more effective and used less protein.

Several recent studies have indicated that a modest increase in body temperature sustained for several hours, i.e. a condition comparable to common febrile response, can significantly affect certain immunological endpoints and immune function (22-25). We therefore exposed mice to 39.5 °C (i.e. core temperature) whole body hyperthermia (WBH) for a period of 8 hours to determine if hsp/grp vaccine efficiency might also be altered as a result of a fever-like thermal condition. As shown in Figure 7, hsc70 or hsp110 are significantly more efficient when purified from tumors derived from animals receiving prior fever-range WBH. However, the prior fever-range thermal treatment is seen to reduce the vaccine efficiency of grp170. This data indicates that fever-like exposures can influence the antigen presentation pathway and/or peptide binding properties of these two (heat inducible) hsps purified from CT 26 tumors but not a heat insensitive grp.

Because our goal is to generate more effective therapeutic strategies, the therapeutic efficacy of the grp170 immunization was tested in mice with advanced lung metastasis. Syngeneic C57BL/6 mice were inoculated i.v. with B16F10 melanoma cells. Following i.v. injection, the B16F10 cells rapidly migrate to the lungs, and mice die from metastatic lung tumor within 4 weeks. On day one after inoculation of tumor, immunotherapy was started. Each mouse was given one injection of 40 μg grp170 weekly. Mice were then sacrificed and lungs were removed, visually inspected. Figure 8. Control mice had a mean of 116 colonies in the lungs, while mice that received grp170 immunization developed less lung colonies with means of 62(±12.6) colonies per mouse. The greatest reduction in lung metastasis can be seen in mice treated with grp170. Therapy with liver-derived grp170 does not have a measurable effect. Statistical analysis demonstrates a significant difference between tumor grp170 treatment group and untreated group or the group treated with liver grp170.

To determine the subset of lymphocytes important for antitumor immunity, antibody depletion assays were carried out. In vivo depletion of CD4+, CD8+ or NK cells with MABs was performed every other days beginning 5 days prior to tumor inoculation, followed by depletion twice weekly thereafter until mice developed progressive tumors. Treatment with grp170 started one day after tumor establishment (Figure 9). When CD4+, CD8+ or NK cells were depleted, the therapeutic effects of grp170 immunization were partially lost. The depletion of NK cells has a less dramatic effect on tumor metastasis, although it is statistically distinct from vaccinated, undepleted animals. In comparison, all mice injected with control antibody or the mice without depletion showed reduced lung metastasis. Clearly, NK cells in conjunction with CD4+ and CD8+ T cells were involved in the protective immunity elicited by HSP vaccination.

In order to develop hsp110 based breast cancer vaccines for immunotherapy, we chose the intracellular domain (ICD) of Her-2/neu, a well known tumor associated antigen which is highly expressed in 40% of breast cancers and is also present in many prostate, ovarian, colon and lung cancers as well (26). We took advantage of the property of hsp110 and grp170 to bind large proteins to develop a novel approach to hsp vaccine therapy, i.e. utilizing recombinant hsp110 or

grp170 non-covalently bound to a well-known, full length, tumor antigen *in vitro* as a vaccine. As shown in Figure 10, we immunized A2/Kb neu transgenic animals with the hsp110-ICD complex and evaluated their cellular immune response using ELISPOT techniques. We observed that the hsp110-ICD complex was highly potent in eliciting a response, i.e. as efficient as Complete Freund's Adjuvant in stimulating an antigen specific immunity as evaluated by production of IFN-γ.

Finally, one of the goals of our grant was to strip peptides from hsp110 for further characterization, including sequencing. As of the present time, no laboratory has successfully stripped and sequenced any peptides from any heat shock proteins, although this has been an important goal of several groups in the field. We have invested significant effort in this area during the period of funding. Only recently have we been successful in stripping peptides from hsp110 which could then be admissible for further analysis, i.e. sequencing. Current studies have been successful in sequencing a peptide bound to hsp110 purified from a mouse colon carcinoma (Figure 11). This is the first time a peptide has been stripped from any hsp/grp and sequenced. This study now opens up important new avenues for the investigation of the nature and antigenicity of hsp bound peptides. At the time of writing we have purified hsp70, hsp110 and grp170 from Her-2/neu overexpressing, transgenic mice. These animals spontaneously develop tumors and this "FVB" mouse model is considered to be an optimal model for the study of breast cancer. Peptides bound to these hsps are presently being analyzed. This work will continue into the next year, without additional funding from DOD.

KEY RESEARCH ACCOMPLISHMENTS

1. Optimization of purification scheme for hsp110 and its ER homologue grp170.
2. Immunization of Meth A tumor derived hsp110 or grp170 cause the complete regression of tumor.
3. Vaccination with tumor hsp110 or grp170 results in significant tumor growth in colon26 tumor model. However, normal liver derived hsp110 or grp170 has no effect.
4. Hsp110 or grp170 immunization significantly improves the survivals of colon26 tumor-bearing mice.
5. HSP therapy reduces experimental lung metastasis in melanoma model.
6. HSP immunization elicits tumor specific CTL response.
7. NK cells in conjunction with CD4+ and CD8+ T cells were involved in the protective immunity elicited by HSP vaccination.
8. Immunization with dentritic cells loaded with tumor derived hsp110 or grp170 induces anti-tumor response
9. Fever-like WBH enhances the vaccination efficiency of tumor-derived hsp110 or hsc70.
10. Hsp110 complexed with breast tumor associated antigen Her2/neu can be used as breast cancer vaccines.
11. Based on the equal molar ratio, grp170 is more potent than hsp110 and hsc70 as anti-tumor vaccine.

REPORTABLE OUTCOMES

Publications:

Wang, XY., Chen, X., Oh, Hyun-Ju., Repasky, E. A., Kazim, L., Subjeck, J. (2000). Characterization of native interaction between hsp110 with hsp25 and hsc70 chaperones. FEBS Letter. 465(2-3): 98-102.

Wang, XY., Kaneko, Y., Repasky, E. A., Subjeck, J.R. (2000). Heat shock proteins and immunotherapy. Immunol Invest. 29(2): 131-137.

Wang, XY., Kazim, L., Repasky, EA., Subjeck, J. (2001). Characterization of hsp110 and grp170 as cancer vaccines and the effect of fever-range hyperthermia on vaccine activity. 165: 490-497.

Presentation:

Subjeck, J. R. Heat shock proteins and cancer immunotherapy. The 14th International Conference on Immunology, Cancer Immunotherapy: Pitfalls/Solutions. Buffalo, New York. 1999.

Subjeck, J. R. II International Conference on Heat Shock Proteins in immune Response. Farmington, CT. October 8-12, 2000

Subjeck J., Kazim L., Chen X, Wang X., Easton D., Manjili M.H. and Repasky E. The HSP110 and GRP170 stress proteins. International symposium on heat shock proteins in biology and medicine. Woods Hole MA, November 6-8, 2000.

Subjeck, J. R. Fourth Annual Regional Cancer Center Consortium for Biological Treatment of Cancer. Pittsburgh, PA. February 22024, 2001

Abstract:

Wang, XY., Repasky, E.A., Kazim, L., Manjili, MH., Li, Y., Subjeck, J.R. Antitumor immunity induced by the high molecular weight stress proteins. Meeting on Molecular Chaperones & the Heat shock Response. Cold Spring Harbor laboratory, Cold Spring Harbor, New York. May 2000

Wang, XY., Kaneko, Y., Li, Y., Repasky, E.A., Kazim, L., Subjeck, J.R. Anti-tumor immunity elicited by two high-molecular-weight heat shock proteins. DoD Breast Cancer Research Program, Era of Hope Meeting. (by Department of Defense, U.S. Army Medical Research and Materiel Command). Atlantic, Georgia. June 8-11, 2000

CONCLUSIONS:

- Tumor-derived high molecular weight stress protein, hsp110 or grp170 both stimulate tumor specific immunity. But HSPs from normal tissue do not induce anti-tumor response.
- Hsp vaccination induces tumor specific CTL response, indicating that induction of immunity with tumor-derived HSPs requires functional host CD8 cells.
- Antigen presenting cells (i.e. dentritic cells) are capable of representing HSP chaperoned peptides, suggesting that APCs are involved in HSP immunization elicited anti-tumor response.
- Hsp110 or grp170 from tumor cells can be used in heat shock protein based immunotherapy, Vaccination with Hsp-peptide complexes derived from tumor circumvents the need to identify a large number of CTL epitopes of cancers, because HSP chaperone and re-present all antigenic repertoire of cancer cells. Thus, tumor derived HSP could become a safe and reliable source of tumor-specific antigens for clinical application.
- Using natural chaperone functions of heat shock protein, specific tumor antigen can be complexed with HSP to develop concentrated and targeted heat shock vaccines.

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APPENDICES

Figure 1. Hsp110 and grp170 preparations from colon 26 tumor or liver of BALB/c mice. Hsp 110 (A) and grp170 (B) purified from colon26 tumor (lane 1, 3) and liver of BALB/c mice (lane 2, 4) were separated by SDS-PAGE, followed by silver staining (lane 1, 2) or immunoblotting analysis (lane 3, 4) using antibodies for hsp110 and grp170 respectively.

Figure 2. Immunization of mice with hsp110 or grp170 protects mice against Meth A tumor challenge. Mice were immunized subcutaneously with 40 μg of hsp110 or grp170 and boosted with the same amounts of these proteins 1 week later. 7 days after the second immunization the mice were challenged with 100,000 live Meth A tumor cells intradermally. Each group contained 5 mice and each line represents the kinetics of tumor growth in one mouse.

Figure 3. Immunogenicity of hsp110 and grp170 preparations purified from Colon 26 tumor. Mice were immunized twice with varying doses (20, 40 and 60 μg) of hsp110 and grp170 from Colon 26 tumor or liver of BALB/c mice as indicated. 1 week after the second immunization, mice were challenged with 20,000 live Colon 26 cells subcutaneously.

Figure 4. Effects of immunization with tumor derived hsp on the survival of tumor-bearing mice. Mice were first inoculated s.c. with 500,000 Colon 26 cells. After the tumor was palpable, mice were treated with or without 40 μg hsp110 or grp170 at weekly interval. The survival of mice was recorded as the percentage of mice surviving after the tumor challenge.

Figure 5. Tumor specific CTL response elicited by immunization with tumor derived hsp110 or grp170. Mice were immunized twice PBS, hsp110 or grp170 (40 μg) at weekly intervals. 1 week after second immunization, splenocytes were isolated as effector cells and re-stimulated with irradiated Colon 26 or Meth A tumor cells in vitro for 7 days. The lymphocytes were analyzed for cytotoxic activity using ^{51}Cr-labeled Colon 26 or Meth A cells as target cells.

Figure 6. Immunotherapy with DCs pulsed with hsp110 or grp170. DCs (1×10^7) were generated from bone marrow of BALB/c mice and incubated with hsp110 or grp170 (200 μg/ml) in vitro for 3 hrs. DCs were washed with introduced to mice (10^6 cells in 100 μl PBS per mouse) by i.v. injection. The whole immunization process was repeated 10 days later. Mice were challenged with 20,000 Colon 26 cells 10 days after second immunization.

Figure 7. Fever-like WBH enhances the vaccination efficiency of tumor-derived hsp110 or hsc70. Mice were first inoculated subcutaneously with 100,000 Colon 26 tumor cells on the frank area. After the tumor reached a size of approximately 1/1 cm, WBH was carried out as described in Materials and Methods. Tumors were collected next day, and grp170, hsp110 and hsc70 were isolated. Mice were immunized twice at weekly intervals and then challenged with 20,000 live Colon 26 tumor cells.

Figure 8. Mice carrying established i.v. B16F10 melanoma have reduced lung metastases following treatment with tumor-derived grp170. Syngeneic C57BL/6 mice were challenged i.v. on day 0 with 1×10^5 B16F10 tumor cells. 1 day after tumor inoculation, mice were treated with grp170 subcutaneously once a week for following 3 weeks. The mice were sacrificed and lungs

Figure 8. Mice carrying established i.v. B16F10 melanoma have reduced lung metastases following treatment with tumor-derived grp170. Syngeneic C57BL/6 mice were challenged i.v. on day 0 with 1×10^5 B16F10 tumor cells. 1 day after tumor inoculation, mice were treated with grp170 subcutaneously once a week for following 3 weeks. The mice were sacrificed and lungs were counted for tumor metastases.

* Statistically significantly different from the untreated groups and the groups treated with liver-derived grp170

Figure 9. Involvement of lymphocyte subset in grp170 elicited antitumor immunity. Depletion of T or NK1.1 cells was accomplished by injection of antibodies to CD4+(GK1.5), CD8+(2.43) and NK1.1 (PK136) on day 5, day 3 and day 1 before establishment of lung metastasis and maintained by injection twice weekly during the experiment. 1 day after tumor inoculation, mice were treated with grp170 subcutaneously once a week for following 3 weeks. The mice were sacrificed and lungs were counted for tumor metastases.

Figure 10. Frequency of IFN-gamma producing T cells by A2/Kb transgenic animals. Five animals/group were immunized i.p. with 25 μg of the recombinant mouse hsp110-ICD, or CFA-ICD complexes. Animals were boosted after 2 weeks and sacrificed 2 weeks thereafter. Control groups were injected with 25 μg of ICD, hsp110, or left non-immunized. 10^7 cells/ml were cultured *in vitro* with 25 μg/ml PHA or 20 μg/ml ICD overnight and IFN-gamma secretion was detected in an ELISPOT assay.

Figure 11. Analysis of hsp110-derived peptides. The hsp110-peptide complexes purified from CT26 tumor were acidified with acetic acid (0.2 M) and boiled for 5 min to dissociate bound peptide. The "stripped" peptides were recovered in the flow-through using a Centricon-10 filter and then analyzed by mass spectrometry.

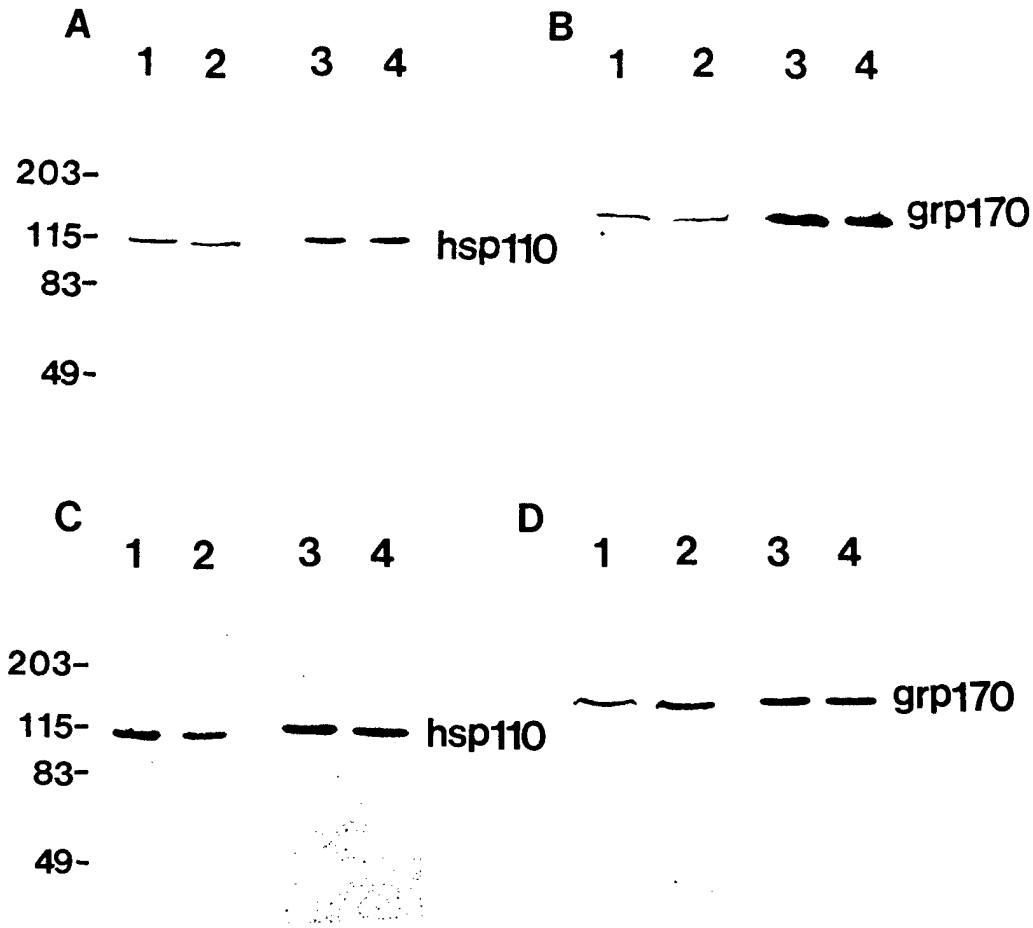
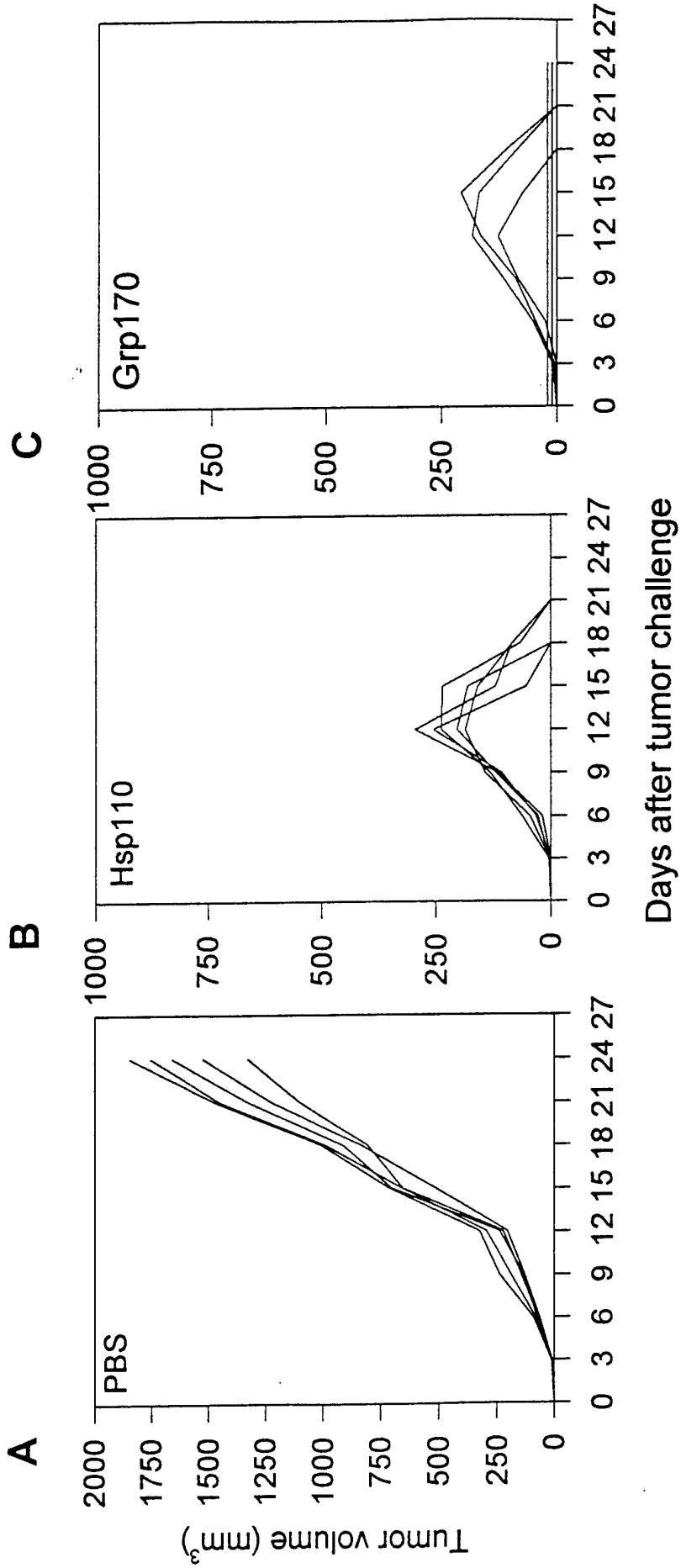
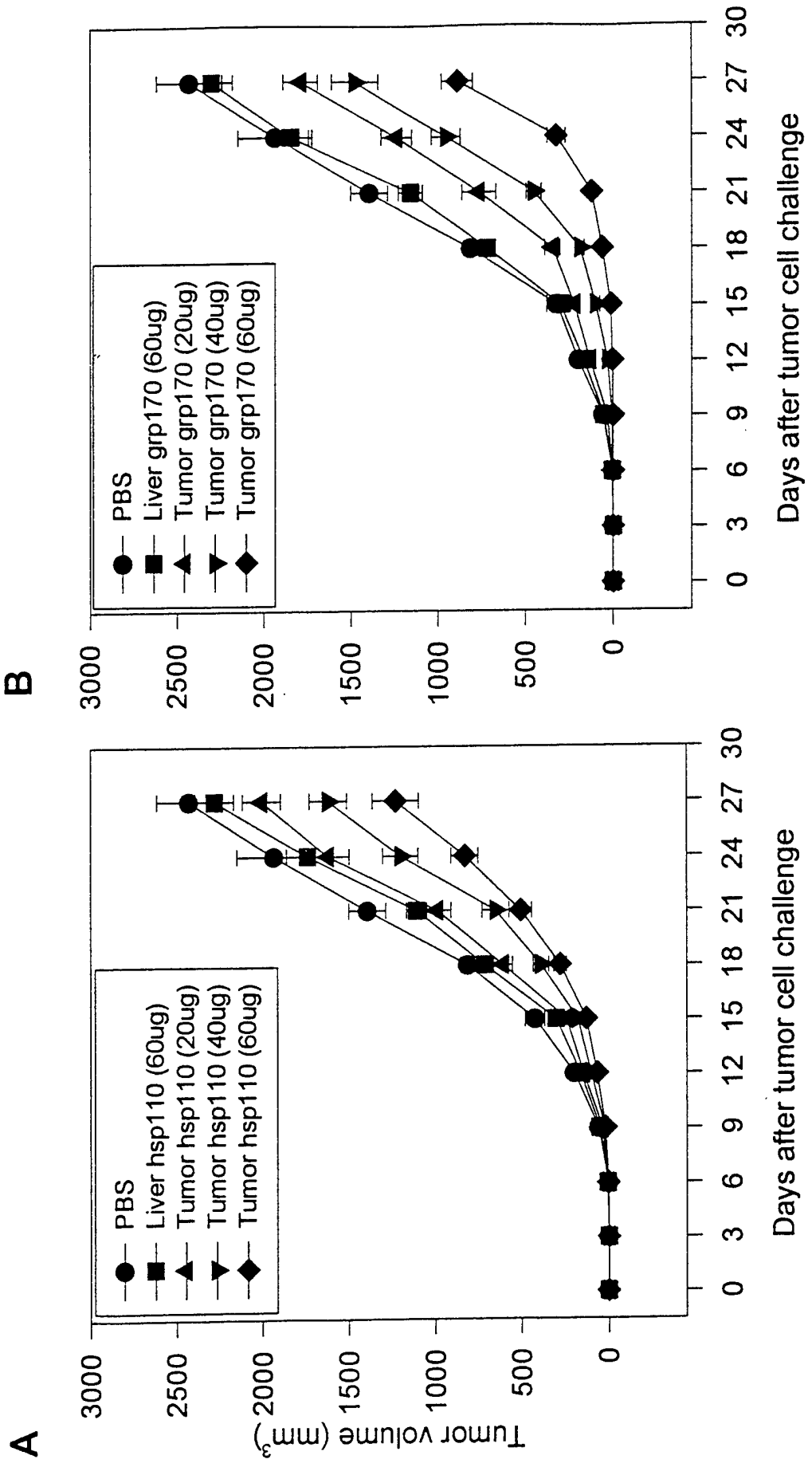
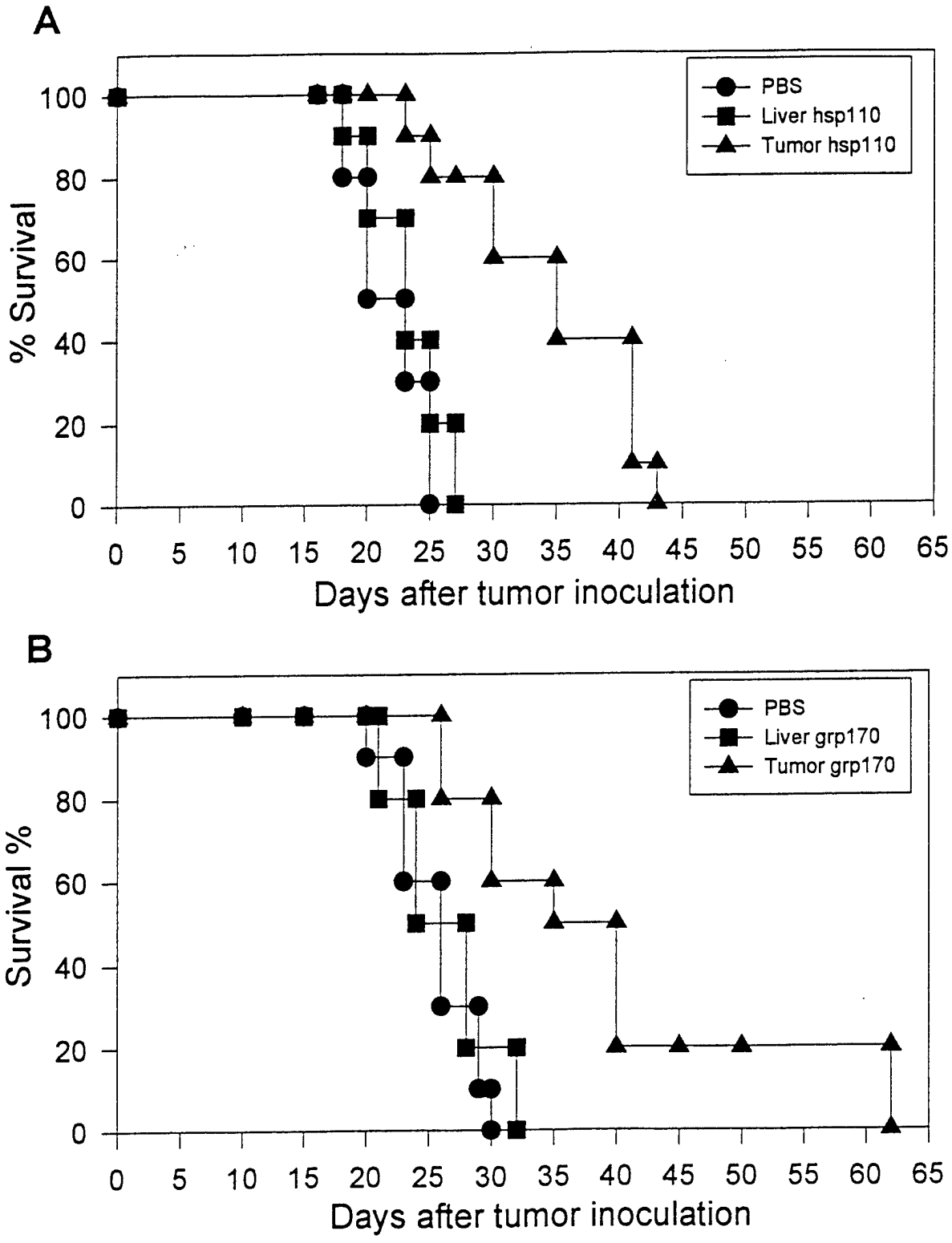

