

Immunoprotective Activities of Multiple Chaperone Proteins Isolated from Murine B-Cell Leukemia/Lymphoma¹

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ABSTRACT

Although the use of tumor-derived heat shock/chaperone proteins (HSPs) as anticancer vaccines is gaining wider study and acceptance, there have thus far been no reports concerning chaperone antitumor activities against disseminated hematological malignancies. We have devised an efficient and effective method for purification of the chaperone proteins grp94/gp96, HSP90, HSP70, and calreticulin from harvested A20 murine leukemia/lymphoma tumor material. We have demonstrated that these purified proteins, when used as vaccines, can induce potent and specific immunity against a lethal tumor challenge. Individual chaperone proteins were differentially effective in their abilities to provide immune protection. The increase in survival generated by the most effective chaperone vaccine, HSP70, resulted from at least a 2-log reduction in tumor burden. Syngeneic granulocyte macrophage colony-stimulating factor producing fibroblasts were injected at the site of vaccination in an attempt to augment the immune response. Surprisingly, localized granulocyte macrophage colony-stimulating factor production inhibited the protective effects of chaperone vaccination. These studies provide evidence that chaperone proteins can be isolated from B-cell tumors and used effectively to immunize against disseminated lymphoid malignancies.

INTRODUCTION

The activity of purified tumor-derived chaperone proteins as adjuvants in cancer immunotherapy has become increasingly well-documented (1–5). It is clear from these reports that immunization of mice with chaperones purified from a given tumor can induce specific immunoprotection against a subsequent challenge with the same tumor, whereas chaperones purified from normal murine tissues provide no such protection.

Received 6/16/99; revised 12/7/99; accepted 12/8/99.

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¹ Supported in part by the American Cancer Society Grant IM-785, the Arizona Disease Control Research Commission, the W. M. Keck Foundation, the Enid and Mel Zuckerman Fund, the Arizona Elks Program in Transplantation Research, and the Michael Landon Fund.

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The data indicate that the chaperones are not immunogenic *per se*, but rather serve as carriers of tumor-derived peptides (6–8). The molecular and cellular mechanisms behind this immunoprotection are not clear, but it appears that chaperone-peptide complexes are taken up by host professional APCs³ and are directed toward the endogenous (MHC class I) presentation pathway (3, 9, 10). Presentation of the (formerly chaperoned) peptides to T cells may lead to the generation of tumor-specific cytotoxic T cells.

A variety of tumor types growing as s.c. masses have been reported to be responsive to tumor-derived chaperone vaccination (1). However, none of these studies have addressed the effectiveness of chaperone vaccines in generating antitumor immunity against disseminated hematological malignancies such as leukemias or lymphomas. This report describes our attempts to elicit antitumor immune responses via chaperones purified from the spontaneously derived murine A20 B-cell leukemia/lymphoma (11). When A20 cells are injected i.v. into BALB/c mice, they induce a disseminated disease characterized by infiltration of lymph nodes, liver, and spleen and the presence of malignant cells in bone marrow and peripheral blood. This leukemia is radioresistant, with even myeloablative doses of irradiation followed by syngeneic bone marrow transplantation failing to cure A20 bearing mice (data not shown; Ref. 12). A20 is poorly immunogenic; vaccination with irradiated wild-type tumor cells confers little protection to subsequent tumor challenge. Systemic T-cell immunity against A20 may be generated, however, following immunization with genetically modified tumor cells engineered to secrete GM-CSF and other cytokines (13).

Herein we report a method for sequential purification of the major immunologically active chaperone proteins HSP70, HSP90, grp94/gp96, and calreticulin from a single A20 tumor sample and demonstrate the relative efficacy of these protein preparations in generating specific antitumor responses in this aggressive murine leukemia model. Syngeneic GM-CSF-secreting fibroblasts were injected at the immunization site in an attempt to stimulate antigen uptake and presentation by professional APCs. Interestingly, production of GM-CSF at the vaccination site by transduced 3T3 fibroblasts actually abrogated the protective effect.