A
1 2 3 4
203-
115-
83-
49-
hsp110
B
1 2 3 4
grp170
C
1 2 3 4
203-
115-
83-
49-
hsp110
D
1 2 3 4
grp170

Figure 2


A
B
C
PBS
Hsp110
Grp170
Tumor volume (mm³)
2000
1750
1500
1250
1000
750
500
250
0
0 3 6 9 12 15 18 21 24 27
Days after tumor challenge


A
B
Tumor volume (mm³)
3000
2500
2000
1500
1000
500
0
0 3 6 9 12 15 18 21 24 27 30
Days after tumor cell challenge
PBS
Liver hsp110 (60ug)
Tumor hsp110 (20ug)
Tumor hsp110 (40ug)
Tumor hsp110 (60ug)
PBS
Liver grp170 (60ug)
Tumor grp170 (20ug)
Tumor grp170 (40ug)
Tumor grp170 (60ug)


A
100
80
60
40
20
0
% Survival
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Days after tumor inoculation
PBS
Liver hsp110
Tumor hsp110
B
100
80
60
40
20
0
Survival %
0 5 10 15 20 25 30 35 40 45 50 55 60 65
Days after tumor inoculation
PBS
Liver grp170
Tumor grp170

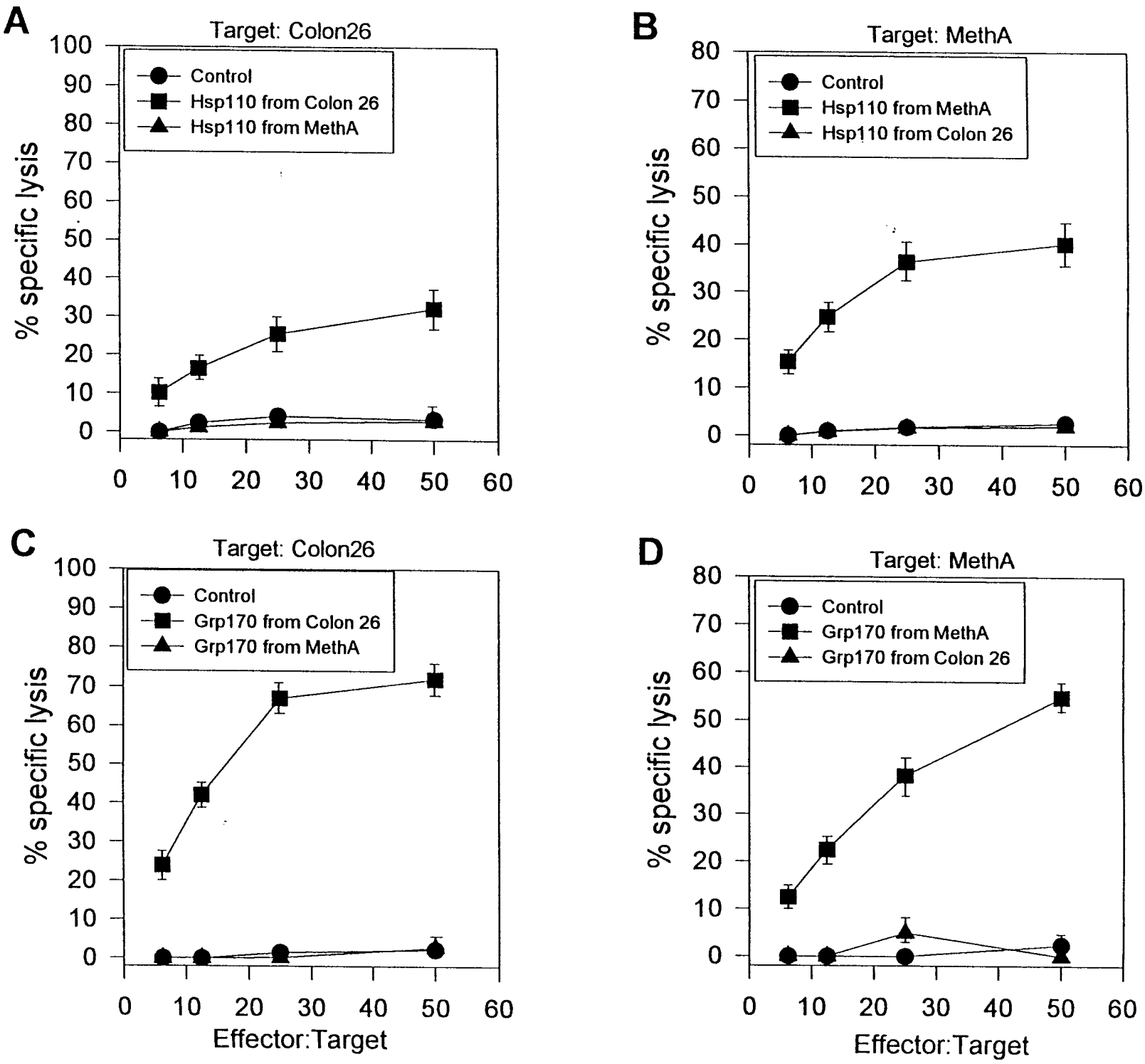
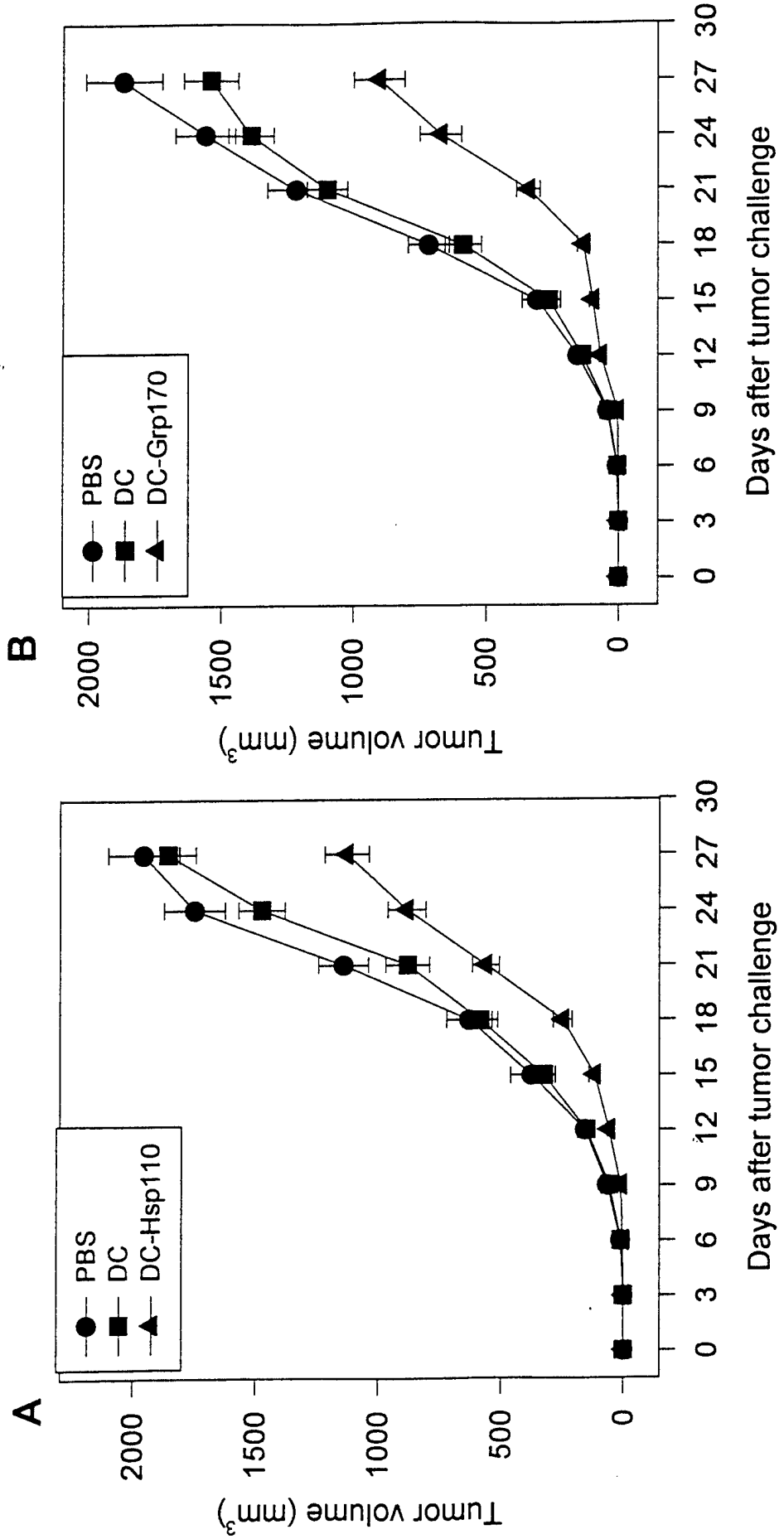

A
Target: Colon26
Control
Hsp110 from Colon 26
Hsp110 from MethA
% specific lysis
B
Target: MethA
Control
Hsp110 from MethA
Hsp110 from Colon 26
% specific lysis
C
Target: Colon26
Control
Grp170 from Colon 26
Grp170 from MethA
% specific lysis
Effector:Target
D
Target: MethA
Control
Grp170 from MethA
Grp170 from Colon 26
% specific lysis
Effector:Target

Figure 5


A
B
Tumor volume (mm3)
2000
1500
1000
500
0
0 3 6 9 12 15 18 21 24 27 30
Days after tumor challenge
PBS
DC
DC-Hsp110
DC-Grp170

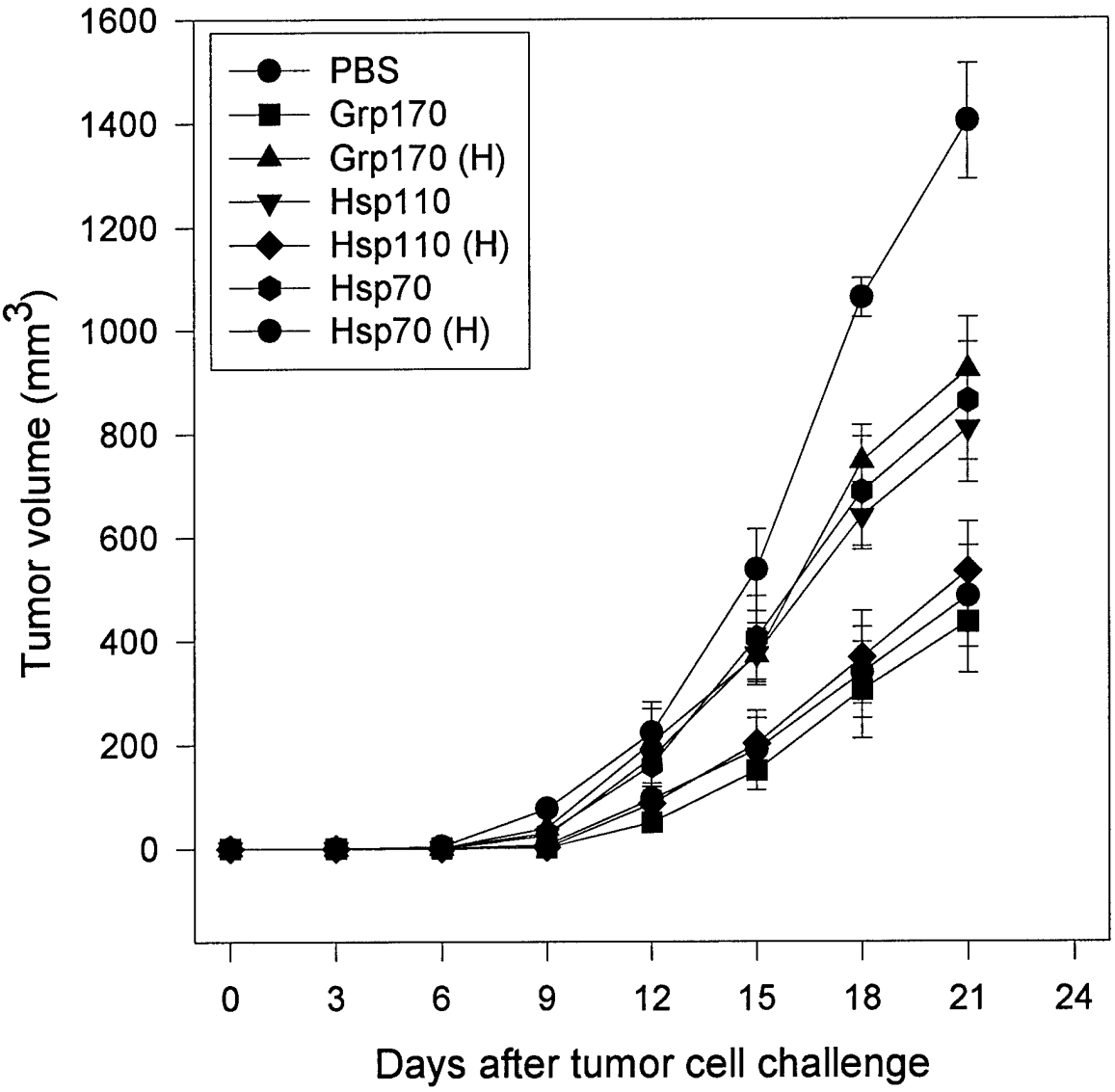
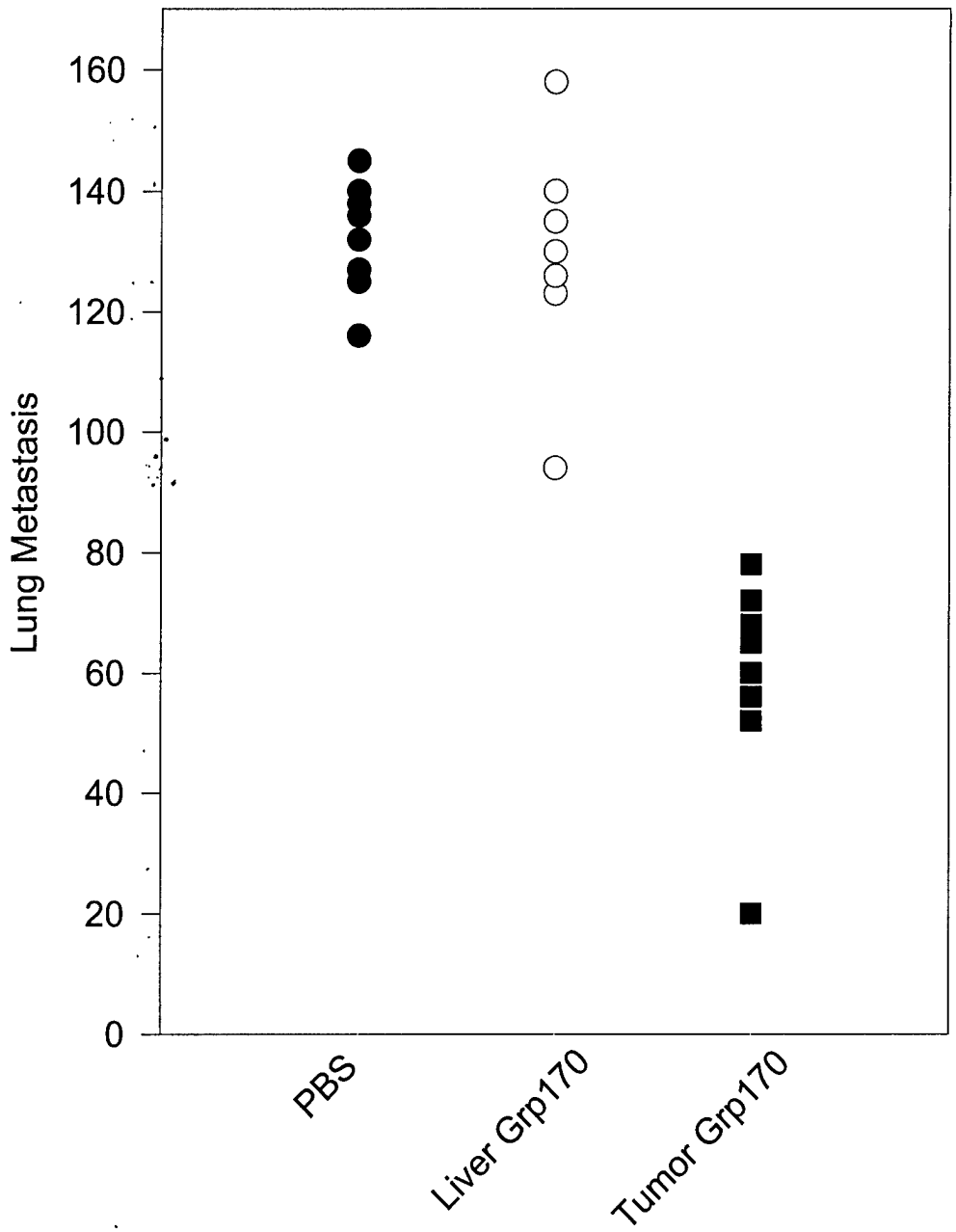

PBS
Grp170
Grp170 (H)
Hsp110
Hsp110 (H)
Hsp70
Hsp70 (H)
Tumor volume (mm3)
1600
1400
1200
1000
800
600
400
200
0
0
3
6
9
12
15
18
21
24
Days after tumor cell challenge

Figure 8


Lung Metastasis
160
140
120
100
80
60
40
20
0
PBS
Liver Grp170
Tumor Grp170

Effect of Lymphocyte Depletion on Grp170 Vaccination

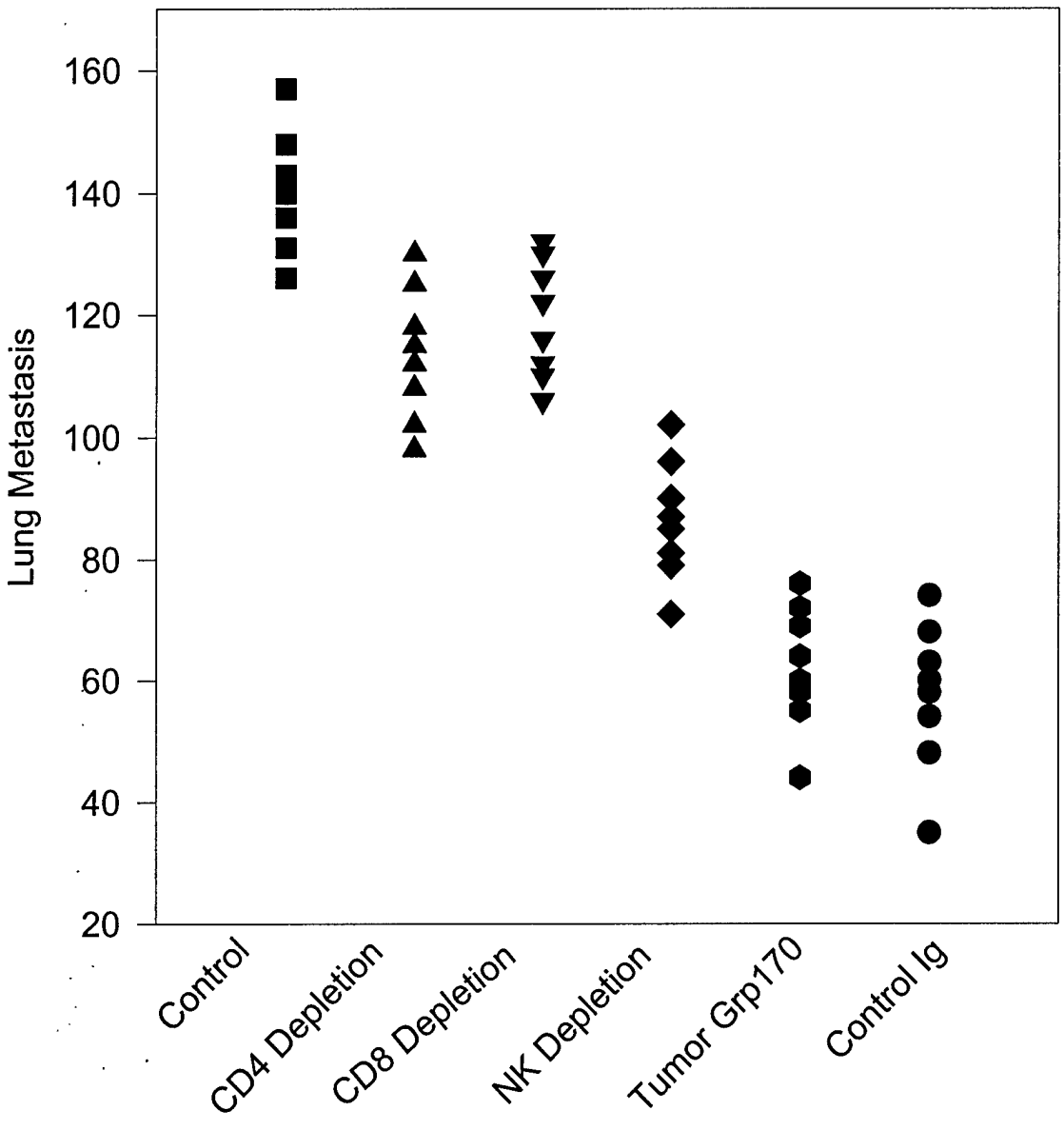
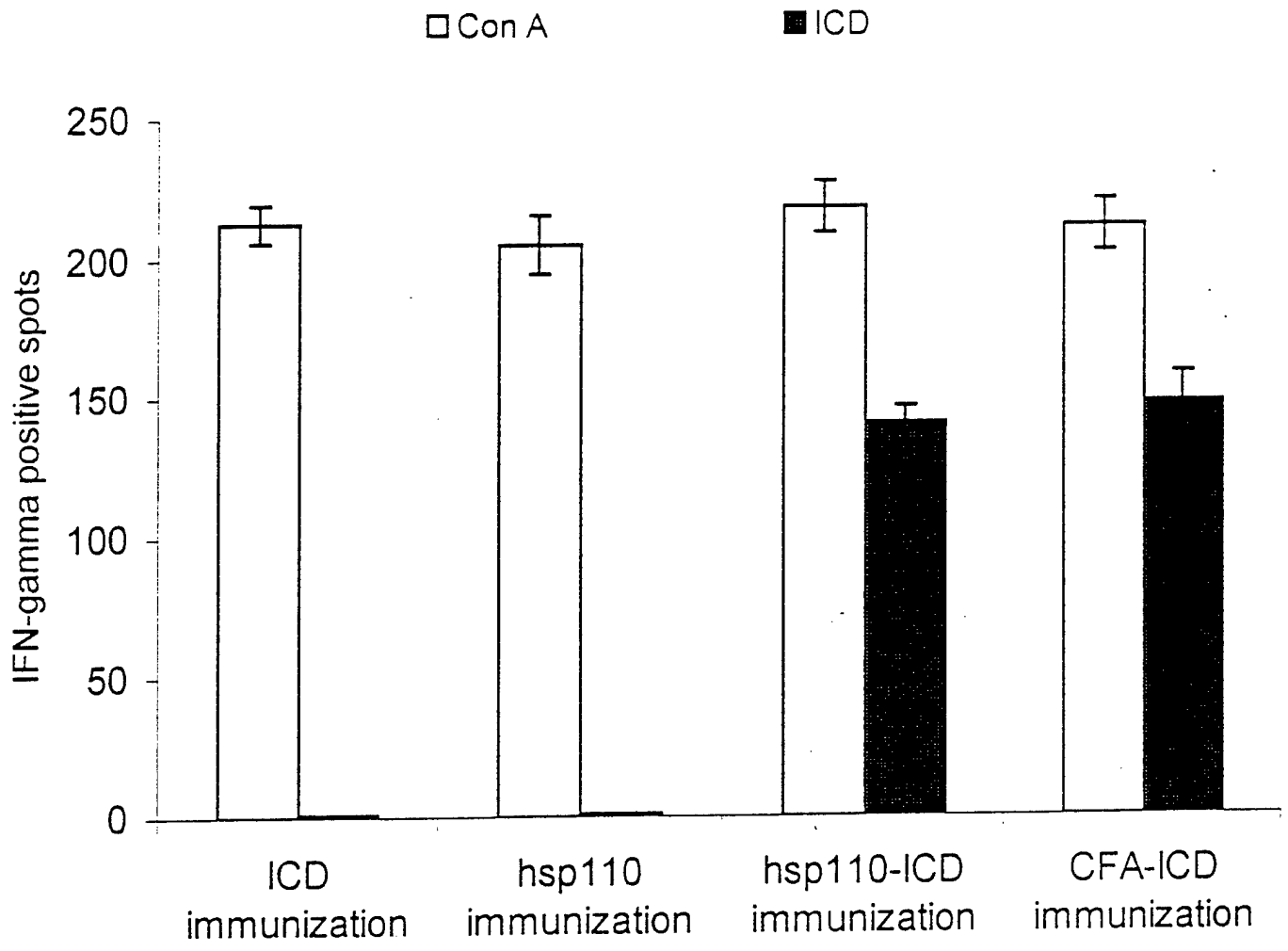
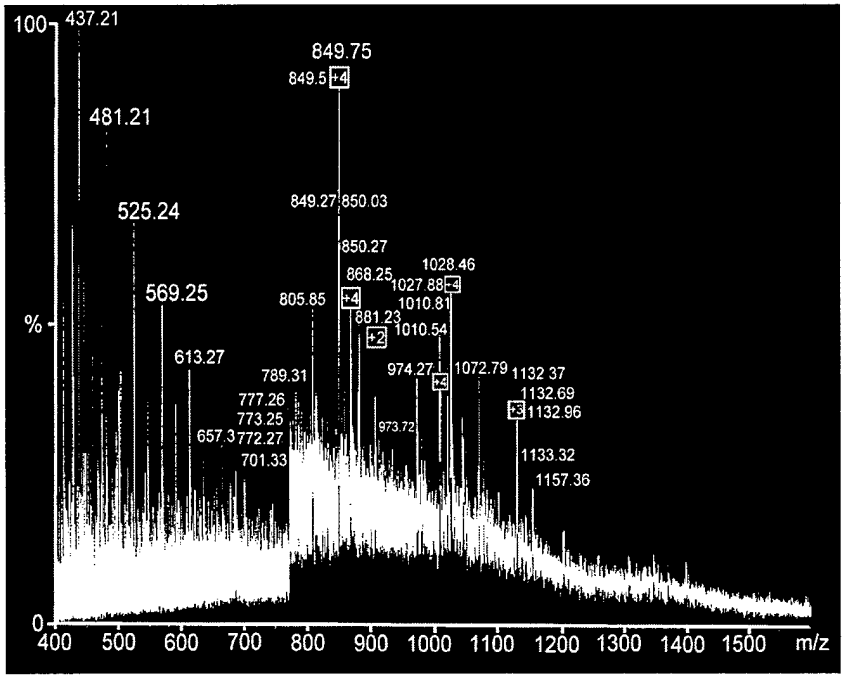


Figure 10


Con A
ICD
IFN-gamma positive spots
250
200
150
100
50
0
ICD immunization
hsp110 immunization
hsp110-ICD immunization
CFA-ICD immunization

Q-TOF MS of Hsp110-Derived Peptides and MS/MS Scan of a Selected Peptide



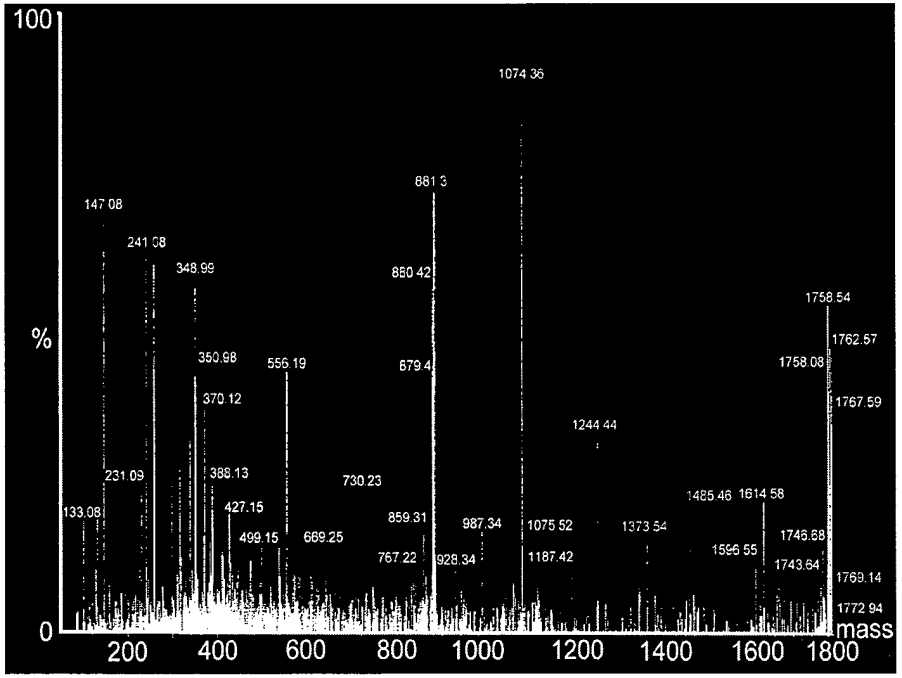


Figure 11

ANTI-TUMOR IMMUNITY ELICITED BY TWO HIGH-MOLECULE-WEGHT HEAT SHOCK PROTEINS

Xiang-Yang Wang[1], Yoshiyuki Kaneko[1], Ying Li[2], Elizabeth Repasky[2], Latif Kazim[3], John Subjeck[1]

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Tumor derived peptide-HSP complexes (particularly HSP70 and GRP96) have already been demonstrated to provide an effective vaccine in animal models, which is related to their general peptide-binding properties. Two high-molecule-weight heat shock proteins, hsp110 and grp170, were recently cloned in our laboratory (1,2). The chaperoning properties of hsp110 has also been characterized (3, 4) and indicate that it not only exhibits similarities in function to HSP70, but also presents significant differences in its peptide binding properties. Here, we analyzed the potential of hsp110 and grp170 derived from murine tumors to serve as cancer vaccines. We show that immunization with these two HSPs purified from tumors results in the growth inhibition of colon carcinoma in Balb/c mice while HSP preparations from liver of same animals have no effect on tumor growth. In the colon26 tumor model, hsp110 or grp170 immunization significantly extended the life span of tumor-bearing mice. We also show that vaccination with hsp110 or grp170 derived from MethA fibrosarcoma caused the complete regression of this tumor. A tumor specific cytotoxic T lymphocyte (CTL) response developed in mice immunized with HSPs from both tumor types. Furthermore, treatments of the mice with bone marrow-derived dendritic cells pulsed with these HSPs from colon26 tumor also elicited an anti-tumor response, indicating that dendritic cells are able to re-present HSP-chaperoned peptides. These studies strongly suggest that hsp110 and grp170 can be used in HSP-based cancer immunotherapy. We are presently examining breast cancer antigens which are complexed with hsp110 or grp170 as potential anti-cancer vaccines.

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The U.S. Army Medical research and Materiel Command under DAMD 17-98-1-8104 supported this work.

ANTI-TUMOR IMMUNITY ELICITED BY THE HIGH MOLECULAR WEIGHT STRESS PROTEINS: HSP110 AND GRP170.

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Recently, it has been demonstrated that the isolation of heat shock proteins (hsps) from a tumor could allow the co-purification of antigenic peptides from the tumor itself. Tumor derived peptide-HSP complexes (particularly hsp70 and grp94/gp96) have already been demonstrated to provide an effective vaccine in animal models, which is related to their general peptide-binding properties. Basic research in our laboratory has led to the cloning and analysis of two long recognized heat shock proteins known as hsp110 and grp170. Here, we analyzed the potential of hsp110 and grp170 derived from murine tumors to serve as cancer vaccines. We showed that immunization with either of these stress proteins purified from tumor results in significant growth inhibition of colon carcinoma in Balb/c mice. Liver preparations of the same animals have no effect on tumor growth. In the colon 26 tumor model, hsp110 or grp170 immunization significantly extended the life span of tumor-bearing mice. We also show that vaccination with hsp110 or grp170 derived from MethA fibrosarcoma caused the complete regression of this tumor. A tumor specific cytotoxic T lymphocyte (CTL) response developed in the mice immunized with HSPs from both tumor types. Furthermore, treatments of the mice with bone marrow-derived dendritic cells pulsed with these HSPs from colon 26 tumor also elicited an anti-tumor response, indicating that dendritic cells are able to re-present HSP-chaperoned peptides and antigen presenting cells are involved in the HSP immunization induced anti-tumor response. These studies strongly suggest that hsp110 and grp170 can be used in HSP-based cancer immunotherapy.

IMMUNOLOGICAL INVESTIGATIONS, 29(2), 131–137 (2000)

Heat Shock Proteins and Cancer Immunotherapy

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ABSTRACT

Vaccination with heat shock proteins from tumor have been shown to elicit an anti-tumor response. Current studies indicate that the immunogenecity of HSPs is derived from the antigenic peptides which they associate with. Mechanisms by which the HSP-peptide complexes induce an immune response and the possible role of HSPs in antigen presentation is discussed in this article. The use of HSP-peptide complexes can be used as tumor vaccines for cancer immunotherapy is reviewed.

Heat shock proteins (HSPs) were first recognized as a set of polypeptides induced in *Drosphila* by elevated temperatures. They are highly conserved and abundant proteins in both eukaryotes and prokaryotes (1). Heat shock proteins are divided into several major families, Hsp110, 90, 70, 60/GroEL and the small HSPs based on their size and structure (2, 3, 4). HSPs have been found to be induced by environmental stress (e.g. heat shock, ethanol, heavy metal, glucose deprivation, inhibitor of glycosylation), pathological stress (viral infection, inflammation, fever and tissue trauma) (5) and even non-stressful conditions (cell cycle, cell differentiation and development) (6). Many HSPs also function as molecular chaperones that prevent irreversible aggregation and assist protein folding, unfolding, assembly and transport. These functions are based on the abilities of HSPs to bind unfolded peptide chains. The heat shock proteins are primarily localized in the cytoplasm and nucleus. A parallel set of stress proteins which are differentially inducible (e.g. by anoxia) are called GRPs and are localized in the endoplasmic reticulum. Primary GRPs fall into families parallel to the major HSPs: i.e. grp78, grp94(gp96) and grp170. Recently, HSPs and GRPs derived from tumors were shown to be able to protect mice against the subsequent challenge with tumor from which the HSPs were

purified. This has been shown to be related to the general peptide binding properties of HSPs. Because of these findings, the role of stress proteins in tumor immunology has attracted significant attention.

Tumor-derived HSPs elicit protective immunity against cancers

In the early 1990's, Srivastava and his colleagues first found that a tumor rejection antigen, isolated by biochemical fractionation of tumor cells, was a heat shock protein (grp94/gp96) (7, 8, 9, 10). There is unequivocal evidence today that the HSPs, including hsp70, hsp90 and grp94/gp96, derived from a given cancer, can elicit protective immunity specific to that particular cancer. HSPs derived from normal tissues do not protect against any cancer tested. In the last several years, immunogenecity of HSP preparations from tumors has been repeatedly seen in different experimental tumor systems of distinct histological origins, which range from chemical or UV-radiation induced tumors to spontaneous tumors (11, 12, 13). Furthermore, two high-molecular-weight heat shock proteins, hsp110, grp170 derived from CT26 and MethA tumors (Wang et al., unpublished data) were recently found to induce an anti-tumor response against these tumors. Most importantly, it has been shown that the immunization of mice with gp96 resulted in the induction of memory T cells (14). Based on these observations, it is apparent that HSPs prepared from tumor are able to act as tumor vaccines.

Immunogenicity of HSPs is due to their peptide-binding properties as chaperones

Molecular cloning and sequence studies indicated that the genes coding for HSPs in the tumor cells and normal tissues did not exhibit any difference in nucleotide sequence (9). It was hypothesized that immunogenicity of HSPs lies not in the HSPs isolated from tumor or normal tissues, but rather in the peptides that they bind (8).

Studies of HSP structure and chaperoning properties by molecular biologists have provided significant evidence for peptide binding activities of heat shock proteins. It was shown that peptide-binding sites consisting of several β sheets exist in heat shock protein 70 (15). Furthermore, hsp70 association with peptides has been demonstrated in vitro (16, 17). Although the peptide-binding structure for gp96 is unknown, peptide-binding activities of gp96 have also been demonstrated. gp96 has been shown to transfer peptides from the transporter associated with antigen processing (TAP) to MHC class I molecules (18, 19, 20). Recently, TAP-independent peptides were also found to bind gp96 (21). In our laboratory, analysis of secondary structure indicated that, while exhibiting similarities to hsp70, hsp110 and grp170 appear to exhibit peptide-binding clefts with a significantly enlarged "lid" domain. This suggests that hsp110/grp170 binding affinities and/or capacities differ from hsp70. Recent studies have

confirmed that hsp110 exhibits a different peptide-binding capacity (22, 23). While little is known about GRP170 functions, it is evident that it is involved in binding immunoglobulin chain in the endoplasmic reticulum (ER) (24). Most notably, GRP170 may be the ATPase responsible for peptide import into the ER from TAP (18, 25, 26).

Recent studies from different laboratories also provide convincing evidence for the binding of antigenic peptide as the basis of the immunogenic activities of HSPs using a number of well-defined systems. Immunization with gp96 isolated from vesicular stomatitis virus (VSV) infected cells primed VSV-specific cytotoxic T lymphocytes (CTL) (27). Similarly, the gp96 isolated from β-galactosidase (β-gal) transfected cells elicited CTLs specific for β-gal and minor histocompatibility antigens expressed in these cells (28). This hypothesis is also directly supported by the observation that peptide-depleted hsp70 by ATP treatment was unable to elicit immunity against tumor challenge (29, 30). Most importantly, hsp70-peptide and gp96-peptide complexes can be reconstituted in vitro, and these complexes can induce peptide-specific CTLs (31). Young and colleagues also reported that immunization with recombinant hsp70-OVA fusion proteins protected mice against challenge with an OVA-expressing tumor (32). In contrast, fusion proteins not containing hsp70 were ineffective. All of these studies and others indicate that HSP-chaperoned peptides are responsible for the antigen specific immune response. Thus, HSPs have been suggested to be the first physiological mammalian adjuvant and they may be used as an antigen delivery vehicle for immunotherapy (31).

Mechanism of HSP-peptides elicited immune response

The mechanism through which immunization with HSP-peptide complex elicits antigen-specific CD8+ T cells is being worked out by a number of investigators. By using macrophage and T cell depletion studies, Udono et al demonstrated that this priming of the immune response by Hsp-peptide complex was sensitive to the functional abrogation of phagocytic cells (33). Macrophages were shown to internalize gp96-peptide complex and re-present the gp96-chaperoned peptide on the MHC I molecules (34). It was also found that peptide was actually recycled through a nonacidic compartment in the cell and not simply transferred to MHC class I molecule on the cell surface directly. Most interestingly, it seems that HSP-chaperoned peptides are independent of the MHC-type of the tumor from which they are derived, whereas, their presentation to the CTL is defined by the MHC phenotype of the APCs (34). Recently, it has been shown that immunization with bone morrow generated dendritic cells which were pulsed with tumor-derived HSPs elicited an anti-tumor response, suggesting that antigen presenting cells (APC) are critical for HSP-peptide complex mediated immune responses (Wang et al unpublished data). Furthermore, hsp70 released from tumor cells was seen to be internalized

directly into DCs and enhanced the capability of DCs to take up proteins/peptides, indicating that in addition to the function of chaperoning antigenic peptides from tumor, heat shock proteins themselves might act as a messenger to deliver an immunological signal to the host system (35). However the details of the intercellular events involved in the transfer of HSP-chaperoned peptides onto MHC class I molecules remains unknown.

Suto and Srivastava demonstrated that brefeldin A inhibited the presentation of HSP-chaperoned peptides (34). This study suggested that transport between endoplasmic reticulum (ER) and Golgi apparatus is necessary for the antigen re-presentation pathway. KDEL receptors recycle between Golgi and ER, thereby retrieving resident ER proteins that escaped from the ER (36). Also exogenous toxins such as Psedomonoas exotoxin and ricin are transported from Golgi to ER by interacting with KDEL receptors (37, 38). Thus, whether or not exogenous HSPs, especially ER resident GRPs, require KDEL receptors for their retrograde transport is an interesting possibility. In addition, the high efficiency of small quantities of HSPs to elicit an immune response indicates that there may exist HSP receptors on the surface of antigen presenting cells which are capable of taking up HSP-peptide complexes specifically. Arnold-Schild et al provided supporting evidence showing that gp96 and hsp70 bind specifically to the surface of APCs and are internalized spontaneously by receptor-mediated endocytosis. Furthermore, internalized HSPs were observed to co-localize with surface MHC class I molecules in early and late endosomal structures, indicating that HSPs are involved in the processes of antigen presentation (39) The HSP receptor may take up the HSP-peptide complex in a manner similar to that used for antigen uptake by the mannose receptor or the FCγ receptor, which are also expressed on dendritic cells (40, 41, 42). Whether the uptake of HSPs is a specific or non-specific process still requires further study. Identification of the receptors responsible for the internalization of HSPs would contribute significantly to our understanding of the mechanism of the HSP-peptide complex elicited immune response.