MATERIALS AND METHODS

Mice. Six-to-10-week-old female BALB/c (H-2^d) mice (Jackson Laboratories, Bar Harbor, ME) were used for the experiments. The animals were housed in a specific pathogen-

³ The abbreviations used are: APC, antigen-presenting cell; GM-CSF, granulocyte macrophage colony-stimulating factor; ConA, concanavalin A; HSP, heat shock/chaperone protein; ns, not significant.

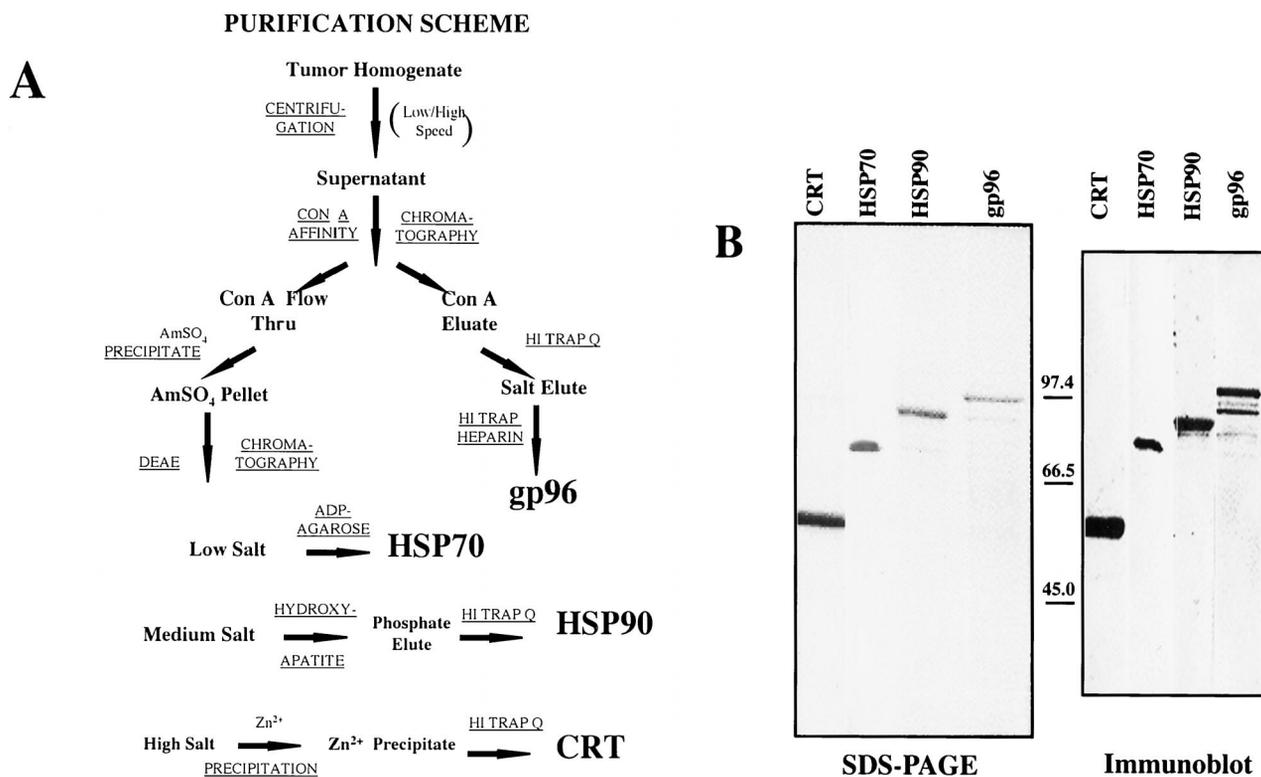


Fig. 1 Chaperone protein purification scheme and resulting purified proteins. A, A20-derived chaperone proteins (calreticulin, HSP70, HSP90, and grp94/gp96) were purified as described in "Materials and Methods" following the outline. B, SDS-PAGE (left panel) and confirmatory Western blot analyses (right panel) of the final purified materials are shown.

free facility and cared for according to the University of Arizona Institutional Animal Care and Use Committee guidelines.