Potential of using HSP as tumor vaccines

Each cancer has a specific antigenic fingerprint which consists of a large repertoire of mutated or non-mutated peptides (43). HSP vaccines are unique because of their ability to chaperone and represent a broad antigenic repertoire of tumor cells. Thus, vaccination with HSPs isolated from tumor cells circumvents the need to identify specific tumor antigens, and hence extends the use of HSP-based immunotherapy to the majority of cancers where specific tumor antigens have not yet been characterized. Moreover, since HSP vaccines are directed against the entire antigenic repertoire of that tumor, this avoids the possibility of immunological escape. All these studies suggest a promising future for HSPs as cancer vaccines.

Many HSPs are believed to be located in intracellular compartments, but cytosolic HSPs were recently found to be present on the cell surface and to be involved in the anti-tumor response. These HSPs seem to function as a target structure which can be recognized by γδ T cells and NK cells (44, 45, 46, 47). More interestingly, it was reported that the recognition of hsp70 on the target cells can be blocked by anti-hsp70 antibodies, but not by anti-MHC class I antibodies or anti-NK antibodies (48, 49). The endoplasmic reticular HSP grp94/gp96 was also seen to localize on the cell surface of tumor cells (50) and exposure of gp96 to microphages resulted in the secretion of a low level of cytokines, regardless of the peptides which gp96 binds (34). These observations are consistent with the idea that HSPs might act as antigen-presenting molecules themselves and possibly HSPs are involved in both antigen-specific and antigen-nonspecific immune responses. However, the mechanisms of HSP surface expression and its roles in the immune response still require further investigation. It is also conceivable that HSP-based immunotherapy may not only promote T cell-dependent anti-tumor immunity but also directly induce NK cell activation in vitro. Indeed, HSPs may be involved in the interaction between adaptive and innate immune responses.

It has been known that heat shock proteins not only protect cells from heat, but also render cells resistant to cell death induced by oxidative stress, TNF, and chemotherapeutic drugs (51, 52, 53). All of these data suggest that HSP expression in the tumor could enhance tumorigenesis and limit the efficacy of cancer therapy (54, 55). In addition, although HSP expression was recognized as a prognostic value in certain tumors, the data are limited and the results are contradictory (56, 57). Consistent with the observations that immunization with tumor-derived HSPs elicited tumor-specific immunity, it has been shown that immunogenicity of tumor cells co-segregate with the expression of heat shock proteins (58). Stable transfection of autologous HSP70 in tumor cells significantly enhances the immunogenicity of tumor, suggesting that increased levels of HSP may provide an immunostimulatory signal in vivo which helps break tolerance to tumor antigens (59). Based on the observations described above, heat shock proteins seem to play multiple functions in the tumor cell. It could increase the immunogenicity of tumor cells, while it could also help the tumor cell survive. Several questions arise: Is the high expression of HSP in the tumor a good or bad prognostic indication? What is the role of heat shock proteins in the tumor immunogenicity? Is it possible to manipulate tumor immunogenicity therapeutically if HSP expression correlates to immunogenicity of tumor? Do all HSP members perform similar immunological functions in the tumor? Can they all be used as tumor vaccines?

Collectively, the capability of HSPs to chaperone antigenic peptides and induce CTL has profound immunological implications. Although many questions remain unanswered, there is now unequivocal evidence from many laboratories that heat shock proteins (HSPs) can serve as

vaccines. Further studies of the physiological and immunological roles of HSPs in cells, including tumor cells, will help the translation of HSP-based immunotherpay into a new generation of anti-cancer vaccines against cancers.

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Characterization of native interaction of hsp110 with hsp25 and hsc70

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Abstract The 110 kDa heat shock protein (HSP) (hsp110) has been shown to be a diverged subgroup of the hsp70 family and is one of the major HSPs in mammalian cells [1,2]. In examining the native interactions of hsp110, we observed that it is found to reside in a large molecular complex. Immunoblot analysis and co-immunoprecipitation studies identified two other HSPs as components of this complex, hsc70 and hsp25. When examined in vitro, purified hsp25, hsp70 and hsp110 were observed to spontaneously form a large complex and to directly interact with one another. When luciferase was added to this in vitro system, it was observed to migrate into this chaperone complex following heat shock. Examination of two deletion mutants of hsp110 demonstrated that its peptide-binding domain is required for interaction with hsp25, but not with hsc70. The potential function of the hsp110-hsc70-hsp25 complex is discussed.

Key words: Heat shock protein; Chaperone complex; Luciferase; Peptide-binding domain

1. Introduction

Heat shock proteins (HSPs) are a number of conserved proteins that can be induced in all organisms upon the exposure to stress conditions like high temperature. Many of them also function as molecular chaperones that prevent irreversible aggregation and assist protein folding or assembly. HSPs are divided into several major families based on their size and structure [3–6], the most well known being hsp110, hsp90, hsp70, hsp60/GroEL, hsp40/DnaJ and the small HSPs (sHSP, e.g. hsp25). All of these HSPs, except hsp110, have been extensively studied and their functions in cellular processes are broadly recognized today. Although the cloning of the hsp110 from hamster, mouse, yeast, *Arabidopsis*, fungi and a variety of other species has been recently described [7–14], the cellular functions of hsp110 are still not understood. Studies in our laboratory have been focusing on this large stress protein. We have previously shown that hsp110 is a significantly enlarged relative of hsp70 family, which also contains unique sequence elements not present in the hsp70s. We have also shown that hsp110 inhibits the aggregation of heat-denatured proteins in a highly efficient manner, sustains denatured proteins in a folding competent state, and confers thermotolerance when overexpressed in vivo [2]. In order to better understand this major stress protein in mammalian cells, its native interactions were investigated. We describe here studies indicating that hsp110 interacts in vivo and in vitro with two other major stress proteins, hsc70 and hsp25, and define the domains of hsp110 involved.

2. Materials and methods

2.1. Reagents

The rabbit anti-hsp110 antibody has been characterized previously [1]. Affinity-purified mouse anti-hsc70 monoclonal antibody, rabbit anti-murine hsp25 antibody, rat anti-hsp90 antibody and rat anti-TCP-1a monoclonal antibody, as well as recombinant hsc70 and murine hsp25, were all obtained from Stressgen Biotechnological Corp (Victoria, Canada). Anti-His Antibody was purchased from Amersham. Colon 26 tumor cells were cultured in DMEM supplemented with 10% calf serum in a 5% CO_2 incubator.

2.2. Plasmid construction and expression

Purification of recombinant His-tagged hsp110 and two deletion mutants used here has been described elsewhere [2,15]. Briefly, for the construction of hsp110 mutants, primers 5′-GCTAGAGGATCCTGTGCATTGCAGTGTGCAATT−/−CAGCGCAAGCTTACTAGTCCAGGTCCATATTGA-3′ (mutant #1, amino acids 375–858) and 5′-GACGACGGATCCTCTGTCGAGGCAGACATGGA−/−CAGCGCAAGCTTACTAGTCCAGGTCCATATTGA-3′ (mutant #2, amino acids 508–858) were used in the polymerase chain reaction (PCR). The PCR products were cloned into pRSETA vector (Invitrogen), and a His_6-(enterokinase recognition sequence) and additional Asp-Arg-Trp-Gly-Ser (for mutant #1) or Asp-Arg-Trp (for mutant #2) were added to the N-terminal of hsp110 mutants. Plasmids were transformed into *Escherichia coli* strain JM109 (DE3) and expression products were purified by Ni_2-nitrilotriacetic acid-agarose column (QIAGEN). The protein concentration was measured using the Bio-Rad protein assay kit.

2.3. Purification of native hsp110

Cells were washed with phosphate-buffered saline (PBS) and homogenized with a Teflon homogenizer with five volumes of buffer (30 mM $NaHCO_3$, pH 7.5, 1 mM phenylmethylsulfonyl fluoride). The homogenates were centrifuged for 20 min at 12 000×*g*, supernatants were further centrifuged for 2 h at 100 000×*g*. Cell extracts were first applied to Con-A-Sepharose column, unbound proteins were collected and loaded on ion exchange column (Mono Q, Pharmacia) equilibrated with 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.1 mM dithiothreitol (DTT). Bound proteins were eluted with a linear salt gradient (200 mM ~ 350 mM NaCl). hsp110 pooled fractions were concentrated using centricon 30 (Amicon) and applied to size exclusion column (Superose 6, Pharmacia) for high performance chromatography equilibrated with 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM DTT), then eluted with at a flow rate of 0.2 ml/min. Thyroglobulin (669 kDa), ferritin (440 kDa), catalase (158 kDa), albumin (67 kDa) and ovalbumin (43 kDa) were used as protein markers.

2.4. Western blot analysis

Cells were washed with PBS and lysed in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and protease inhibitors. After incubation on ice for 30 min, cell extracts were boiled with an equal volume of sodium dodecyl sulfate (SDS) sample buffer

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(50 mM Tris–HCl, pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol) for 10 min and centrifuged at 10 000×*g* for 20 min. Equivalent protein samples were subjected to 7.5–10% SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto Immobilon-P membrane (Millipore, UK). Membranes were blocked with 5% non-fat milk in TBST (20 mM Tris–HCl, pH 7.4, 137 mM NaCl, 0.05% Tween-20) for 1 h at room temperature, and then incubated for 2 h with primary antibodies diluted 1:1000 in TBST. After washing, membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG diluted 1:2000 in TBST. Immunoreactivity was detected using the enhanced chemiluminescence detection system [16,17] (Amersham, Arlington Heights, IL, USA).

2.5. Immunoprecipitation

Immunoprecipitation was performed as previously described [18,19]. In brief, cells were washed three times with cold PBS and lysed in buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 10 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM ABESF, 0.025% NaN_3). The lysates were centrifuged and supernatant was presorbed with 0.05 volume pre-immune serum together with 30 ml protein A beads for 1 h. The lysates were incubated overnight at 4°C with hsp110 antibody (1:100) or hsc70 antibody (1:200) or hsp25 antibody (1:100). For in vitro analysis of interaction within chaperones, recombinant wild-type hsp110 and hsp110 mutants were first incubated with hsc70 or hsp25 at 30°C. Then, hsc70 antibody or hsp25 antibody were added and further incubated overnight at 4°C. Immune complex was precipitated with protein A-agrose (30 μl) for 2 h. Precipitates were washed three times with 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP40, 30–40 μl SDS sample buffer was added and boiled for 5 min. Supernatants were loaded to 7.5–12% SDS-PAGE and analyzed by immunoblotting.

2.6. Interaction between luciferase and HSPs

Luciferase (Boehringer Mannheim) was incubated with hsp110, hsc70 and hsp25 (150 nM each) in 25 mM HEPES, pH 7.9, 5 mM magnesium acetate, 50 mM KCl, 5 mM β-mercaptoethanol, and 1 mM ATP at room temperature or 43°C for 30 min. The solution was centrifuged at 16 000×*g* for 20 min, the supernatant was loaded on the Sephacryl S-300 column (Pharmacia) equilibrated with 20 mM Tris–HCl, pH 7.8, 150 mM NaCl and 2 mM DTT. The protein was eluted at a flow rate of 0.24 ml/min at 4°C. Fractions were collected and analyzed by Western blotting.

3. Results

3.1. Existence of hsp110 as a large complex containing hsc70 and hsp25

In order to investigate the physiological role of hsp110, our efforts have focused on the characterization of native hsp110 in Colon 26 cells. After cell extracts were applied to successive chromatography on Con-A-Sepharose and Mono Q columns, partially purified hsp110 fraction was loaded onto the Superose 6 size exclusion column (maximum resolution of 5000 kDa). It was observed that the Con-A and ion exchange-purified hsp110 fraction eluted from the Superose column in those fractions of size range between 200 and 700 kDa (Fig. 1A). Work was repeated using Sephacryl 300 (allyl dextran/bisacrylamide matrix) column and analysis provided similar data (data not shown).

Since hsp110 was eluted as one broad peak of high molecular mass, it is reasonable that this large in situ hsp110 complex might also contain additional components, potentially including other molecular chaperones and/or cellular substrates that may interact with hsp110. In order to investigate this possibility, we examined the purified hsp110 fraction derived from both ion exchange and size exclusion columns by immunoblotting for other HSPs using available antibodies. As shown in Fig. 1B, antibodies for hsp90, hsc70, T-complex polypeptide 1 (TCP-1) and hsp25 were used. All four proteins were readily detectable in the total cell lysate (lanes 1, 3, 5 and 7). When the hsp110 fraction was examined, TCP-1 and hsp90 were not observed (lanes 2 and 6). However, both hsc70 and hsp25 were found to co-purify with hsp110 with a significantly greater fraction of total cellular hsc70 present than of hsp25. Having found that hsc70 and hsp25 were also present, we determined their chromatographic profiles in the purified system (also shown in Fig. 1A).

To determine whether this co-purification also reflected an interaction between these three molecular chaperones, a recip-

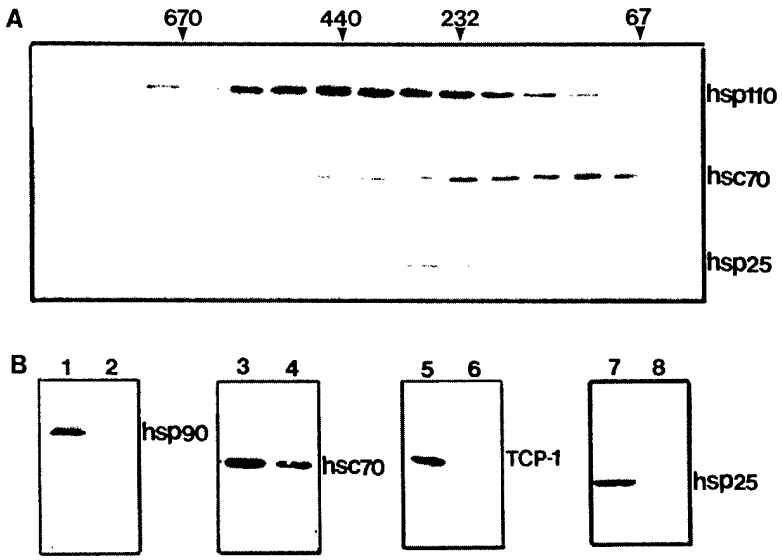

Fig. 1. Characterization of hsp110 complex. A: Chromatography profiles of native hsp110 separated by size exclusion column for FPLC. hsp110 was partially purified by successive chromatography on Con-A-Sepharose and Mono Q column. Pooled fraction was loaded on the Superose 6 column, proteins in each fraction were detected by immunoblotting with antibodies for hsp110, hsc70 and hsp25 (1:1000). B: Composition analysis of native hsp110 complex. Purified hsp110 fraction was detected by antibodies for hsp90 (lane 1, 2), hsc70 (lane 3, 4), TCP-1 (lane 5, 6) and hsp25 (lane 7, 8). Total cell extracts were also used as a positive control (lane 1, 3, 5 and 7).

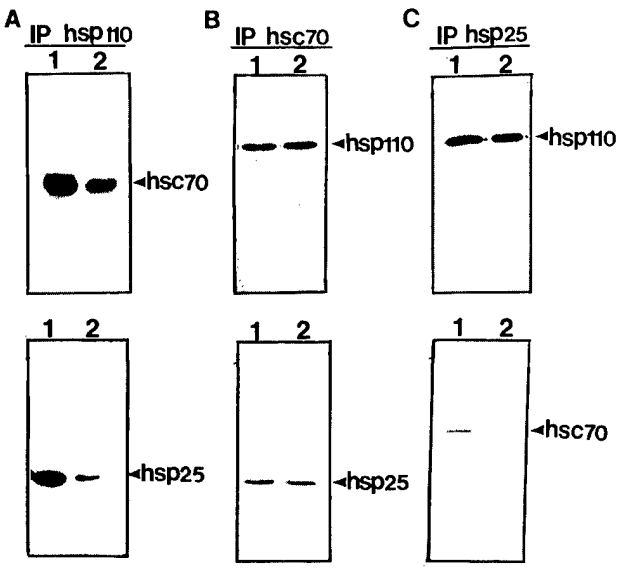

Fig. 2. Reciprocal immunoprecipitation between hsp110 and hsp70, hsp25. Cell lysates (lane 2) were incubated with antibodies for hsp110 (1:100) (A), hsp70 (1:200) (B) and hsp25 (1:100) (C), protein A-Sepharose was added and further incubated at 4°C overnight, immunoprecipitates were examined by immunoblotting with hsp110, hsp70 and hsp25 antibodies. Total cell extracts were also used as a positive control (lane 1).

rocal co-immunoprecipitation analysis was conducted with Colon 26 cell extracts and hsp110 fractions. Hsc70 and hsp25 were shown to precipitate with hsp110 using an anti-hsp110 antibody (Fig. 2A). Conversely, hsp110 was co-precipitated by an anti-hsc70 antibody or anti-hsp25 antibody (Fig. 2B,C, top). Pre-immune serum was also used to perform immunoprecipitation as a negative control with a correspondingly negative outcome (data not shown). Finally, interaction between hsc70 and hsp25 was analyzed by using antibodies for hsc70 and hsp25. Again, these two proteins were observed to co-immunoprecipitate with one (Fig. 2B,C, bottom). From the above study, we can conclude that hsp110, hsc70 and hsp25 interact in situ, either directly or indirectly.

3.2. *Analysis of interaction of hsp110 with hsc70 and hsp25 in vitro*

Next, we wished to determine whether hsp110, hsc70 and hsp25 interacted in vitro and whether they also were capable of forming a large molecular weight complex by using purified protein components. We then added luciferase as a potential substrate to this mixture since we have previously shown that hsp110 can solublize this reporter protein following heat denaturation. Luciferase, with hsp110, hsc70 and hsp25 mix (at 1:1 molar ratio) were incubated at room temperature or at 43°C for 30 min. The soluble fractions were loaded onto a Sephacryl S-300 column, eluted fractions were run on SDS-PAGE and analyzed by immunoblotting with antibodies for hsp110, hsc70, hsp25 and luciferase. The results of this study are presented in Fig. 3. It was found that hsp110, hsc70 and hsp25 are again present in high molecule weight fractions, however, these fractions were eluted at a significantly larger molecular size than that seen in vivo (Fig. 3A). Moreover, it was seen that heat treatment does not change the elution pattern for hsp110, hsc70 or hsp25. However, luciferase, which does not co-elute with the hsp110 complex prior to heating (being present as a monomer), was observed to move into a high molecule weight structure after the heat

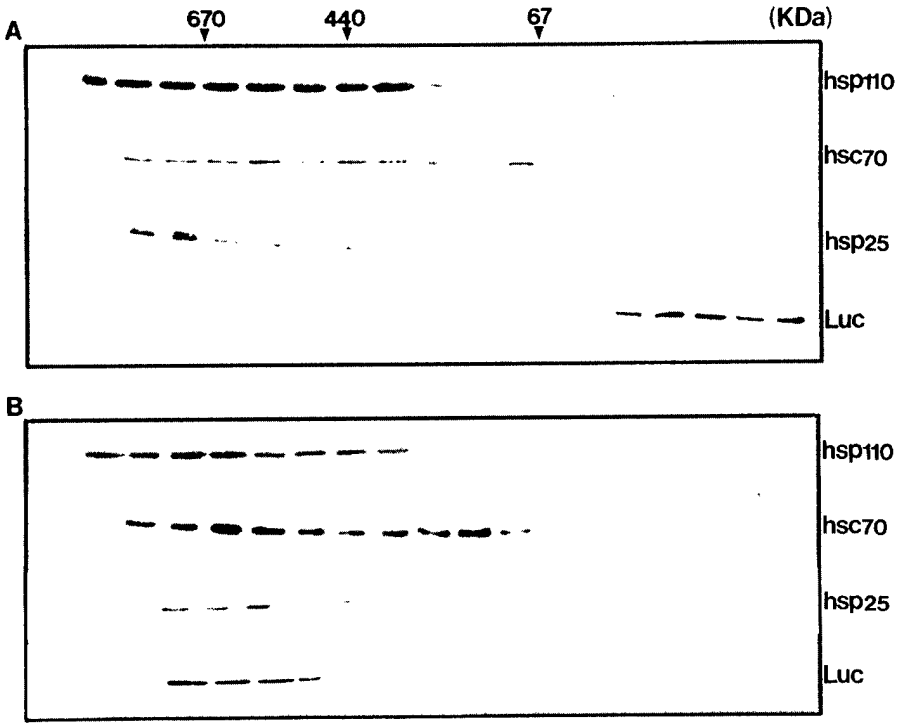

Fig. 3. Interaction between luciferase and HSPs complex. Luciferase and HSPs were incubated at room temperature (A) or 43°C (B) for 30 min and soluble fraction after centrifugation at 16000×*g* was loaded on Sephacryl S-300 column. The eluted fractions were analyzed by immunoblotting with antibodies for HSPs and luciferase.

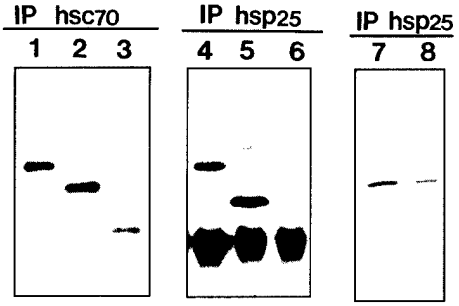

Fig. 4. Interaction analysis of hsp110 mutants and hsp70, hsp25 in vitro. *E. coli* expressed full-length hsp110 (lane 1, 4) and mutant #1 (lane 2, 5), mutant #2 (lane 3, 6) were incubated with hsc70 or hsp25 at 30°C for 1 h, then anti-hsc70 or anti-hsp25 antibodies were added. Immunoprecipitates were detected by anti-His antibody. In vitro interaction between hsc70 and hsp25 was also analyzed by the same method described above, hsc70 antibodies were used to test immunoprecipitate (lane 8). Total cell lysate was used as a positive control (lane 7). Equal amounts of protein (2 μg) for wild-type hsp110, hsp110 mutants, hsc70 and hsp25 were included in each assay.

exposure (Fig. 3B). Almost all of the luciferase was sustained in a soluble form in these experiments. When heated alone, luciferase became rapidly insoluble ([2] and data not shown). Heat shock did not affect the solubility of the three hsp110, hsc70 or hsp25.

The above data indicate that hsp110, hsc70 and hsp25 co-purify in a large molecular weight structure in vitro, as does luciferase (if present) after heating. This does not indicate how these proteins interact with themselves or that any two of them interact at all, although, that heated luciferase remains soluble is evidence for its interaction with at least one of the chaperones. Specifically, do both hsp110 and hsp25 bind to hsc70 but not to one another or can hsp110 and hsp25 interact on their own, etc.? To determine how these proteins interact, we again performed co-immunoprecipitation experiments using the pairs of purified proteins. Hsc70 and hsp110 were found to interact in the absence of hsp25 (Fig. 4, lane 1) and correspondingly hsp110 was observed to precipitate with hsp25 alone, in the absence of hsc70 (lane 4). Lastly, hsc70 and hsp25 also co-precipitate in the absence of hsp110 (lane 8).