Tumor Cell Lines. A20 is a B-cell leukemia/lymphoma that arose spontaneously in an old (>15 months) BALB/c mouse (11). BDL-2 is a murine B-cell lymphoma line (H-2^d) established by the long-term *in vitro* culture of murine lymphoid tissue and was kindly provided by Dr. Jonathan Braun, University of California, Los Angeles School of Medicine (Los Angeles, CA; Ref. 14). The 3T3 fibroblasts were purchased from American Type Culture Collection (Manassas, VA). All tissue culture reagents were from Life Technologies, Inc. (Gaithersburg, MD). The cell lines were cultured at 37°C under 5% CO₂ in air using RPMI media containing 10% heat-inactivated FCS and supplemented with 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 0.025 µg/ml amphotericin B, 0.5× MEM nonessential amino acids, 1 mM sodium pyruvate, and 50 µM 2-mercaptoethanol.

Transduction of 3T3 Cells. GM-CSF gene transfer was performed using the MFG-GM-CSF retroviral vector (kindly provided by Drew Pardoll, Johns Hopkins University, Baltimore, MD), which is a Moloney murine leukemia virus-based vector that does not contain selection genes (15, 16). Supernatants from the retroviral producer cell line, CRIP, were collected and used to transduce 3T3 cells in the presence of 8 µg/ml polybrene for 4 h and repeated after 24 h.

GM-CSF Bioassay. The GM-CSF-dependent cell line FDCP2-1D was used to determine the amount of GM-CSF

bioactivity present in the supernatants of transduce 3T3 cells (17). Samples were added in triplicate to 96-well flat-bottomed plates (Costar, Cambridge, MA) and serially diluted 3-fold. FDCP2-1D cells (5000 cells/well), washed free of GM-CSF, were then added to the plates. The microtiter plates were incubated for 24 h at 37°C and 5% CO₂, then pulsed for 24 h with 2 µCi [³H]thymidine (Dupont New England Nuclear, Boston, MA). The cells were then harvested using a Packard plate harvester, and the radioactivity was measured on a Packard β counter. The GM-CSF concentrations were calculated by comparing to a known GM-CSF standard (PeproTech, Inc., Rocky Hill, NJ).

Purification of Tumor-derived Chaperone Proteins.

A20 cells were prepared for injection by washing and resuspending them in HBSS. Tumors >1 cm in diameter were surgically harvested after euthanizing the mice. Resected tumor tissue was homogenized in a hypotonic buffer plus protease inhibitors. Fig. 1 schematically outlines the purification and may prove helpful in following the steps described from this point. Following a low-speed centrifugation (12,000 × g; 30 min; 4°C) to remove debris, a high-speed supernatant (100,000 × g; 90 min; 4°C) was obtained. Albumins were removed via a Cibacron Blue 3GA (Sigma, St. Louis, MO) resin. The Ciba Blue flow-through material was chromatographed over ConA-Sepharose (Sigma, St. Louis, MO); the lectin column was eluted using 10% α-D-methylmannose hypotonic buffer. Elution fractions containing grp94/gp96 (as determined by SDS-PAGE and