Finally, we extended this in vitro study defining the interactions between hsp110, hsc70 and hsp25 by examining two deletion mutants of hsp110 which have previously been shown to represent the most simplistic (i.e. functional and non-functional) forms of this chaperone [15]. The first mutant examined (#1) lacks the N-terminal ATP-binding domain of hsp110, but contains the remaining sequence: i.e. the adjacent β sheet peptide-binding domain and other C-terminal sequences (size: 75 kDa and containing amino acids 375–858). This mutant has been shown to be fully functional in its ability to stabilize heat-denatured luciferase in a folding competent state. The second mutant used here (#2) again lacked the ATP-binding domain as well as the adjacent β sheet (peptide-binding) domain, but contained the remaining C-terminal sequence (size: 62 kDa and containing amino acids 508–858). This mutant has recently been shown to be incapable of performing the chaperoning function of sustaining heat-denatured luciferase in a soluble state. Mutant #1 (no ATP-binding domain) was observed to co-precipitate with both hsp70 (lane 2) and hsp25 (lane 5), indicating that these interactions do not involve its ATP-binding domain. However, mutant #2 (lacking both the ATP region and the peptide-binding region of hsp110) was observed to only associate with hsp70 (lane 3). This indicates that hsp25 and hsp70 can interact with hsp110 at different sites and that the association of hsp110 with hsp25 requires the peptide-binding domain of hsp110.

4. Discussion

The present study describes investigations into the native interactions of hsp110 in Colon 26 cells. We have found that hsp110 co-purifies with both hsc70 and hsp25 and further, that the three proteins can be co-immunoprecipitated. To determine that the co-immunoprecipitation results can reflect direct interactions between these chaperones and to also define these interactions, in vitro studies using purified hsp110, hsc70 and hsp25 were undertaken. It was found that these three chaperones also spontaneously form a large molecular complex in vitro. Moreover, this complex forms in the absence of an added substrate, but substrate (luciferase) can be induced to migrate into the complex by a heat stress. It is also shown that each pair of these proteins can interact directly, i.e. hsc70 with hsp110, hsc70 with hsp25, and hsp110 with hsp25. This, together with the co-precipitation data obtained from cell lysates, strongly argues that these interactions naturally occur in situ. Moreover, use of two deletion mutants of hsp110 demonstrates that its peptide-binding domain is required for hsp25-binding, but not for hsc70-binding and that its ATP-binding domain is not required for the interaction with either hsc70 or hsp25. This suggests that hsp110 may bind to hsp25 through its peptide-binding domain. That hsc70-hsp110-binding occurs in the absence of the hsp110 peptide-binding domain suggests that hsc70 may be actively binding to hsp110 through its (i.e. hsc70's) peptide-binding domain, but does not exclude the possibility that the two proteins interact via the involvement of other C-terminal domains.

These interactions between hsp110 and hsc70 raise questions as to how these proteins may function cooperatively. Since the peptide-binding domain of hsc70 and hsp110 appears to represent the 'business end' of these chaperones in performing chaperoning functions, it would be anticipated that their peptide-binding domains would be actively associated with substrate and not one another. This raises the possibility that this complex represents a chaperone 'storage compartment' which awaits cellular requirements. However, the migration of heat-denatured luciferase into this fraction following heat shock argues for an active chaperoning activity of the complex itself. It is possible that hsc70 may piggy-back hsp110 in a manner which allows transfer of substrate from hsp110 to hsc70 with subsequent folding in conjunction with DnaJ homologs and other chaperones. hsp110 has not yet been shown to have a folding function in conjunction with DnaJ co-chaperones, as is the case with hsc70 [2,15]. However, hsp110 exhibits different ATP-binding properties than do the hsp70s [15] and possible co-chaperones of hsp110 may be awaiting discovery. Previous in vitro studies have demonstrated that while sHSPs (e.g. hsp25) bind non-native protein [20–23], refolding still requires the presence of hsp70 [24]. Perhaps, hsp110 and sHSPs may act in the differential binding of a broad variety of substrates for subsequent shut-

tling to hsp70-DnaJ containing chaperone machines. Studies indicate, however, that an in vitro hsp110-hsc70-hsp25 complex (at a 1:1:1 molar ratio) is slightly less effective than is hsp110 alone in inhibiting luciferase aggregation following heat shock and further studies of this nature are necessary to determine or exclude a direct chaperoning function (Wang et al., data not shown).

That these three chaperones interact may represent a general phenomenon. Plesofsky-Vig and Brambl have recently shown that the small HSP of *Neurospora crassa*, called hsp30, binds to two cellular proteins, hsp70 and hsp88. Cloning and analysis of hsp88 have shown that it represents the hsp110 of *N. crassa* [25], suggesting that the interactions described here are phylogenetically conserved. In addition, Hatayama has described an interaction between hsp110 (referred to as hsp105) and hsp70 in FM3A cells [26]. The size of the hsp110 complex and the interaction with hsc70 observed in the present study (which also employed the added step of ion exchange chromatography) are clearly similar to, and in excellent agreement with this recent report. That hsp110 and hsc70 interact is also suggested by earlier studies from our laboratory which have demonstrated that hsp110 and hsc70 can interact and are functional in the folding heat-denatured luciferase [2]. Finally, it is noteworthy that hsp90 and TCP-1 were not observed in the hsp110 complex in the present study, despite its previously identified association with hsc70 and other proteins in the steroid hormone receptor [27–31]. However, it has recently been shown that SSE1 encoding a yeast member of the hsp110 family is required for the function of glucocorticoid receptor and physically associates with the hsp90 [32].

Several important questions are raised by the observations described here. For example, does this complex offer an enhanced capacity to hold a greater variety of substrate proteins in a folding competent state and/or to do so more efficiently, and is there an enhanced ability gained to refold denatured proteins in the presence of additional chaperones? Further studies are needed to define the function(s) of this hsp110-hsc70-hsp25 complex and how these chaperones interact with one another in the processing of substrate.

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Characterization of Heat Shock Protein 110 and Glucose-Regulated Protein 170 as Cancer Vaccines and the Effect of Fever-Range Hyperthermia on Vaccine Activity[1]

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Several studies have confirmed that certain stress proteins can function as potent vaccines against a specific cancer when purified from the same tumor. Recent studies of two long-recognized but unstudied stress proteins, heat shock protein (hsp) 110 and glucose-regulated protein (grp) 170, have shown them to be efficient peptide chain-binding proteins. The present investigation examines the vaccine potential of hsp110 and grp170. First, it is shown that prior vaccination with hsp110 or grp170 purified from methylcholanthrene-induced fibrosarcoma caused complete regression of the tumor. In a second tumor model, hsp110 or grp170 purified from Colon 26 tumors led to a significant growth inhibition of this tumor. In addition, hsp110 or grp170 immunization significantly extended the life span of Colon 26 tumor-bearing mice when applied after tumor transplantation. A tumor-specific cytotoxic T lymphocyte response developed in the mice immunized with tumor-derived hsp110 or grp170. Furthermore, treatments of the mice with bone marrow-derived dendritic cells pulsed with these two proteins from tumor also elicited a strong antitumor response. Last, we showed that mild, fever-like hyperthermic conditions enhance the vaccine efficiency of hsp110 as well as heat shock cognate 70, but not grp170. These studies indicate that hsp110 and grp170 can be used in hsp-based cancer immunotherapy, that Ag-presenting dendritic cells can be used to mediate this therapeutic approach, and that fever-level hyperthermia can significantly enhance the vaccine efficiency of hsps. *The Journal of Immunology,* **2001, 165: 490–497.**

Tumor-derived heat shock protein (hsp)[3]-peptide complexes (particularly hsp70 and glucose-regulated protein (grp) 94/gp96) have been demonstrated to serve as effective vaccines, producing antitumor responses in several animal models (1–4). This approach takes advantage of the peptide-binding properties of stress proteins that are responsible for their functions as molecular chaperones in numerous processes such as protein folding, transport, assembly, and peptide trafficking in Ag presentation (5–8). Indeed, since hsps purified from cells bind a spectrum of cellular peptides (9), purification of some stress proteins copurifies a cell-specific peptide "fingerprint" of the cell of origin. In the case of cancer cells, this presumably includes a subset of antigenic, tumor-specific epitopes. By virtue of these antigenic peptides, the hsp (or grp) preparation can be used as a vaccine. Vaccination with hsp-/grp-peptide complexes derived from tumors circumvents the need to identify a large number of CTL epitopes of a cancer and the technical limitations associated with that approach.

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[3] Abbreviations used in this paper: hsp, heat shock protein; grp, glucose-regulated protein; hsc, heat shock cognate; DC, dendritic cell; Meth A, methylcholanthrene-induced fibrosarcoma; ER, endoplasmic reticulum; WBH, whole-body hyperthermia.

The hsps of mammalian cells can be classified into several families of sequence-related proteins. The most obvious mammalian hsps, based on protein expression levels, are cytoplasmic/nuclear proteins with masses of ~25 kDa (hsp25), 70 kDa (hsp70), 90 kDa (hsp90), and 110 kDa (hsp110). However, in addition to hsps, a second set of stress proteins has been long observed that are localized in the endoplasmic reticulum (ER). The induction of these stress proteins is not readily responsive to hyperthermic stress, as is that of the hsps, but is regulated by stresses, which disrupt the function of the ER (e.g., glucose starvation and inhibitors of glycosylation, anoxia and reducing conditions, or certain agents that disrupt calcium homeostasis). These stress proteins have been historically referred to as grps to clearly distinguish them as a group. The principal grps on the basis of expression have approximate sizes of 78 kDa (grp78), 94 kDa (grp94), and 170 kDa (grp170). grp78 is homologous to cytoplasmic hsp70, whereas grp94 is homologous to hsp90 (10, 11). Although individual stress proteins have been studied for several years (in some cases intensively studied, e.g., hsp70), the largest of the above hsp and grp groups, hsp110 and grp170, have been almost entirely ignored. These stress proteins have only been cloned within the last few years, and their characterization remains at a very preliminary level (12–16). Curiously, they have both been found by sequence analysis to represent large and highly "diverged" relatives of the hsp70 family. It is recognized today that the hsp70 "family," the hsp110 family, and the grp170 family comprise three distinguishable stress protein groups in eukaryotic cells that share a common evolutionary ancestor (11, 17). The existence of hsp110 in parallel with hsp70 in the cytoplasm and of grp170 in parallel with grp78 in the ER of (apparently) all eukaryotic cells argues for important differential functions for these distantly related protein families. Indeed, present data indicate important functional differences between these large and small stress protein groups; e.g., hsp110 appears to be significantly more efficient than hsp70 in binding peptide chain

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but does not bind to ATP agarose, as does hsp70 (15, 18); grp170 binds peptide from TAP, whereas grp78 does not (19, 20).

Because of the above points and the previously demonstrated effectiveness of a few other stress proteins as vaccines, we undertook an analysis of effectiveness of the vaccine potential of hsp110 and grp170. In the present report, we describe the procedure for purification of hsp110 and grp170 and begin to evaluate their use as cancer vaccines using two mouse tumor models. In addition, we examined the use of hsp110 and grp170 in the preparation of dendritic cell (DC) anticancer vaccines. Finally, several recent studies indicate that fever-like therapy can have significant effects on several immunological end points. We also examine the effect of a fever-like thermal exposure on the effectiveness of these stress proteins as well as hsc70 as vaccines.

Materials and Methods

Mice and Abs

BALB/c mice (viral Ag free) were obtained from The Jackson Laboratory (Bar Harbor, ME) and were maintained in the mouse facilities at Roswell Park Cancer Institute. Abs to hsp110 and grp170 were made in our laboratory (13, 20). Abs to hsc70 were purchased from StressGen Biotechnologies (Victoria, British Columbia, Canada). Colon 26 carcinoma cells were maintained in DMEM supplemented with 10% heat-inactivated FCS (Life Technologies, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Methylcholanthrene-induced fibrosarcoma (Meth A) was kindly provided by Pramod K. Srivastava (University of Connecticut School of Medicine, Farmington, CT) and maintained in ascites in BALB/c mice by weekly i.p. passage of 2 million cells.

Purification of hsp110, grp170, and hsc70

Both tumor tissue and culture cells were used for hsp isolation. A cell pellet or tissue (40–60 ml) was homogenized in 5 vol of hypotonic buffer (30 mM sodium bicarbonate (pH7.2) and protease inhibitors) by Dounce homogenization. The lysate was centrifuged at 4,500 × *g* and then 100,000 × *g* to remove unbroken cells, nuclei, and other tissue debris. The supernatant was further centrifuged at 100,000 × *g* for 2 h. Supernatant was applied to a Con A-Sepharose column (Pharmacia Biotech, Piscataway, NJ) previously equilibrated with binding buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, and 15 mM 2-ME). The bound proteins were eluted with binding buffer containing 15% α-D-methylmannoside (Sigma, St. Louis, MO). For purification of hsp110, Con A-Sepharose unbound material was first dialyzed against 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 15 mM 2-ME and then applied to a DEAE-Sepharose column and eluted by salt gradient from 100 to 500 mM NaCl. Fractions containing hsp110 were collected, dialyzed, and loaded onto a Mono Q (Pharmacia) 10/10 column equilibrated with 20 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 15 mM 2-ME. The bound proteins were eluted with a 200–500 mM NaCl gradient. Fractions were analyzed by SDS-PAGE followed by immunoblotting with an Ab for hsp110, as described previously (21). Pooled fractions containing hsp110 were concentrated by Centriplus (Amicon, Beverly, MA) and applied to a Superose 12 column (Pharmacia), and proteins were eluted by 40 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 15 mM 2-ME with a flow rate of 0.2 ml/min. For purification of grp170, Con A-Sepharose-bound material was first dialyzed against 20 mM Tris-HCl (pH 7.5) and 150 mM NaCl and then applied to a Mono Q column and eluted by a 150 to 400 mM NaCl gradient. Pooled fractions were concentrated and applied on the Superose 12 column (Pharmacia). Fractions containing homogeneous grp170 were collected. Hsp70 was purified as described previously (22). Con A-Sepharose unbound proteins were loaded on an ADP-agarose column (Sigma) equilibrated with binding buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 15 mM 2-ME, 3 mM $MgCl_2$, and protease inhibitors). The column was then incubated with buffer containing 5 mM ADP at room temperature for 1–2 h. Proteins were subsequently eluted with the same buffer. The elute was resolved on a fast protein liquid chromatography system using a Mono Q column and eluted by a 20–500 mM NaCl gradient. For purification of hsps or grps from liver, the 100,000 × *g* supernatant was first applied to a blue Sepharose column (Pharmacia) to remove albumin. All protein was quantified with a Bradford assay (Bio-Rad, Richmond, CA). In these studies, it should be noted that although grp170 was purified using a Con A-Sepharose column, contamination with Con A can be largely ruled out, because the protective immunity was only observed in mice immunized with tumor-derived grp170 preparations and not in normal liver preparations that also utilized Con A columns.

Immunoblot analysis

Equivalent protein samples were subjected to 7.5–10% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) (21). Membranes were blocked with 5% nonfat milk in TBST (20 mM Tris-HCl (pH 7.4), 137 mM NaCl, and 0.05% Tween 20) for 1 h at room temperature and then incubated for 2 h with primary Abs diluted 1:1000 in TBST. After washing, membranes were incubated with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG diluted 1:2000 in TBST. Immunoreactivity was detected using the ECL detection system (Amersham, Arlington Heights, IL).

Tumor rejection assays

Mice (6- to 8-wk-old female) were immunized s.c. with hsp110, grp170, or PBS twice at weekly intervals. Seven days after the second immunization, mice were challenged by s.c. injections of 20,000 Colon 26 tumor cells or intradermal injections of 100,000 Meth A tumor cells (viability of tumor cells is >99%). s.c. injections were administered in the flank area, and intradermal injections were given in the skin on the ventral aspect of the trunk. The shortest diameter (*A*) and the longest diameter (*B*) were measured with a caliper every 2 days to monitor tumor growth. The volume (*V*) was calculated using the formula $V = (A^2B)/2$.

Immunotherapy of mice bearing Colon 26 tumor

All mice were first inoculated s.c. with 500,000 live Colon 26 cells. After tumors were palpable and visible, mice were treated every week with PBS, liver hsp110 (40 μg), and tumor hsp110 or grp170 (40 μg). A total of five injections were performed during the protocol. The survival of mice was monitored and recorded as the percentage of mice surviving after the tumor challenge. Mice that appeared moribund were killed and seen as "not surviving."

Generation and assay of CTLs

Mice were immunized as described before. Ten days after the second immunization, spleens were removed and spleen cells (1×10^7) were cocultured in a mixed lymphocyte-tumor culture with irradiated (12,000 rad) tumor cells (5×10^5) for 7 days and supplemented with 10% FCS, 1% penicillin/streptomycin, 1 mM sodium pyruvate, and 50 μM 2-ME. Splenocytes were purified by Ficoll-Paque (Pharmacia) density centrifugation and used as effector cells. Cell-mediated lysis was determined in vitro using a standard ^{51}Cr release assay. Briefly, effector cells were serially diluted in 96-well V-bottom plates (Costar, Cambridge, MA) in triplicate with varying E:T ratios of 50:1 25:1, 12.5:1, and 6.25:1. Target cells (5×10^6) were labeled with 100 μCi of sodium [^{51}Cr]chromate at 37°C for 1–2 h. ^{51}Cr-labeled tumor cells (5000) were added to a final volume of 200 μl/well. Wells containing only target cells with either culture medium or 0.5% Triton X-100 served as spontaneous or maximal release controls, respectively. After a 4-h incubation at 37°C and 5% CO_2, 150 μl of supernatant was analyzed for radioactivity in a gamma counter and percentage of specific lysis was calculated by the formula: percent specific lysis = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release). The spontaneous release was <10% of maximum release.

Vaccination with DCs pulsed with hsps from tumor

Bone marrow was flushed from the long bones of the limbs and depleted of RBC with ammonium chloride. Leukocytes were plated in bacteriological petri dishes at 2×10^6/dish in 10 ml of RPMI 10 supplemented with 20 ng/ml murine GM-CSF (R&D Systems, Minneapolis, MN), 10 mM HEPES, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 mM 2-ME. The medium was replaced on days 3 and 6, and on day 8 the cells were harvested for use. The quality of DC preparation was characterized by cell surface marker analysis and morphological analysis. DCs (1×10^7/ml) were pulsed with tumor-derived hsps (200 μg) for 3 h at 37°C. The cells were washed and resuspended in PBS (10^6 pulsed DCs in 100 μl PBS per mouse) for i.v. injection. The entire process was repeated 10 days later, for a total of two immunizations per treated mouse. Ten days after the second immunization, mice were challenged with Colon 26 tumor cells (2×10^4).

Whole-body hyperthermia (WBH) exposure

Mice were first inoculated s.c. with 500,000 Colon 26 tumor cells on the flank area. After the tumor reached a size of ~1 × 1 cm, WBH was

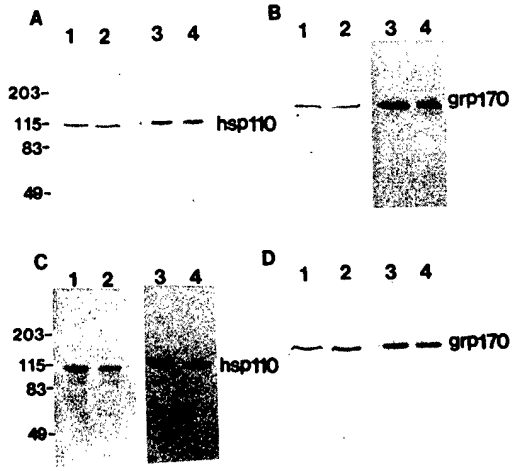

FIGURE 1. Hsp110 and grp170 preparations from tumor or liver of BALB/c mice. Hsp110 and grp170 purified from Meth A tumor (*top*), Colon 26 tumor (*bottom, lanes 1* and *3*), and liver of BALB/c mice (*lanes 2* and *4*) were separated by SDS-PAGE, followed by silver staining (*lanes 1* and *2*) or immunoblotting analysis (*lanes 3* and *4*) using Abs for hsp110 and grp170, respectively.

conducted as described before (22). Briefly, mice were placed in the microisolater cages preheated to 38°C that contained food, bedding, and water. The cages were then placed in a gravity convection oven (Memmert model BE500; Memmert, East Troy, WI) with preheated incoming fresh air. The body temperature was gradually increased 1°C every 30 min until a core temperature of 39.5°C (±0.5°C) was achieved. Mice were kept in the oven for 6 h. The core temperature of the mice was monitored with the Electric Laboratory Animal Monitoring System from Biomedic Data Systems (Maywood, NJ).

Results

Purification of hsp110 and grp170

Hsp110 and grp170 were purified simultaneously from tumor and liver. Purification protocols were developed as described in *Materials and Methods*, and homogeneous preparations for these proteins were obtained. The purity of the proteins was assessed by SDS-PAGE and silver staining as shown in Fig. 1. Approximately 20–50 μg hsp110 and 10–40 μg grp170 were obtained from each gram (wet weight) of tumor or tissue. The yield of grp170 from tumor is usually higher than that from normal tissue as a result of a higher level of grp170 expression in the tumor, possibly due to a hypoxic tumor fraction.

Hsp110/grp170 immunization causes the complete regression of Meth A tumors

We then investigated whether immunization with purified hsp110 and grp170 could protect mice against tumor challenge. For this purpose, the Meth A tumor model was initially used. We immunized mice twice with 40 μg (dose based on preliminary data) hsp110 or grp170 and then challenged them with Meth A cells by intradermal injection as described in *Materials and Methods*. Fig. 2 shows the results of this study. Separate lines present tumor growth data on individual animals, since some individual differences in the grp170-treated animals were observed. It is seen that mice immunized with hsp110 and grp170 were protected from the Meth A tumor challenge. Interestingly, and similarly to studies of others, most hsp110/grp170-vaccinated animals transiently developed tumors that then regressed and disappeared. However, in the mice that were immunized with grp170, two of five mice failed to develop any measurable tumor mass. To see whether this antitumor activity induces a long-term immunity against tumor, we challenged mice that survived with 100,000 Meth A tumor cells 5 months after the first challenge, and none of the mice was found to have developed tumor (data not shown).