Western blotting) were pooled and dialyzed into a Tris-acetate/NaCl buffer. Grp94/gp96 was further purified on a Hi Trap Q strong anion exchange column (Pharmacia Biotech, Piscataway, NJ), which was eluted in NaCl. Fractions containing grp94/gp96 were finally purified by Hi Trap Heparin chromatography (Pharmacia Biotech) treated as an anion exchange resin and eluted in a NaCl gradient. Fractions containing the purified gp96 were identified by SDS-PAGE and Western blotting. Flow-through and wash fractions from the ConA column were pooled and differentially precipitated with ammonium sulfate; precipitated proteins were pelleted by centrifugation, resuspended in Tris-Acetate/NaCl, and dialyzed. Proteins from the 70% ammonium sulfate cut were separated on a DEAE-Sephacel resin (Pharmacia Biotech). Fractionated proteins were analyzed by SDS-PAGE and Western blotting. Fractions enriched for proteins of interest were pooled and concentrated. HSP70 was purified from DEAE-pooled fractions via ADP-agarose affinity chromatography (N-6 linkage, Sigma, St. Louis, MO) essentially as described previously (18). HSP70 was identified and purity was assessed by SDS-PAGE and Western blotting. HSP90 was further purified from DEAE elution fractions by hydroxyapatite chromatography. Proteins were dialyzed into phosphate buffer, concentrated, loaded onto the column, and eluted in a step gradient of 100 mM, 200 mM, 300 mM, and 400 mM sodium phosphate (pH 7.0). Fractions containing HSP90 were identified via Western blotting; positive fractions were pooled, and the buffer exchanged to Tris-acetate/NaCl. The pooled material was chromatographed on a Hi Trap Q column and eluted in NaCl. Fractions containing purified HSP90 were identified by SDS-PAGE and Western blotting. Calreticulin was purified from the 85% ammonium sulfate precipitate and high salt DEAE elutions by dialysis into 10 mM 3-[*N*-morpholino]propanesulfonic acid, 100 mM NaCl (pH 7.1), and precipitation with 10 mM ZnCl₂. The precipitate was recovered by centrifugation, resuspended and dialyzed into Tris-acetate/NaCl, and chromatographed on Hi Trap Q, eluted in NaCl. Fractions containing purified calreticulin were identified by SDS-PAGE and Western blotting.

Analytical Methods. SDS-PAGE was performed according to Laemmli (19). Gels were stained in Coomassie Blue. Western blotting was performed as described by Towbin *et al.* (20), beginning with transfer using an Idea Scientific (Madison, WI) Genie electroblotter. Blots were then processed via standard protocols. Primary antibodies (StressGen Biotechnologies, Victoria, British Columbia, Canada) included anti-Hsc70/anti-HSP70 (SPA-820); anti-grp94/anti-gp96 (SPA-850); anti-HSP90 (SPA-830); and anti-calreticulin (SPA-600). Secondary antibodies (goat anti-mouse, goat anti-rat, and goat anti-rabbit alkaline phosphatase conjugate) were from Chemicon International (Temecula, CA). Immunoreactive signals were detected by color deposition of the alkaline phosphatase substrates nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim, Indianapolis, IN).

In Vivo Immunoprotection Experiments. BALB/c mice were immunized s.c. in the flank with A20-derived HSP70, grp94/gp96, calreticulin, or HSP90 in 0.2 ml PBS. Control mice received PBS only. Mice were vaccinated twice at weekly intervals (*i.e.*, day -14 and day -7). Some experiments involved prevaccination using 3T3 cells transduced (or not) with the gene encoding murine GM-CSF. Fibroblasts were then in-

jected s.c. on day -15 and day -8, whereas chaperone vaccinations were given at the same site on day -14 and day -7. Seven days after the last immunization (day 0), mice were challenged via tail vein injection with 1×10^6 viable A20 or BDL-2 B-cell leukemia/lymphoma cells using a 27-gauge needle. Both A20 and BDL-2 cells used for challenge were harvested from tumor-bearing mice 2-3 h before injection. s.c. tumors were removed under sterile conditions, placed in PBS, finely minced with a scalpel, gently crushed with the piston of a syringe, gathered in a pipette, filtered through a 100- μ m Nytex mesh, washed twice in PBS, and resuspended at a concentration of 5×10^6 /ml.

Statistical Analysis. The Kaplan-Meier product-limit method was used to plot the survival of mice inoculated with the B-cell leukemias and the log-rank statistic to test differences between groups (21, 22).

RESULTS

Purification of the Chaperone Proteins HSP70, HSP90, grp94/gp96, and Calreticulin. We have devised a purification scheme to isolate the major immunologically active chaperone proteins HSP70, HSP90, grp94/gp96, and calreticulin from a single tumor source. Such a scheme is valuable because availability of adequate amounts of tumor is often a limiting factor in the preparation of vaccination reagents. Therefore, purification of multiple immunogenic proteins from the single source is critical. Moreover, it is possible that each of the chaperone proteins escorts a distinct set of peptides, implying that the full repertoire of potentially antigenic material can only be represented if multiple chaperone family members are isolated from the tumor.

The protein purification scheme outlined herein represents a modification of previous techniques used to purify chaperones. These methods have been revised and refined to allow stepwise progression through the protocol with high efficiency and recovery. Additionally, the procedure employs relatively inexpensive separations, such as centrifugation and precipitation, as well as "low tech" conventional and affinity chromatographies. Fig. 1A outlines the purification scheme, and Fig. 1B shows SDS-PAGE analysis (*left*) and Western blots (*right*) of the resulting final materials. Each of the purified proteins was subjected to Western blotting with antibodies against each of the other chaperones, and no cross-contamination was detected (data not shown).

Concerns about the use of ConA chromatography have arisen previously (23). The assertion is that low levels of ConA contamination insidiously present in the final purified components may be the actual source of a generalized immune response. We do not believe that such a scenario is true for several reasons: (*a*) The chaperone proteins provide specific immunity only against the tumor that served as the chaperone source (see Table 1) and not against a different syngeneic B-cell malignancy (BDL-2); (*b*) We have purified and identified by NH₂-terminal amino acid sequencing a protein that copurifies with calreticulin until separation in the final step (including passage through a ConA column). This protein, identified as mouse serum albumin, is ineffective as a vaccine against A20 tumor challenge (data not shown); (*c*) We have also purified A20 HSP70 to a

Table 1 Immunization with A20-derived chaperone proteins protects mice against A20 but not against BDL-2 B-cell leukemia/lymphoma challenge^a

A20-derived chaperone vaccination	Tumor challenge	Survival (%)	Median survival time (days)	P versus PBS
None (PBS)	A20	0	26.0	
Calreticulin	A20	0	29.0	0.002
grp94/gp96	A20	0	31.0	0.003
HSP70	A20	8	35.0	0.001
None (PBS)	BDL-2	0	16.0	
Calreticulin	BDL-2	0	18.5	ns
grp94/gp96	BDL-2	0	17.0	ns
HSP70	BDL-2	0	18.0	ns

^a Mice were immunized s.c. with 20 μ g of each chaperone protein (A20-derived) on days -14 and -7 and challenged i.v. with 10^6 viable A20 or BDL-2 lymphoma cells on day 0.

high degree using only centrifugation, ADP-agarose affinity chromatography, and anion exchange chromatography, thus bypassing the ConA column. In survival experiments, these preparations provide protection to mice from tumor challenge that is indistinguishable from A20 HSP70 purified by the longer protocol (*i.e.*, including the lectin chromatographic step). These observations lead us to conclude that ConA immune stimulation is not a factor in our assays.

Efficacy of the Various Tumor-derived Chaperone Proteins in Generating A20 Leukemia-specific Immunity. We have used the murine B-cell line A20 to establish a leukemia model. When 10^6 A20 cells are injected i.v., BALB/c mice die at a median time of 20–25 days, displaying lymphoblastic infiltration of spleen, liver, lymph nodes, and bone marrow. This model therefore provides the opportunity to study whether chaperone-based immune stimulation can be effective against a disseminated B-cell malignancy. Although tumor-derived HSP70, HSP90, grp94/gp96, and calreticulin have been shown to induce specific immunity against spontaneous solid tumors, chemically and UV-induced solid tumors and tumors transfected with foreign genes, such as β -galactosidase and chicken ovalbumin (23–29), there are no published data on the use of this strategy against hematological malignancies.

To determine whether or not the tumor-derived chaperone proteins purified by this protocol were effective in stimulating immune responses against A20 leukemia, syngeneic BALB/c mice were immunized with purified chaperone preparations (HSP70, HSP90, grp94/gp96, and calreticulin) from an A20 tumor source on days -14 and -7, followed by i.v. challenge with A20 on day 0.