Immunization of mice with tumor-derived hsp110 or grp170 leads to significant delays in growth of Colon 26 tumor

To test the generality of these observations on the vaccine activity of hsp110 and grp170 in the Meth A tumor system, we next chose the Colon 26 tumor model. This model was chosen since we found it to be generally resistant to various therapies. Groups of mice (five mice per group) were injected with PBS or with varying quantities of tumor-derived hsp110 or grp170 in 200 μl of PBS. These mice were then given booster injections 1 wk later. Hsp110 or grp170 was also isolated from the livers of the same animals, and this or PBS was used as control. Seven days after the last immunization, mice were injected s.c. on the right flank with 20,000 Colon 26 tumor cells. As seen in Fig. 3, all mice that were treated with PBS or liver-derived hsp110 or grp170 developed rapidly growing tumors. In contrast, mice immunized with hsp110 and grp170 from Colon 26 tumor showed a significant tumor growth delay, in general agreement with the above Meth A results. The inhibitory effect of hsp110 or grp170 vaccination on Colon 26 tumor growth was dependent on the dose of hsp110 or grp170 used for immunization. Although mice immunized with 20 μg (per injection) of hsp110 or grp170 showed an only slightly slowed tumor growth, those immunized with 40 or 60 μg of hsp110 or grp170 showed increasingly significant tumor growth delays (Fig. 3). Although tumor growth was not preventable in this highly aggressive

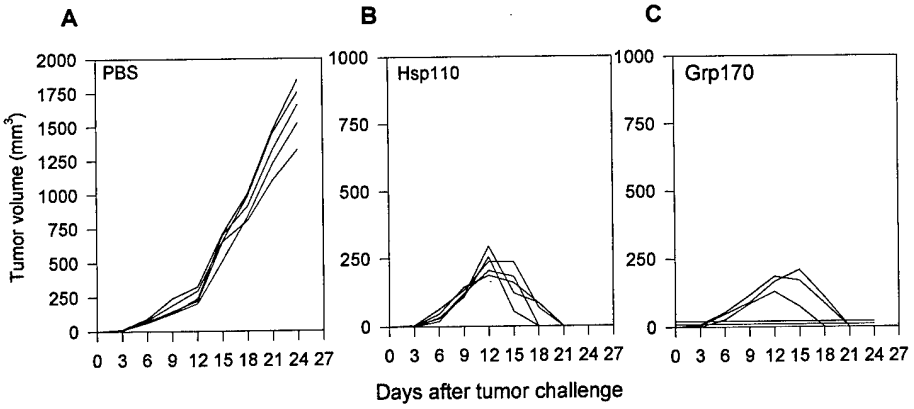

FIGURE 2. Immunization of mice with hsp110 or grp170 protects mice against Meth A tumor challenge. Mice were immunized s.c. with 40 μg of hsp110 or grp170 and boosted with the same amounts of these proteins 1 wk later. Seven days after the second immunization, the mice were challenged with 100,000 live Meth A tumor cells intradermally. Each group contained five mice, and each line represents the kinetics of tumor growth in one mouse.

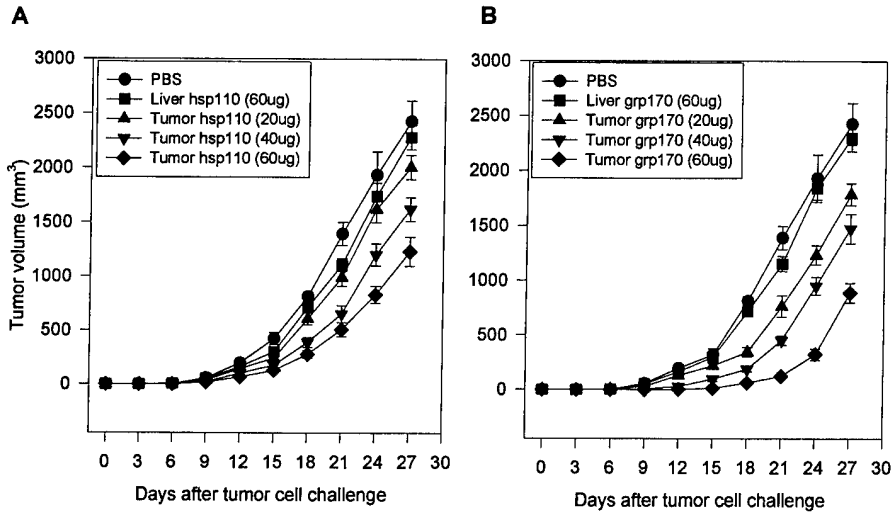

FIGURE 3. Immunogenicity of hsp110 and grp170 preparations purified from Colon 26 tumor. Mice were immunized twice with varying doses (20, 40, and 60 μg) of hsp110 and grp170 from Colon 26 tumor as indicated. Hsp110 or grp170 (60 μg) from liver of BALB/c mice was used as a control. One week after the second immunization, mice were challenged s.c. with 20,000 live Colon 26 cells.

and rapidly growing tumor system, these data demonstrate that hsp110 and grp170 have specific antitumor effects. On each day examined (e.g., 15, 21, and 27 days after challenge), the mean volumes of the tumors that developed in mice immunized with hsp110 or grp170 at doses of 40 and 60 μg were significantly smaller than those of control mice ($p < 0.01$, Student's t test). However, the differences in the mean volumes of the groups injected with PBS or liver-derived hsp110/grp170 preparations were not significant. Last, it was found that mice immunized with Meth A-derived hsp110 or grp170 were not resistant to challenge with Colon 26 tumor cells (data not shown).

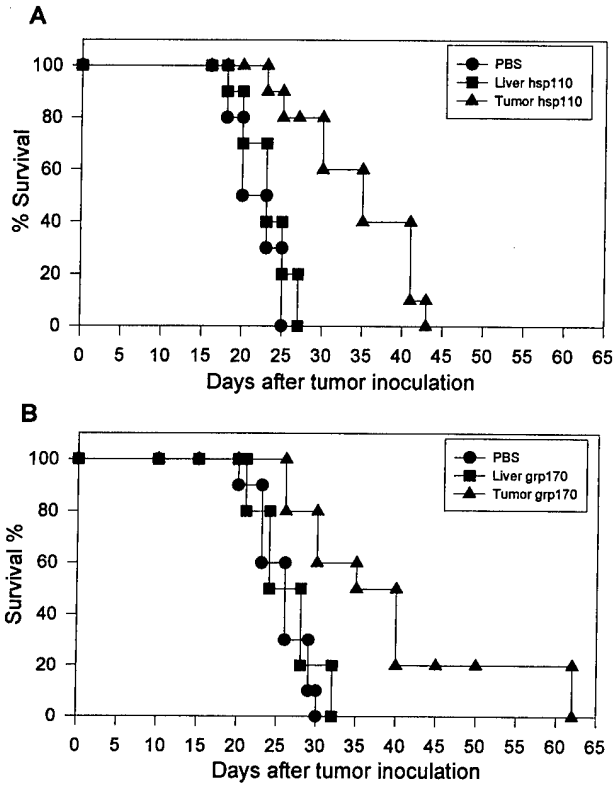

FIGURE 4. Effects of immunization with tumor-derived hsp on the survival of tumor-bearing mice. Mice were first inoculated s.c. with 500,000 Colon 26 cells. After the tumor was palpable, mice were treated with or without 40 μg of hsp110 or grp170 at weekly intervals. The survival of mice was recorded as the percentage of mice surviving after the tumor challenge.

Hsp110/grp170 immunization improves the survival of Colon 26 tumor-bearing mice

In considering the clinical application of a tumor vaccination strategy, it is more realistic to treat animals with tumor present at the time of vaccination. Thus, the aggressive Colon 26 tumor was again examined using a therapy approach. Tumor cells were transplanted into the flank of mice (10 mice in each group). When tumors were readily palpable after inoculation, animals were treated with liver- or Colon 26-derived hsp110 or grp170 on a weekly basis. The survival of mice was recorded as the percentage of mice surviving after the tumor challenge. Tumor-bearing mice treated with autologous hsp110 or grp170 preparations showed significantly longer survival times compared with the untreated mice or mice immunized with liver-derived hsp110 or grp170. As shown in Fig. 4, all control mice died within 30 days, but approximately half of each group survived to 40 days and 20% of grp170-treated mice lived beyond 60 days, clearly demonstrating a beneficial antitumor effect. In parallel with the data shown in Fig. 2, these data suggest that grp170 is more efficient than hsp110 on an equal-mass basis.

Hsp110/grp170 vaccination elicits a tumor-specific CTL response

Since cellular immunity appeared to be critical in mediating the observed antitumor effects, we analyzed the ability of tumor-derived hsp110 and grp170 preparations to elicit a tumor-specific $CD8^+$ T cell response. Mice were immunized twice at weekly intervals with 40 μg of hsp110 or grp170 derived from Colon 26 or Meth A tumors. Splenocytes generated from these immunized mice were then cultured in vitro for 7 days with irradiated tumor cells. These cultured cells were then used as effector cells in the CTL assay. As shown in Fig. 5, a tumor-specific cytotoxicity was observed to occur against the tumor from which the immunogen (hsp110 or grp170) was derived. Splenocytes from mice immunized with Colon 26 cell-derived hsp110 or grp170 preparations showed specific lysis for Colon 26 tumor cells only, but not for Meth A tumor cells; conversely, splenocytes from animals immunized with Meth A tumor cells were only effective against Meth A

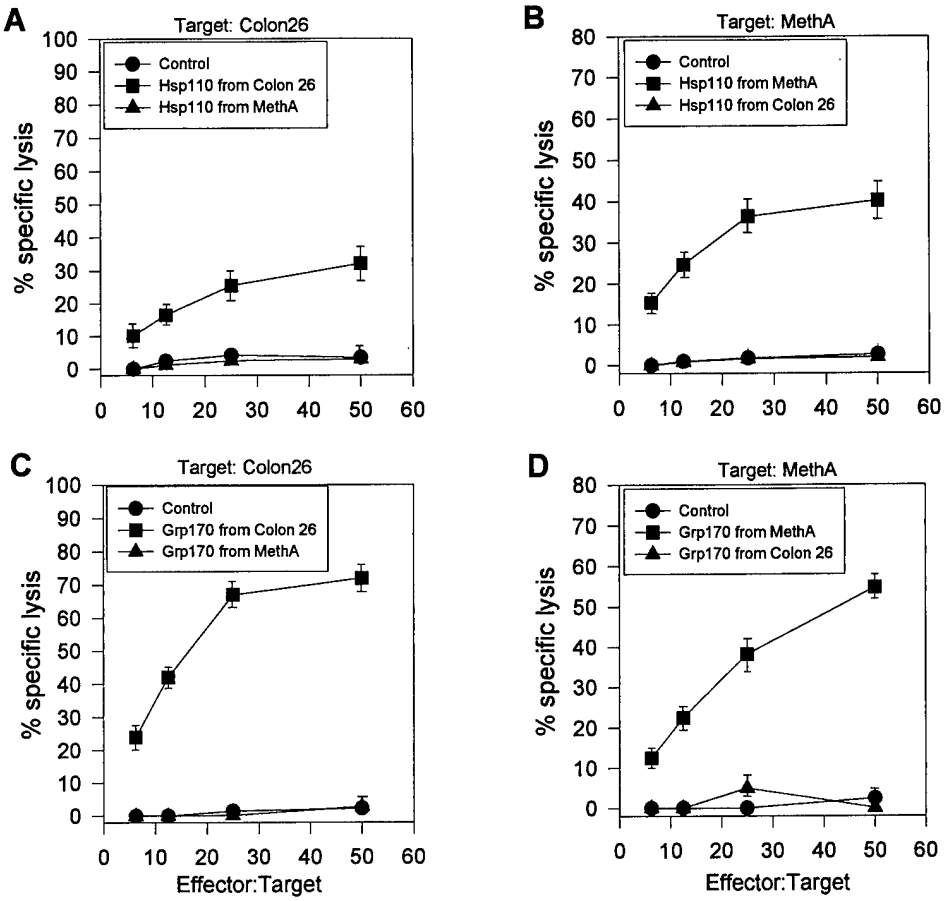

FIGURE 5. Tumor-specific CTL response elicited by immunization with tumor-derived hsp110 or grp170. Mice were immunized twice with PBS, hsp110, or grp170 (40 μg) at weekly intervals. One week after the second immunization, splenocytes were isolated as effector cells and restimulated with irradiated Colon 26 or Meth A tumor cells in vitro for 7 days. The lymphocytes were analyzed for cytotoxic activity using ^{51}Cr-labeled Colon 26 or Meth A cells as target cells.

cells and not against Colon 26 cells. This demonstrates that vaccination with hsp110 or grp170 elicits a tumor-specific CTL response. Splenocytes from naive mice were unable to lyse both target cells (control). Again, spleen cells derived from grp170-immunized animals yielded a greater percentage specific lysis than was obtained from hsp110-immunized animals.

Hsp110/grp170-pulsed DCs mount an effective antitumor response

To investigate whether APCs could be involved in the antitumor response elicited by hsp110 or grp170 immunization, we tested the ability of DCs to acquire an antitumor activity, presumably by presentation of hsp110- or grp170-chaperoned peptides. DCs were prepared from mouse bone marrow as described in *Materials and Methods*. DCs were then incubated with grp170 or hsp110 purified from the Colon 26 tumors for 3 h at 37°C. Cells were washed and resuspended in PBS. Pulsed DCs (10^6) in 100 μl of PBS were used for i.v. injection for each mouse. The entire process was repeated 10 days later. Ten days after the second immunization, mice were challenged with 2×10^4 Colon 26 tumor cells, and tumor growth was monitored by measuring the tumor diameter as shown in Fig. 6. It was observed that tumors grew rapidly in the mice that received PBS or (nonpulsed) DCs alone. However, tumor growth was significantly delayed in mice immunized with DCs pulsed

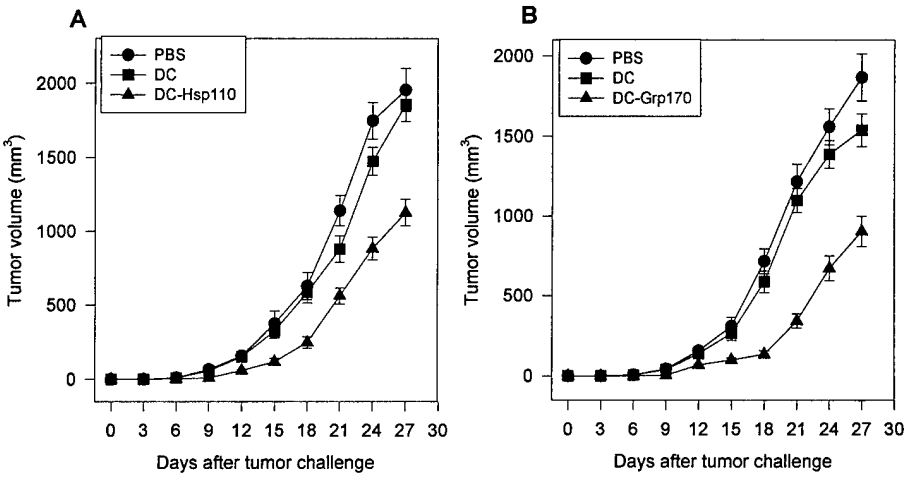

FIGURE 6. Immunotherapy with DCs pulsed with hsp110 or grp170. DCs (1×10^7) were generated from bone marrow of BALB/c mice and incubated with hsp110 or grp170 (200 μg/ml) in vitro for 3 h. DCs were washed and introduced to mice (10^6 cells in 100 μl PBS/mouse) by i.v. injection. The whole immunization process was repeated 10 days later. Mice were challenged with 20,000 Colon 26 cells 10 days after the second immunization.

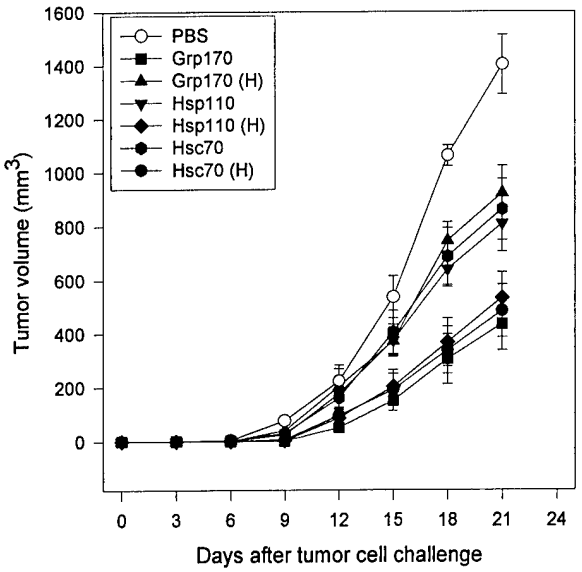

FIGURE 7. Fever-like WBH enhances the vaccination efficiency of tumor-derived hsp110 or hsc70. Mice were first inoculated s.c. with Colon 26 tumor cells on the flank area. After the tumor reached a size of ~1 × 1 cm, WBH was conducted as described in *Materials and Methods*. Tumors were collected on the next day, and grp170, hsp110, and hsc70 were isolated. Mice were immunized twice at weekly intervals and then challenged with 20,000 live Colon 26 tumor cells.

with hsp110 or grp170. Grp170, once again, appeared to be more effective. Moreover, based on the immunization effects in the mice that received 10^6 DCs pulsed with 20 μg of protein and those that received two doses of 40 μg of protein by s.c. injections, it was found that less stress protein was required for DC-based immunotherapy.

Fever-like thermal conditions significantly enhance the efficiency of hsp110 or hsc70 as anticancer vaccines

Several recent studies have indicated that a modest increase in body temperature sustained for several hours, i.e., a condition comparable with common febrile response, can significantly affect certain immunological end points and immune function (22). We therefore exposed mice to 39.5°C (i.e., core temperature) WBH for a period of 8 h to determine whether hsp/grp vaccine efficiency might also be altered as a result of a fever-like thermal condition. Fig. 7 compares the effectiveness of hsp110 and grp170, as well as hsc70 (40 μg each), derived from Colon 26 tumors taken from both normothermic (control) animals and animals previously exposed to this fever-like thermal treatment. This figure illustrates several points. First, hsc70 or hsp110 is significantly more efficient when purified from tumors derived from animals receiving prior fever-range WBH. However, the prior fever-range thermal treatment is seen to reduce the vaccine efficiency of grp170. These data indicate that fever-like exposures can influence the Ag presentation pathway and/or peptide-binding properties of these two (heat-inducible) hsps purified from Colon 26 tumors but not a heat-insensitive grp. In addition to these observations, this figure also shows that grp170 purified from unheated control tumors (mice) is significantly more efficient in its vaccine efficiency when compared on an equal-mass basis with either hsc70 or hsp110 (without heat). This increased efficiency of grp170 compared with hsp110 is also reflected in the studies described above. This comparison is based on administration of equal masses of these proteins, and the enhanced efficiency of grp170 is further exacerbated when molecular size is taken into account (i.e., comparisons made on a molar basis). Third, hsc70 is seen here to be approximately equivalent in its vaccine efficiency (again, on an equal-mass but not equal-molar basis) to hsp110.

Discussion

It has long been recognized that the major hsps of mammalian cells are observed at 25–28, 70, 90, and 110 kDa, and other hsp families, e.g., hsp60 and hsp40, have been subsequently identified. These heat- or oxidative stress-inducible stress proteins principally reside in the cytoplasm and nucleus, excepting hsp60, which is in the mitochondria. It has also been long recognized that a second set of stress proteins called grps resides in the ER. This group of proteins is not responsive to typical heat shocks or oxidative stress but to reducing conditions (e.g., anoxia) or other states that interfere with the function of the ER. Principal grps have been observed at 78, 94, and 170 kDa. Both of the large stress protein species, hsp110 and grp170, have only recently been cloned. Their sequences have, surprisingly, shown them to be very large and greatly diverged relatives of the hsp70 family. They appear to possess many of the secondary structural features of hsp70 and are peptide chain-binding proteins. Although little is known about the cellular functions of hsp110, deletion mutational studies have defined its basic domains and indicate that it has a peptide-binding domain generally analogous to that of hsp70, while it also exhibits major functional differences from those of hsp70 (15, 18). Less is understood at the molecular level of grp170 structure and function; however, cellular studies have shown that it binds to Ig chain in the ER, may be the ATPase responsible for protein import into the ER, and actively binds peptides from TAP (i.e., the transporter associated with Ag processing; Refs. 11, 19, 20, 23, 24).

There is now considerable evidence from different laboratories that stress proteins (i.e., hsps and grps) can serve as vaccines that produce a tumor-specific CTL response and a protective antitumor immunity in animals (3–5, 25–29). We have examined here the capacity of hsp110 and grp170 to also function as stress proteins (or "heat shock") vaccines. We report that immunization with these two high molecular weight stress proteins leads to an antitumor immune response. It was found that hsp110 or grp170 immunization leads to a complete regression of Meth A tumor. In addition, either of these stress proteins was found to significantly inhibit Colon 26 tumor growth and significantly prolong the life span of mice with previously established tumors. These findings indicate that hsp110 and grp170 are both active anticancer vaccines.

Cytotoxicity assays described here demonstrate that hsp110 or grp170 immunization results in $CD8^+$ T lymphocyte response that correlates the in vivo tumor rejection observed. This is consistent with earlier studies concerning the antitumor immunity elicited by immunization with gp96 (25–29). In addition, the hsp-peptide complex, reconstituted in vitro, also elicits an Ag-specific CTL response (30). The capacity of hsp/grp to elicit an immune response is seemingly independent of the MHC type of the tumor, whereas the (presumed) presentation of the hsp-chaperoned peptides to CTL is MHC I restricted and is therefore defined by the MHC phenotype of the APC (9, 26, 31, 32). In addition, it is observed that priming of mice with Colon 26-derived hsp110 or grp170 only results in the lysis of Colon 26 tumor cells and not Meth A tumor cells. Conversely, a similar Meth A-targeted response was also obtained in the mice immunized with Meth A tumor-derived hsp110 or grp170. These observations are again consistent with earlier studies with other stress proteins showing that hsp immunization induces tumor-specific immune response (25, 28, 32, 33). Therefore, hsp-chaperoned peptides, even though

they are provided exogenously, are apparently capable of entering the class I Ag-presenting pathway. To investigate the molecular mechanism involved in hsp immunization-mediated antitumor immunity, additional experiments (i.e., T cell subset depletion) need to be performed.

DCs have been known to be highly specialized APCs and to be the principal activators of naive T cells in vitro and in vivo (34–37). Many have demonstrated that DCs pulsed in vitro with tumor Ag, tumor extracts, or mRNA (38–41) are capable of stimulating specific CTL activity and protect animals against subsequent tumor challenge. In the present study, we have shown that immunization with DCs pulsed with tumor-derived hsp110 or grp170 results in tumor growth inhibition in vivo, strongly suggesting that APCs are involved in the hsp-elicited antitumor response. It is suggested that hsp110- or grp170-peptide complexes can be targeted to APCs through a putative receptor. The hsp-chaperoned peptides are thus processed and re-presented by the MHC class molecules that stimulate Ag-specific $CD8^+$ T lymphocytes. Recently, it has been reported that hsp70 and gp96 receptors on the cell surface are involved in endocytosis of these stress proteins by APCs (42, 43). Further studies are needed to determine whether there exists a specific Ag internalization pathway mediated by these receptors and how hsp110- or grp170-associated peptides gain access to the ER of APCs.