Fig. 2A depicts Kaplan-Meier plots comparing the protective effect of different chaperone vaccinations. In these experiments, mice were given two 20- μ g doses of purified chaperones before tumor injection. HSP70, grp94/gp96, and calreticulin all provided significant improvement in survival over controls, with A20-derived HSP70 being the most effective chaperone protein followed by grp94/gp96 and calreticulin. A20-derived HSP90, given in 30- μ g doses, also provided substantial protection compared to PBS-treated controls (Fig. 2B).

In an attempt to quantify the effects of tumor-derived chaperone vaccinations in terms of reduction in tumor burden,

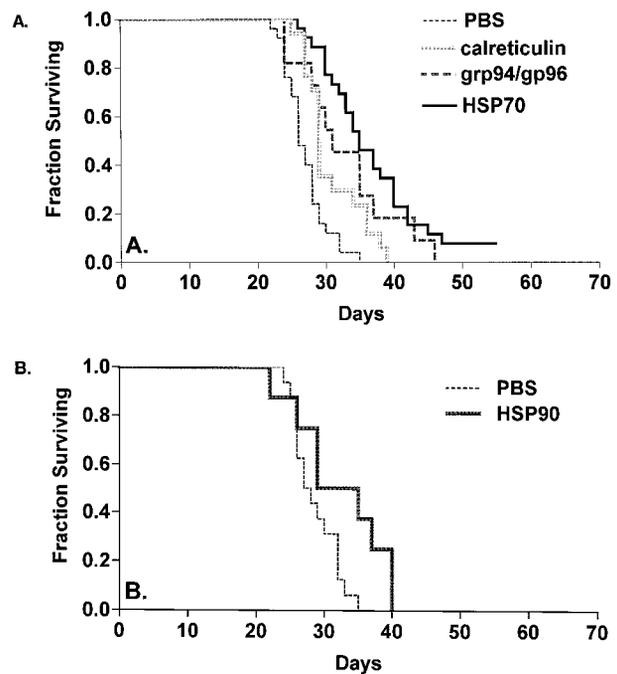


Fig. 2 Immunization with A20-derived chaperone proteins increases the survival of mice challenged with A20 B-cell leukemia. **A**, mice were immunized s.c. with 20 μ g of the listed chaperone protein or with PBS (controls) on days -14 and -7 and challenged i.v. with 10^6 viable A20 cells on day 0. Survival was thereafter monitored. Data represent pooled data from three experiments ($n = 11$ –26 mice/group). *P* versus control; calreticulin $P < 0.002$; grp94/gp96 $P < 0.003$; HSP70 $P < 0.001$. Survival differences between immunized groups were significant only for Hsp70 versus calreticulin $P < 0.002$. **B**, mice were immunized with 30 μ g of HSP90 on days -14 and -7 and challenged i.v. with 10^6 viable A20 cells on day 0. $P < 0.003$ versus control. Data represent pooled data from two experiments ($n = 8$ –16 mice/group).

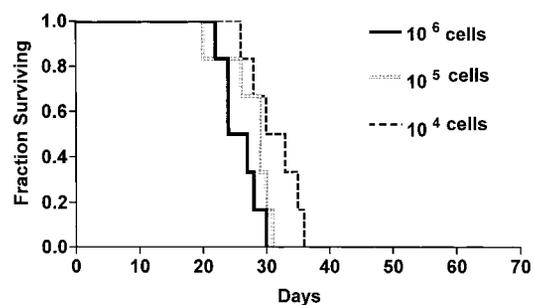


Fig. 3 A20 leukemia dose titration effect on survival. Groups of unimmunized mice were i.v. injected with 10^6 , 10^5 , or 10^4 viable A20 cells, and survival thereafter was monitored ($n = 6$ mice/group).

we generated survival curves for groups of mice that were given decreasing numbers of A20 cells (Fig. 3). Naïve mice were i.v. challenged with 10^6 , 10^5 , or 10^4 A20 cells. Of note, A20 cells derived from *in vivo*-propagated tumor cells are more tumorigenic than those cultured *in vitro* (data not shown; Ref. 30). Only *in vivo* grown B-cell leukemia/lymphoma cells were used in our studies. Based on the survival curves generated, A20-derived HSP70 vaccinations provide protection in terms of in-

creased survival equivalent to at least a 2-log reduction in tumor burden (Fig. 3). There is obvious clinical significance in the degree of immune-mediated cytotoxicity induced by chaperone protein vaccination. If used in the setting of minimal residual disease, such as following autologous stem cell transplantation, chaperone protein vaccination may increase leukemia/lymphoma cure rates without toxicity.

Tumor Specificity of the Immunizing Chaperones. To demonstrate the specificity of chaperone protein-stimulated immune response, mice were twice vaccinated with 20 μ g of either HSP70, grp94/gp96, or calreticulin isolated from A20 tumors (amounts that significantly prolonged survival against autologous tumor; Fig. 2A). However, on day 0, the vaccinated mice and untreated controls were i.v. challenged with the BDL-2 B-cell leukemia/lymphoma, which is syngeneic to BALB/c mice but antigenically unrelated to A20. As outlined in Table 1, A20-derived chaperone protein vaccinations did not generate significant protection against BDL-2. These data confirm that the immunity provided by A20-derived chaperone proteins is tumor-specific.

Localized GM-CSF Cytokine Production Inhibits Chaperone-induced Protection. Because tumor-derived chaperone protein vaccinations are capable of generating CTL responses against the tumor of origin, it is posited that professional APCs are involved in transiting antigenic peptides from the vaccinating chaperones into effective antigen presentation pathways. We sought to augment the immune response resulting from chaperone injections by providing a localized production of GM-CSF, which is known to stimulate dendritic cells and macrophages at the site of immunization (15, 31). 3T3 fibroblasts were transduced with the murine GM-CSF gene and were found to produce 3–5 ng/ml of the cytokine/ 10^6 cells/24 h (data not shown). On days –15 and –8, GM-CSF-transduced 3T3 fibroblasts or unmodified 3T3 cells were injected s.c. into a marked shaven site of each mouse. Twenty four h later (days –14 and –7), mice were immunized with chaperone preparations (20 μ g of either HSP70 or grp94/gp96) into the same site. i.v. A20 tumor challenge was given on day 0, and survival was monitored. Surprisingly, production of GM-CSF at the vaccination site abrogated the effects of chaperone immunization (Fig. 4, A and B). Similar inhibitory effects of GM-CSF were evident in the case of calreticulin vaccination (data not shown). As discussed later, this may be secondary to unopposed production of GM-CSF inhibiting T-cell responses by dysregulating APC maturation (32).

DISCUSSION

We have demonstrated the efficacy of tumor-derived chaperone protein vaccinations against a disseminated hematological malignancy. We have purified the chaperone proteins in a manner that allows us to efficiently obtain the four major chaperones known to generate tumor specific immunity, namely grp94/gp96, HSP90, HSP70, and calreticulin. In using these chaperone proteins as vaccines, our results indicate that these proteins are not comparable in their capacity to provide protection against a lethal tumor challenge. HSP70 immunizations offered the most potent antitumor effect. In fact, mice immunized with HSP70 and challenged with 10^6 A20 cells survived longer than naïve