Comparing the results of immunization of hsp110 and grp170 as immunogens in Colon 26 and Meth A tumor models and in the DC study, it is seen that grp170 appears to be more efficient than is hsp110 when administered on an equal-mass basis (i.e., Figs. 2–4). In addition, Fig. 7 further indicates that grp170 is also more effective on an equal-mass basis than is tumor-derived hsc70. We have also examined grp78, another relative of this stress protein superfamily. Curiously, grp78 appears to be largely ineffective as an anticancer vaccine when derived from tumors (data not shown). This latter observation is also consistent with data obtained by others (33). In this scheme, the approximate relative vaccine efficiency (least to most on an equal-mass basis for Colon 26 tumors) is as follows: grp78 (ineffective), hsp110 and hsc70 (similar effectiveness), and grp170 (most effective).

It has been shown that the immunogenicity of hsc70 can be attributed to the peptides chaperoned by it and that its properties as a vaccine are lost if the bound peptides are released (25, 45–47). Hsp110 and grp170 both appear to exhibit a peptide-binding cleft (11, 18, 44). However, hsp110 and grp170 differ dramatically from the hsc70s in their C-terminal domains, which, in the case of hsc70 proteins, appear to function as a "lid" for the peptide-binding cleft and may have an important influence on the properties of the bound peptide/protein and/or the affinity for the associated peptide/protein. Both hsp110 and grp170 appear to be more significantly efficient in binding to and stabilizing thermally denatured proteins relative to hsc70. This may reflect these structural differences and influence peptide-binding properties, a factor that is a key element in the ability of stress proteins to function as vaccines. Although hsc70 and hsp110 are approximately similar in vaccine efficiency, they may bind differing subsets of peptides (e.g., hsp110 may carry antigenic epitopes, which do not readily bind to hsc70); i.e., they may exhibit differing vaccine potential if not differing (mass) efficiencies. A similar argument can be made for grp170. The significant differences in molar efficiencies of these stress proteins may result from differing peptide-binding affinities, differing properties of peptides bound to each stress protein family, or differing affinities of APCs to interact with each of these four stress protein groups. It may also be noteworthy that grp170, the most efficient vaccine in this group, is the only gp.

Finally, reports in the last few years have suggested that a mild, fever-level thermal treatment can significantly stimulate various features of the immune response. At the cellular level, it has been shown that fever-like treatments of lymphocytes (39.5°C for 6–8 h) leads to activation of protein kinase C, massive cytoskeleton changes characteristic of a heightened activation status, and the induction of hsps including hsc70 and hsp110 (22, 48–49). In mice, fever-level hyperthermia has been shown to lead to an antitumor effect involving both the innate and specific immune systems (50). It is possible that mild hyperthermia, which is nontoxic, may lead to several changes in immunological parameters. We have shown here that the vaccine potential of hsc70 and hsp110 are significantly enhanced following fever-level therapy. This could result from enhanced proteosome activity, enhanced peptide binding of the hsp, altered spectrum of peptides bound to the hsp, or other factors. Since the hsps were purified 16 h after the 8-h hyperthermic exposure, the effect is maintained for some time at 37°C. It would seem that the factors leading to this enhanced immunogenicity would derive from an altered and/or enhanced antigenic profile of hsp-bound peptides. Stability following the hyperthermic episode suggests upstream changes in Ag processing that are still present many hours later, e.g., stimulation of proteosome activity. Another feature of fever-like hyperthermia is the highly significant induction of hsps in Colon 26 tumors (X.-Y. Wang and J. R. Subjeck, unpublished observations). Therefore, fever-like heating not only provides a more efficient vaccine in the case of the hsps examined, but also a lot more of it. Finally, it is intriguing that the observed increase in vaccine efficiency resulting from hyperthermia is seen only for hsp110 and hsc70. grp170, which is regulated by an alternative set of stress conditions such as anoxia and other reducing states, but not heat, is diminished in its vaccine potential by heat. It is not clear why grp170 efficiency as a vaccine is depressed by this heat shock condition. Further studies are required to determine how these changes arise.

Hsp vaccines are unique because of their promiscuous ability to chaperone and present a broad antigenic repertoire of tumor cell peptides. Thus, vaccination with hsps isolated from tumor cells circumvents the need to identify specific tumor Ags and hence extends the use of hsp-based immunotherapy to the majority of cancers of which specific tumor Ags have not yet been characterized (51). The administration of hsp/grp vaccines or hsp-/grp-pulsed DCs for cancer treatment might be safer than using whole tumor cell or cell lysates, specifically genetically modified cells, as tumor vaccines that could introduce transforming DNA or potentially immunosuppressive factors. The present study demonstrates that hsp110 and grp170 can both function as potent anticancer vaccines and provides strong additional supporting evidence for the development of hsp-/grp-peptide complexes as a basis for a new approach to cancer immunotherapy. Further investigation of mechanisms underlying the hsp-elicited antitumor response may help us to better understand the powerful immunological potential that is associated with hsp-mediated immunotherapy.

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Development of a recombinant HSP110-HER-2/neu vaccine using the chaperoning properties of HSP110

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Abbreviations: intracellular domain (ICD), antigen presenting cells (APCs), heat shock protein (HSP)

Abstract

HSP110 is a major heat shock protein of eukaryotic cells, which has been long observed but only recently cloned and studied. In contrast to other HSPs, evidence indicates that HSP110 is a highly efficient chaperone in binding to a full-length protein substrate. Other studies have shown that some stress proteins bind antigen presenting cells through specific receptors and can induce activation and maturation of dendritic cells. Having had these properties, these HSPs can exhibit potent anti-tumor vaccine activity when purified from the tumor. The present study utilizes the potent protein binding property of HSP110 to form a natural chaperone complex with the intracellular domain (ICD) of HER-2/neu as a substrate. This natural, non-covalent complex is then assessed for its immunogenicity. The HSP110-ICD complex elicits a cell-mediated immune responses against ICD, which is not obtained with ICD alone as determined by an antigen-specific IFN-γ production. The complex also significantly enhances the humoral immune response against ICD relative to that seen with ICD alone. *In vivo* depletion studies revealed that both $CD4^-$ and $CD8^-$ T cells were involved in antigen-specific IFN-γ production, and $CD8^+$ T cell response was independent of $CD4^+$ T cell help. Although both IgG1 and IgG2a antibodies were observed following the HSP110-ICD immunization, IgG1 antibody titer was more vigorous than IgG2a antibody titer. Interestingly, neither $CD8^-$ T cell nor antibody response was detected against the HSP110 itself. The use of HSP110 to form natural chaperone complexes with full-length proteins opens up a new approach for the design of protein-targeted vaccines.

Introduction

A few major heat shock proteins have been observed in countless studies over the last twenty years that include HSP27, HSP60, HSP70, HSP90 and HSP110 (1, 2). All of these stress proteins have been extensively cloned and studied except for the HSP110 group has been essentially ignored. Only in recent years has HSP110 been cloned from numerous organisms from yeast to man. These studies show that HSP110 is representative of a newly discovered stress gene family which shares an evolutionary ancestor with members of the HSP70 stress protein family. Initial analysis of its molecular chaperoning properties indicates that HSP110 differs functionally from the HSP70s. Studies indicate that HSP110 is considerably more efficient than HSC70 in stabilizing denatured peptide chain (3, 4). Surprisingly HSP110 complexes with reporter proteins and totally inhibits their heat induced aggregation at a 1:1 molar ratio. A second major difference involved ATP binding properties. HSP70 has been long known to avidly bind ATP. However, full length HSP110 does not bind ATP, or binding requires other co-chaperones.

In recent years, certain heat shock proteins have been shown to act as effective vaccines against a cancer when they are purified from that specific cancer (5-8). This approach takes advantage of the peptide binding properties of these stress proteins which is also responsible for their functions as molecular chaperones in numerous processes (9). The purification of these stress proteins would then co-purify a peptide "fingerprint" of the cell of origin. In the case of cancer cells, this presumably includes a small subset of antigenic, tumor specific

epitopes. Importantly, HSPs not only bind peptides but also possess other immunological properties, some of which are receptor-mediated endocytosis by dendritic cells (DCs), maturation of DCs, induction of proinflammatory cytokine secretion, and up-regulation of MHC class II molecule (10-12). By virtue of bound antigenic peptides and these excellent adjuvant properties, the HSP or GRP preparation can be used as a vaccine in the absence of any other adjuvant. The success of HSP vaccines in pre-clinical animal studies has led to clinical phase I/II trials of tumor-derived HSP preparations (specifically GRP94) as autologous tumor vaccines (13, 14). Only a few stress proteins have been shown to exhibit vaccine activity in animals when prepared in this way (most notably, GRP94/gp96 and HSC70). In addition, recent studies have shown that HSP110 also exhibits excellent vaccine activity against a tumor when purified from the same tumor. HSP110 or GRP170 (a homologue of HSP110 in the endoplasmic reticulum) purified from Meth A fibrosarcoma caused complete regression of the tumor, and when purified from Colon 26 led to a significant growth inhibition of this tumor (15).

Srivastava and others (16-18) have shown that immunogenic HSP-peptide complexes could also be generated *in vitro*. However, neither tumor-derived epitopes nor a full-length antigenic protein has been utilized *in vitro* to complex with HSPs as a vehicle for antigen delivery. The present study describes a new approach in the preparation of heat shock protein vaccines. This takes advantage of the property of HSP110 to naturally and efficiently bind large proteins (3). In this approach, recombinant HSP110 is non-covalently complexed during heat

shock with a well-known, full-length, tumor antigen *in vitro* as a vaccine. This molecular targeting approach would not be patient specific, as is the case with tumor-derived HSP/GRP, but could be applied to any patient with a tumor expressing that protein antigen. Importantly, this approach would present to the immune system a complex representative of the natural chaperoning function of HSP110.

HER-2/neu has been selected as a protein antigen of choice since it is clinically relevant to breast cancer and could well be applicable to other tumor systems such as ovarian, prostate, lung, and colon cancers expressing this protein. Importantly, some patients with breast cancer have preexisting cellular and humoral immune responses directed against intracellular domain (ICD) of HER-2/neu (19). Thus, an effective cancer vaccine targeting HER-2/neu, ICD in particular, would be able to boost this immunity to potentially therapeutic levels in humans (19). Moreover, the results from clinical trials targeting HER-2/neu have been promising (20).

We decided to explore whether this novel approach, which uses HSP110, may be able to elicit both cell-mediated and humoral immune responses against this bound protein antigen. We show that HSP110 was as efficient as Complete Freund's Adjuvant (CFA) in eliciting an antigen-specific $CD8^+$ T cell response both in a $CD4^+$-dependent and in a $CD4^+$-independent fashion with no indication of anti-HSP110 cell-mediated or humoral immune responses.

Materials and Methods

Mice. Studies were performed in A2/Kb transgenic animals purchased from Harlan Sprague Dawley (La Julla, CA). This model was used for comparison of data obtained in the present study with peptide immunization approach using the HSP110-peptide complex (HLA-A2 epitopes from HER-2/neu) underway in a separate investigation (unpublished data). In addition, studies were reproduced using C57/BL6 mice (obtained from the Department of Laboratory Animal Resources at Roswell Park Cancer Institute) in a confirmatory experiment. Data obtained using A2/Kb mice is presented. All animals used in this study were 6-8 week old females.

Recombinant proteins. Recombinant mouse HSP110 is being routinely prepared in our laboratory using pBacPAK.His vector (CLONTECH Laboratories Inc., CA). This vector carrying HSP110 gene was co-transfected with BacPAK6 viral DNA into Sf21 insect cells using a BacPAK^{TM} Baculovirus Expression System Kit (CLONTECH Laboratories Inc. CA) followed by amplification of the recombinant virus and purification of HSP110 protein using Ni-NTA-Agarose (QIAGEN, Germany). Concentration of the recombinant HSP110 was determined using Bio-Rad protein assay Kit. Highly purified recombinant human ICD was provided by Corixa Corp. This protein was produced in *E. coli* and purified from solublilized inclusion bodies via High Q anion exchange followed by Nickel resin affinity chromatography. A control recombinant protein was also made in *E. coli* and purified in a similar way as the ICD.

In vitro HSP110-antigen binding. The HSP110-ICD complex (3-6 ug each in 1 ml PBS) was generated by incubation of the mixture in a 1:1 molar ratio at 43°C for 30 min and then at 37°C for 1h. The binding was evaluated by immunoprecipitation as previously described (3), with some modifications. Briefly, the HSP110-ICD complex was incubated with either rabbit anti-mouse HSP110 antiserum (1:200) or rabbit anti-mouse GRP170 antiserum (1:100), as a specificity control, at room temperature for 1-2 h. The immune complexes were then precipitated by incubation with Protein-A Sepharose™ CL-4B (20 ul/ml; Amersham Pharmacia Biotech AB, Upsala Sweden) and rocking for 1 h at room temperature. All proteins were spun for 15 min at 4°C to precipitate any aggregation before use. Samples were then washed 8 times with washing buffer (1 M Tris-Cl pH 7.4, 5 M NaCl, 0.5 M EDTA pH 8.0, 0.13% Teriton X-100) at 4°C to remove any non-specific binding of the recombinant proteins to protein-A sepharose. The beads were then added with 2x SDS sample buffer, boiled for 5 min. and subjected to SDS-PAGE (10%) followed by either Gell-blue staining or probing with mouse anti-human ICD antiserum (1:10000, provided by Corixa Corp.) in a western blotting analysis using HRP-conjugated sheep anti-mouse IgG (1:5000, Amersham Pharmacia Biotech, NJ) and 1 min incubation of the nitrocellulose membrane with Chemiluminescence reagent followed by exposure to Kodak autoradiography film for 20 sec.

Immunizations. Preliminary studies showed that s.c. and i.p. routes of injection of the HSP110-ICD complex stimulated comparable levels of cell-mediated immune responses, but i.p. injection was better than s.c. injection in eliciting

antibody responses (data not shown). Thus, all groups were injected i.p. except for mice immunized s.c. with ICD together with CFA and boosted together with Incomplete Freund's Adjuvant (IFA). Mice (5/group) were injected with 25 ug of the HSP110-ICD complex in 200 ul PBS on days 0 and 14. Control groups were injected with 25 ug of the HSP110, ICD, ICD together with CFA/IFA, or left unvaccinated. The splenocytes were removed 14 days after the booster and subjected to ELISPOT assay to evaluate CTL responses. Sera were also collected on days 0, 14, and 28 to measure isotype-specific antibodies (IgG1 and IgG2a) against the ICD or HSP110 using ELISA technique. Groups of animals (5/group) were also depleted from $CD8^+$, $CD4^+$, or $CD4^+/CD8^+$ T cells either 4 days prior to vaccination followed by twice a week injections or one week after the priming. The splenocytes were then subjected to ELISPOT assay.

In vivo antibody depletion. *In vivo* antibody depletions were carried out as previously described (21). The GK1.5, anti-CD4 and 2.43, anti-CD8 hybridomas were kindly provided by Dr. Drew Pardoll (John Hopkins University) and the ascites were generated in SCID mice. The depletions were started 4 days before vaccination. Each animal was injected i.p. with 250 ug of the monoclonal antibodies (mAbs) on 3 subsequent days before and twice a week after immunization. Animals were depleted from $CD4^+$, $CD8^+$, or $CD4^+/CD8^+$ T cells. Depletion of the lymphocyte subsets was assessed on the day of vaccination and weekly thereafter by flow cytometric analysis of spleen cells stained with mAbs GK1.5 or 2.43 followed by FITC-labeled rat anti-mouse IgG (Pharmingen, San Diego CA). For each time point analysis, >98% of the appropriate subset was

achieved. Percent of $CD4^+$ T cells did not change after $CD8^+$ T cell depletion, and neither did percent of $CD8^+$ T cells change after $CD4^+$ T cell depletion. The representative data are shown in Table 1.

Enzyme-linked immunosorbent spot (ELISPOT) assay. Generation of CTL responses by the immunized animals were evaluated using ELISPOT assay as described by others (22). Briefly, the 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 ug/ml of rat anti-mouse IFN-γ antibody (clone R4-6A2, Pharmingen, San Diego, CA) in 50 ul PBS. After overnight incubation at 4°C, the wells were washed and blocked with RPMI-1640 medium containing 10% fetal bovine serum (RF10). Red cells were lysed by incubation of the splenocytes with Tris-NH_4Cl for 5 min at room temperature followed by two times washing in RF10. Fifty ul of the cells (10^7 cells/ml) were added into the wells and incubated with 50 ul of the ICD (10-20 ug/ml) or HSP110 (20 ug/ml) at 37°C in a atmosphere of 5% CO2 for 20 h. Positive control wells were added with Con-A (5 ug/ml) and background wells were added with RF10. A control recombinant protein made in *E. Coli* was also used (10-20 ug/ml) in a confirmatory experiment using the HSP110-ICD or ICD immunized animals (data not shown). The plates were then washed extensively (10 times) and incubated with 5ug/ml biotinylated IFN-γ antibody (clone XMG1.2, Pharmingen, San Diego CA) in 50 ul PBS at 4°C overnight. After six times washing, 0.2 U/ml alkaline phosphatase avidin D (Vector Laboratories, Burlingame CA) in 50 ul PBS, was added and incubated for 2 h at room temperature, and washed on the following day (the last wash was carried out with PBS without Tween-20). IFN-γ spots were developed by adding

50 ul BCIP/NBT solution (Boehringer Mannheim, Indianapolis, IN) and incubating at room temperature for 20-40 min. The spots were counted using a dissecting microscope.

Enzyme-linked immunosorbent assay (ELISA). ELISA technique was carried out as described elsewhere (23). Briefly, 96-well ELISA plates were coated with ICD (20 ug/ml) or HSP110 (20 ug/ml), and then blocked with 1% BSA in PBS after incubation at 4°C overnight. After washing with PBS-0.05% Tween-20, wells were added with five-fold serial dilutions of the sera starting at 1:50, then incubated at room temperature for 1 h, washed 3 times and added with HRP-labeled goat anti-mouse IgG1 or IgG2a Ab (Caltag laboratories, Burlingame CA). The reactions were developed by adding 100 ul/well of the TMB Microwell peroxidase substrate (KPL, Maryland) and reading at 450nm after stopping the reaction with 50 ul of 2 M H_2SO_4. Specificity of the binding was assessed by testing the pre-immune sera or staining of the ICD with the pooled immune sera (1:2000), collected from the HSP110-ICD immunized animals, in a Western blot. Data are presented as mean values for each antibody isotype.

Statistical analysis: Unpaired two-tailed Student's *t* test was used to analyze the results. Data are presented as the ± SE. $p \leq 0.05$ was considered significant (24).

Results

Non-covalent binding of the HSP110 to ICD at 43°C. Based on our previous finding that HSP110 binds to Luciferase and Citrate Synthase at a 1:1 molar ratio at 43°C, we examined whether the same condition was applicable for binding of HSP110 to ICD. Different molar ratios of HSP110 and ICD (1:4, 1:1, 1:0.25) were used and the samples were run on SDS-PAGE. The bands were developed by either Gell-blue staining or Western blot analysis using mouse anti-human ICD antiserum and HRP-conjugated sheep anti-mouse IgG. It was found that excess ICD over HSP110 did not improve the binding efficiency nor did excess HSP110 over the ICD. Approximately a 1:1 molar ratio of the HSP110 to ICD was again found to be optimal for formation of the complex (15). Thus, a 1:1 molar ratio was used to generate the HSP110-ICD binding complex (Figure 1).

Vaccination with the HSP110-ICD complex induces antigen-specific IFN-γ production. ELISPOT assay is a sensitive functional assay used to measure IFN-γ production at the single-cell level, which can thus be applied to quantify antigen-specific $CD8^+$ or $CD4^+$ T cells. Depletion of T cell subsets was also performed to determine the source of IFN-γ production. We first explored whether the HSP110-ICD complex, without any adjuvant, could elicit antigen-specific IFN-γ production. Figure 2 demonstrates that the HSP110-ICD-immunized animals elicited significant IFN-γ production upon stimulation with ICD *in vitro*. No IFN-γ spot was detected in the background wells. The HSP110-ICD complex was as efficient as the CFA-ICD, i.e. there was no significant difference between the two vaccines in their ability to induce IFN-γ production. This shows that IFN-γ

production was specific for ICD. Splenocytes collected from all groups did not produce IFN-γ upon *in vitro* stimulation with *r*HSP110. Mice that immunized with ICD only did not show IFN-γ production upon stimulation with the antigen.

Vaccination with the HSP110-ICD complex induces both CD8⁻ and CD4⁻ T cell-mediated immune responses. To identify which cell populations were involved in the antigen-specific IFN-γ production, *in vivo* lymphocyte subset depletion was performed with injections of the mAb 2.43 or GK1.5 to deplete $CD8^-$ or $CD4^-$ T cells, respectively. A group of animals were also depleted from both $CD8^-$ and $CD4^-$ T cells. Figure 3 shows that all animals vaccinated with the HSP110-ICD complex and depleted from the $CD8^-$ or $CD4^-$ T cells showed IFN-γ production upon *in vitro* stimulation with the antigen. Animals depleted from both $CD8^-$ and $CD4^-$ T cells did not show any IFN-γ production upon either ICD or Con A stimulation *in vitro*. There was also no significant difference between the $CD8^-$-depleted cells and $CD4^-$-depleted cells to produce antigen-specific IFN-γ *in vitro* ($p = 0.95$).

To further explore whether activation of $CD4^-$ T cells may promote activation of $CD8^-$ T cells, we carried out $CD4^-$ T cell depletion in the HSP110-ICD immunized animals one week after the booster. Although frequency of IFN-γ producing cells was slightly higher in these animals than that in animals depleted from $CD4^-$ T cells prior to vaccination, this difference was not statistically significant ($p \geq 0.16$).

Vaccination with the HSP110-ICD complex induces both IgG1 and IgG2a antibody responses against the ICD. It has been reported that non-covalent

binding of HSPs with a peptide could elicit a potent T cell responses to the bound peptide whereas the covalent binding complexes elicit the potent antibody responses (25, 26). Therefore, we decided to examine whether *in vitro* loading of HSP110 with a large tumor antigen, ICD, in a form of non-covalent complex may be able to elicit antibody responses in addition to cell-mediated immunity. We collected blood from animals that were utilized to monitor cell-mediated immunity by ELISPOT assay. Sera were prepared and tested for antigen specific antibody responses by ELISA. Using HRP-labeled anti-mouse isotype specific antibodies, IgG1 or IgG2a, we identified that both IgG1 and IgG2a Abs were elevated remarkably in the immunized animals (Figure 4a). Both IgG1 and IgG2a Ab levels were significantly higher in the HSP110-ICD immunized animals than those in the ICD immunized animals 14 days after immunization ($p \leq 0.0001$). However, IgG2a Ab reached the same levels in the two groups on day 28. The IgG1 was the major antibody, which stayed significantly higher in the HSP110-ICD immunized animals than in the ICD-immunized animals 28 days after immunization ($p \leq 0.0001$). Western blot analysis of the pooled immune sera collected from the HSP110-ICD immunized animals revealed specificity of the Ab for the ICD (Figure 4b, lane 1). Mouse anti-human ICD Ab (1:10000) was used as a control to stain the ICD (Figure 4b, lane 2). No anti-HSP110 antibody was detected before or after immunization.

Discussion

It was recognized approximately twenty years ago that there are only a few major HSPs in mammalian cells. One of these, HSP110, has only recently been cloned and only a few recent studies of its properties have appeared (27, 28). It has been found that HSP110 and its mammalian and non-mammalian relatives are distantly related to HSP70, but do not fall into the previously defined HSP70 "family" (27 - 29). Indeed HSP110 is representative of a family of heat shock proteins conserved from *S. cerevisiae* and *S. pombe* to man (28). Since HSP110 exists in parallel with HSP70 in the cytoplasm of (apparently) all eucaryotic cells, it is expected that HSP110 would carry out functions not performed by members of the HSP70 family. Initial characterization of the chaperoning properties of HSP110 demonstrate that it indeed exhibits major functional differences when compared to HSP70. While HSP70 avidly bind ATP, HSP110 does not. Secondly, in protein binding studies it has been found that HSP110 is significantly more efficient (i.e. approximately four fold more efficient) compared to HSP70 in forming natural chaperone complexes with denatured reporter proteins (3, 4). Surprisingly HSP110 complexes with reporter proteins and totally inhibits their heat induced aggregation at a 1:1 molar ratio.

This unexpected protein binding property of HSP110 is the basis of a new approach for the development of protein vaccines, which uses the binding of the protein antigen to HSP110 in a natural chaperone complex by heat shock. The protein antigen used here was ICD, which is a 84 kDa protein. One advantage of the Her-2/neu antigen is its involvement in the malignant phenotype of the tumor.

Therefore, in the case of tumor escape by antigen loss due to the treatment, it would still be beneficial to patients since HER-2/neu negative cancers are less aggressive than those that overexpress the neu protein and are associated with a more favorable prognosis (19).

As with previous studies using reporter proteins, HSP110 is again found to efficiently bind ICD at approximately a 1:1 molar ratio as seen in Figure 1. This strong protein binding capacity of HSP110 may be a typical and unique property of this stress protein. Immunization with this heat shock HSP110-ICD complex was found to be as potent as adding CFA to the ICD in eliciting specific IFN-γ production in immunized animals. On the other hand, neither naïve nor ICD-immunized animals showed a IFN-γ production upon *in vitro* stimulation with the ICD. Importantly, mice immunized with HSP110 did not show any IFN-γ production upon *in vitro* stimulation with the HSP110, indicating that this heat shock protein, as a self-protein, did not elicit an autoimmune response.

The ability of HSP110 to chaperone and present the ICD of HER-2/neu to the immune system and the strong response indicates that ICD is processed via an intracellular pathway, which requires degradation of ICD in antigen presenting cells (APCs) into a repertoire of antigenic peptides. This would facilitate the presentation of both $CD8^+$ as well as $CD4^+$ T cell epitopes from ICD by APCs since immunization with the HSP110-ICD complex was able to induce both $CD8^+$ and $CD4^+$ T cells to produce IFN-γ. Depletion studies showed that NK cells were not involved in the antigen-specific IFN-γ production since mice depleted of both $CD8^+$ and $CD4^+$ T cells did not produce IFN-γ. Elevation of these T cell subsets

were comparable and also antigen specific, but not due to alteration in the percent of T cell subsets following depletion. Our finding is consistent with previous studies showing that HSPs are able to route exogenous antigens into an endogenous presentation pathway for presentation by MHC class I molecules (30).

Depletion studies also demonstrated that stimulation of the $CD8^-$ T cells did not require help of $CD4^+$ T cells. This finding is consistent with previous studies showing that depletion of $CD4^+$ T cells in the priming phase did not abrogate the immunity elicited by gp96 (10, 31). Udono et al. (31) also showed that depletion of macrophages by treatment of mice with carrageenan during the priming phase resulted in loss of gp96-elicited immunity. One explanation for this phenomenon is that HSPs may replace $CD4^-$ T cells help to convert APCs into the cells that are fully competent to prime $CD8^+$ T cells (32). These findings indicate the central role that HSP-APC may play in activation of $CD8^-$ T cells via expression of CD40 molecule, which may interact with CD40 ligand and provide help for $CD8^+$ T cell activation. This pathway does not necessarily require activation of $CD4^+$ T cells for $CD8^+$ T cell priming. It has been shown that HSP-APCs interaction leads to activation of APCs, and induces proinflammatory cytokines secretion by activated DCs (10 - 12, 33).

Evaluation of the ICD-specific antibody responses in the immunized animals revealed that the HSP110-ICD complex could elicit both T_h1 and T_h2 cells as evaluated by production of IgG2a and IgG1 antibodies, respectively. This finding was consistent with the results obtained from the ELISPOT assay showing

that HSP110-ICD complex could provide the immune system with the $CD4^+$ T cell epitopes. Earlier and more vigorous anti-ICD antibody responses in the HSP110-ICD immunized animals than in the ICD-immunized animals may be due to the chaperon activity of HSP110 to facilitate antibody responses by a better presentation of the antigen through MHC class II molecules and thereby to provide help for B-cells through activation of $CD4^+$ T cells. Western blot analysis of the immune sera revealed the specificity of the antibody for ICD. Elevation of IgG Ab isotype against ICD is important since Herceptin, an anti-HER-2/neu antibody being used to treat breast cancer patients overexpressing HER-2/neu, is also of IgG isotype (34, 35).

While this HSP110-protein vaccine lacks some of the polyvalent benefits of the tumor-derived HSPs, which presumably carries a spectrum of unknown peptides, it also offers important benefits: 1) Since HSP110 is able to efficiently bind large proteins at approximately an equivalent molar ratio, a highly concentrated vaccine would be presented to the immune system compared to a tumor derived HSP/GRP where only a very small fraction of the HSP/GRP would be expected to carry antigenic epitopes. This vaccine would include numerous peptide epitopes (a single copy of each represented in each full-length protein) bound to every HSP110. Thus, such a preparation would not only be "partially polyvalent" as well as being targeted against a specific tumor protein antigen but may also provide both CD4 and CD8 antigenic epitopes. The vaccine would also circumvent HLA restriction since a large reservoir of potential peptides would be available. 2) Such a recombinant protein vaccine would not be an individual

specific vaccine, as are the tumor-derived HSP vaccines (36), but could be applied to any patient with a tumor expressing that tumor antigen. Further, if an antigenic protein is shared among several tumors, the HSP110-protein complex could well be applied to all cancers expressing that protein. For example, in the case of HER-2/neu, HSP110-her-2 vaccines would be applicable to the treatment of numerous patients with breast cancer as well as ovarian, prostate, lung and colon cancers. 3) Lastly, preparation of such protein vaccines would be much less labor intensive than purification of tumor-derived HSP from a surgical specimen. Indeed, a surgical specimen is not required to prepare such a vaccine. The vaccine would also be available in unlimited quantity and a composite vaccine using more than a single protein antigen (e.g. gp100, MART1, etc for melanoma) could be easily prepared.

HSPs have been proposed to be "danger signals" which alarm the immune system of the presence of tumor or damaged tissues (37). This hypothesis envisions the release of HSPs, carrying peptides, from necrotic or damaged cells and their uptake by APCs, thereby providing the immune system with both a "signal 1" (peptide presentation) and a "signal 2" (upregulation of co-stimulatory molecules). Indeed, several studies indicated that HSPs are able to activate APCs (11, 12, 33). We have also recently observed that HSP110 could induce maturation of DCs, up-regulate MHC class II surface expression, and up-regulate the expression of pro-inflammatory cytokines tumor necrosis factor-alpha (TNF-α) and IL-6 in mouse DCs. However, in addition to peptides, it has long been understood that HSPs/GRPs are also essential to protein folding and assembly

events within cells and also bind damaged and mutant proteins *in vivo* (38-39). It is not clear what fraction of an HSP/GRP family (e.g. HSP70 or HSP110) is actually complexed with peptides relative to that fraction complexed with full-length proteins. Thus, the release of HSP as a putative danger signal would also encompass the presentation of HSP-protein complexes, as studied here, in addition to peptide complexes.

Aluminium adjuvants, together with calcium phosphate and a squalene formulation are the only adjuvants approved for human vaccine use. These approved adjuvants are not effective in stimulating cell-mediated immunity but rather stimulate a good Ab response (40). We have shown here that HSP110 is a safe mammalian adjuvant in molecular targeting of a well-known tumor antigen, ICD of HER-2/neu, being able to activate both arms of the immune system. In addition, neither CTL nor antibody responses was found against HSP110 itself. This property of HSP110 is particularly interesting in light of the paucity of adjuvants judged to be effective and safe for human use. We have begun preliminary studies in HER-2/neu transgenic mouse using HSP110-ICD complex as an immunogen. Our initial results demonstrate the possibility that HSP110-ICD complex may inhibit spontaneous breast tumor formation in this transgenic animal model.

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	T cell subsets	
Animals	CD4	CD8
Wild type	22%	14%
CD4 depletion	<2%	15%
CD8 depletion	20%	<2%
CD4/CD8 depletion	<2%	<2%

Table 1. Flow cytometric analysis of the persence of T cell subsets following *in vivo* antibody depletion. Depletion of $CD4^+$ or $CD8^+$ T cells was accomplished by i.p. injection of GK1.5 or 2.43 antibodies (250 ug), respectively. The $CD4^+/CD8^+$ T cells were also depleted by i.p. injection of both GK1.5 and 2.43 antibodies (250 ug of each). The depletion was performed on 3 subsequent days prior to immunization, and followed by twice a week injections. Spleen cells were stained for $CD4^+$ or $CD8^+$ T cells using FITC-labeled rat anti-mouse IgG and subjected to flow cytometry showing that almost 98% of the lymphocyte subsets were depleted without any affect on other T cell subsets.

Figure Legends:

Figure 1. Formation of a non-covalent HSP110-ICD binding complex *in vitro*. Recombinant HSP110 (*r*HSP110) was incubated with recombinant intracellular domain of human HER-2/neu (*r*ICD) at 43°C for 30 min followed by further incubation at 37°C for 1h in PBS. Different molar ratios of HSP110:ICD (1:4, 1:1.or 1:0.25) were used. The complexes were then immunoprecipitated by anti-HSP110 antiserum (1:200) or an unrelated Ab (1:100) using protein A sepharose and incubation at room temperature for 1 h while rotating. The complexes were washed 8 times in a washing buffer at 4°C and subjected to SDS-PAGE (10%). Gells were either stained with Gell-blue or subjected to western blot analysis using HRP-conjugated sheep anti-mouse IgG (1:5000) followed by 1 min incubation of the nitrocellulose membrane with Chemiluminescence reagent and exposure to Kodak autoradiography film for 20 sec.

Figure 2. Frequency of IFN-γ producing T cells following immunization with different vaccine formulations. Five A2/Kb transgenic mice/group were immunized with 25 μg of the HSP110-ICD (i.p.), or CFA/IFA-ICD (s.c.) complexes. Animals were boosted after 2 weeks with the HSP110-ICD or IFA-ICD and sacrificed 2 weeks thereafter. Control groups were injected i.p. with 25 μg of the ICD, HSP110, or left non-immunized. The splenocytes (10^7 cells/ml) were cultured *in vitro* with Con A (5 μg/ml), or ICD (10-20 μg/ml) overnight and IFN-γ secretion was detected in an ELISPOT assay using biotinylated anti- IFN-γ antibody and BCIP/NBT substrate. Control wells were also pulsed with 20 ug/ml of HSP110. Data are presented after subtraction of background IFN-γ secretion

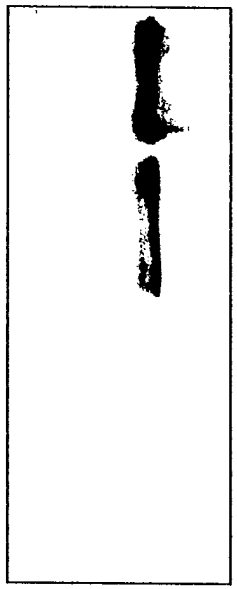
upon *in vitro* stimulation with a control recombinant protein made in *E. Coli* (10-20 ug/ml).

Figure 3. Frequency of IFN-γ producing $CD8^+$ and $CD4^+$ T cells following immunization with the HSP110-ICD complex. Five A2/Kb transgenic mice/group were depleted from $CD8^+$, $CD4^+$ or $CD8^+/CD4^+$ T cells on three sequential days before immunization followed by twice a week i.p. injections (250 ug) using mAbs 2.43 and/or GK1.5. Animals were also depleted from $CD4^+$ T cells one week after the booster to determine whether $CD4^+$ T cells helps to generate stronger antigen-specific CTL responses. They were primed i.p. with the HSP110-ICD (25 ug/mouse) and boosted 2 weeks later. The splenocytes (10^7 cells/ml) were cultured *in vitro* with Con A (5 ug/ml) or ICD (10-20 ug/ml) overnight and IFN-γ secretion was detected in an ELISPOT assay using biotinylated anti- IFN-γ antibody and BCIP/NBT substrate.

Figure 4. Isotype-specific antibody responses against the ICD following immunization with the HSP110-ICD complex or ICD. Five A2/Kb transgenic mice/group were immunized i.p. with 25 ug of the HSP110-ICD complex or ICD alone. Animals were boosted 2 weeks later and their blood samples were collected on days 0, 14 and 28 prior to each injection. The sera were prepared and subjected to ELISA using HRP-labeled anti-mouse IgG1, or IgG2a at dilutions recommended by manufacturers. The reactions were developed by adding TMB Microwell substrate, stopping the reaction by 2 M H_2SO_4 and reading at 450 nm (a). Sera were also collected and pooled from the HSP110-ICD immunized animals and utilized to stain the ICD in a western blot (b). Lane 1 shows specific

staining of the ICD with the immune serum (1:2000) and lane 2 shows the specific staining with mouse anti-human ICD antibody (1:10000).

Western



Anti-HSP110 Ab

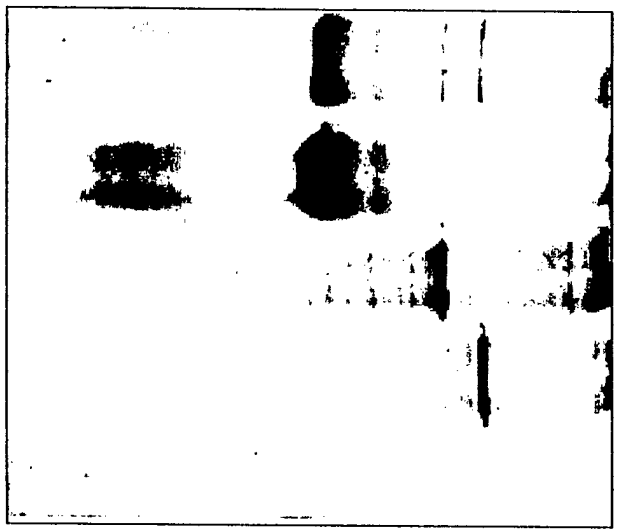
Unrelated Ab

No Ab

Anti-HSP110 Ab

Unrelated Ab

No Ab



HSP110-ICD

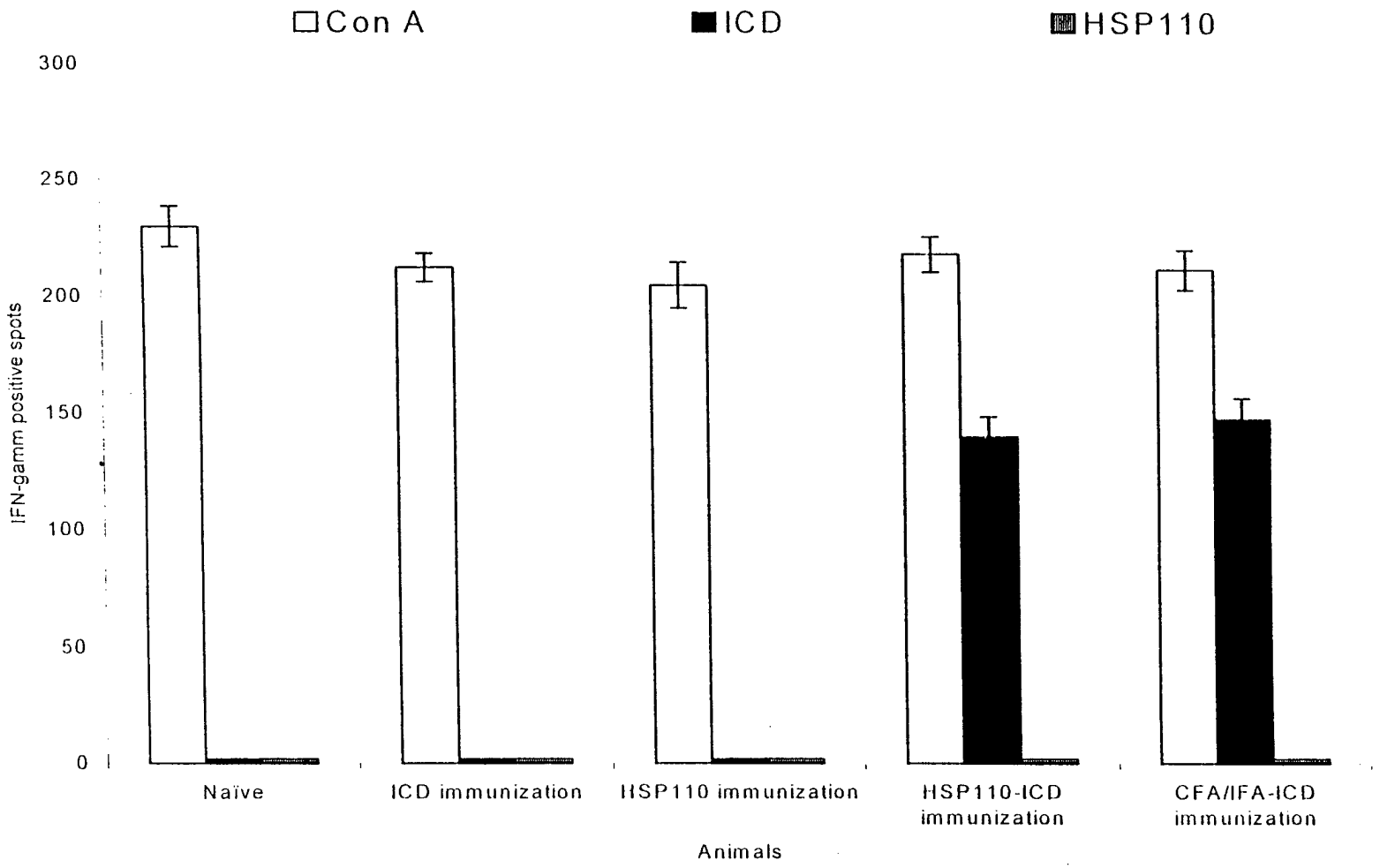
HSP110-ICD

ICD

HSP110

Protein A

Gel


Con A
ICD
HSP110
300
250
200
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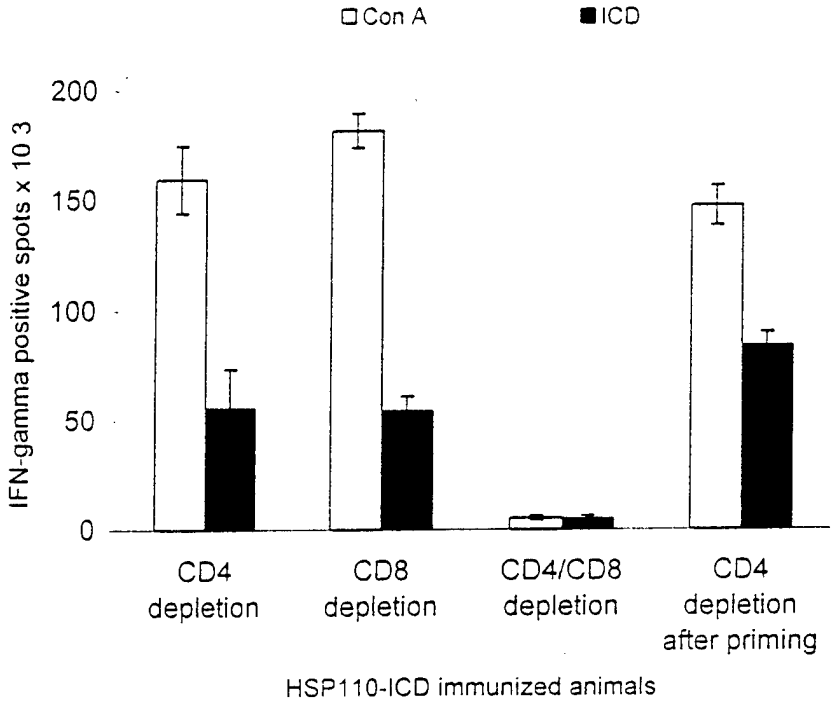
0
IFN-gamm positive spots
Naïve
ICD immunization
HSP110 immunization
HSP110-ICD immunization
CFA/IFA-ICD immunization
Animals


Con A
ICD
IFN-gamma positive spots x 10 3
200
150
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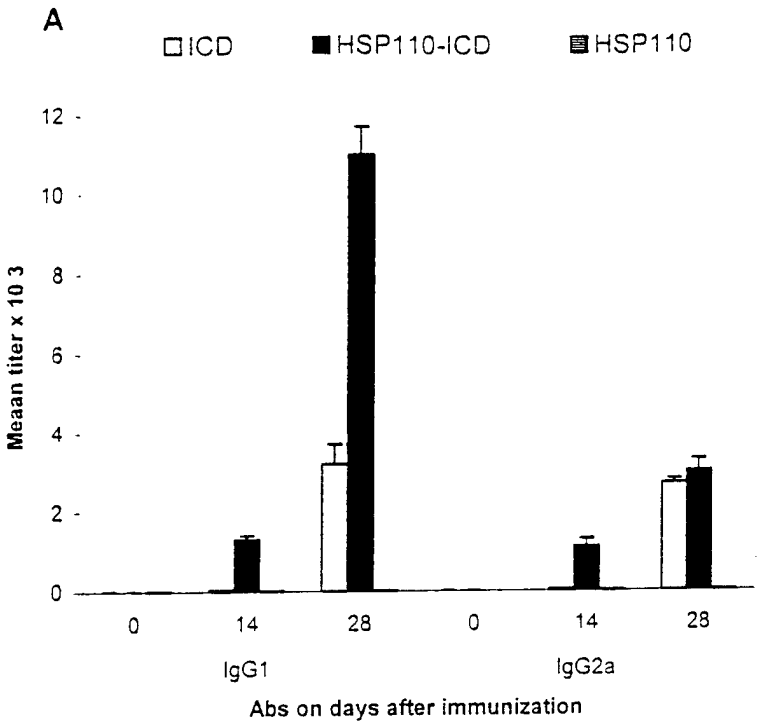
50
0
CD4 depletion
CD8 depletion
CD4/CD8 depletion
CD4 depletion after priming
HSP110-ICD immunized animals


A
ICD
HSP110-ICD
HSP110
12
10
8
6
4
2
0
Meaan titer x 10 3
0
14
28
0
14
28
IgG1
IgG2a
Abs on days after immunization

Personnel involved in the research project

Xiangyang Wang 7/1/98-6/30/2001

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