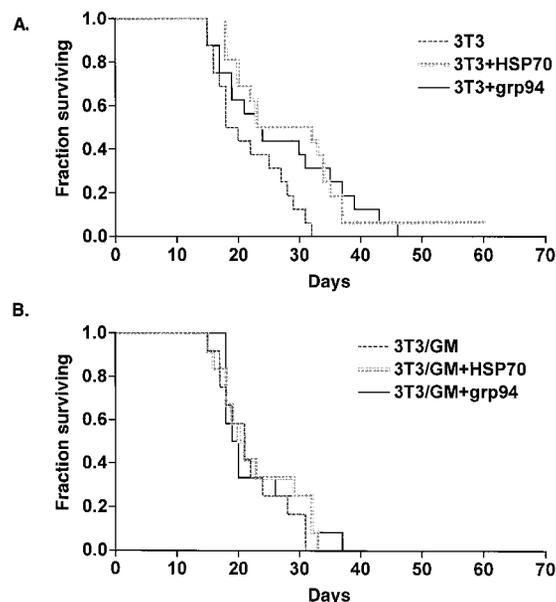


Fig. 4 Localized GM-CSF cytokine production inhibits chaperone-induced protection. Mice were injected with (A) 3T3 fibroblasts or (B) 3T3/GM fibroblasts transduced to produce GM-CSF (3–5 ng/ml/ 10^6 cells/24 h) on days –15 and –8. Twenty-four h later (days –14 and –7), they were vaccinated with 20 μ g of HSP70 or 20 μ g of grp94/gp96 into the same site and challenged i.v. with 10^6 viable A20 cells on day 0. A, *P*s versus 3T3 control; 3T3 + HSP70 < 0.004; 3T3 + grp94/gp96 < 0.05. B, *P*s versus 3T3/GM control; 3T3/GM + HSP70 = ns; 3T3/GM + grp94/gp96 = ns. Data represent pooled data from two experiments ($n = 12$ –16 mice/group).

mice challenged with 10^4 A20, indicating that HSP70 immunization results in at least a 2-log reduction in tumor cell burden. The protection provided by A20-derived chaperones was specific for A20 because A20-derived chaperones did not protect against a syngeneic but antigenically distinct B-cell leukemia/lymphoma, BDL-2.

Although the immunological activities of tumor-derived HSP70, grp94/gp96, and HSP90 family members have been known for some time, calreticulin is a newcomer to this field. Basu *et al.* (23) recently demonstrated that calreticulin purified from a methylcholanthrene-induced fibrosarcoma when used as a vaccine confers protection to subsequent s.c. tumor challenge. Moreover, Nair *et al.* (25) reported that dendritic cells pulsed with calreticulin isolated from B16 melanoma or ovalbumin-transfected EL-4 thymoma elicits tumor-specific CTL responses as assessed by *in vitro* cytotoxicity assays. These investigators ranked calreticulin as equivalent or even better than grp94/gp96 in the assays used. In contrast, in our hands, calreticulin was not as effective against A20 leukemia when compared to HSP70 and gp96/grp94. The number of subcellular localizations and proposed roles for calreticulin within (and outside) the cell have risen dramatically over the past few years (33–37). The discovery of calreticulin's involvement in antigen processing is quite recent, however (38–40). It is possible that calreticulin may be involved in this mechanism of peptide retention, and as such, it is a reasonable candidate for a chaperone of potentially antigenic peptides from a tumor source.

The molecular mechanisms of antigen capture, antigen presentation, and effector cell stimulation following exogenous administration of tumor-derived chaperone proteins are not clear. Suto and Srivastava (9) demonstrated the need for APCs in chaperone-based immune responses in tumor rejection, and *in vitro* studies using chaperone-pulsed APCs further elucidated the role of APCs (3, 25). Actual uptake of an exogenously delivered chaperone protein by APCs was observed recently (2). Together, these studies imply the presence of receptors for chaperones, at least on the surfaces of APCs. Competition experiments indicate that the chaperone proteins HSP70 and grp94/gp96 may have different receptors (2).

In view of the importance of professional APCs in generating tumor-derived chaperone-based immune responses, we introduced syngeneic GM-CSF-producing fibroblasts at the vaccination site in an attempt to augment the immune response by stimulation and local and expansion of APCs before injection of chaperone protein preparations. Surprisingly, the protective effect of tumor-derived chaperone vaccinations was abrogated under these conditions. Bronte *et al.* (32) have reported inhibition of CD8⁺ T-cell responses in tumor microenvironments where the tumor secretes GM-CSF. The inhibition appears to occur due to failure of certain APC progenitors to fully mature in the presence of GM-CSF alone. An inhibitory population of cells is present (CD11b⁺/Gr-1⁺) causing an impairment of CD8⁺ T-cell function and even inducing apoptotic T-cell death (32). This inhibition could be overcome with the administration of IL-4, indicating that a balance between the two cytokines is necessary to fully mature the APCs. These mechanisms certainly seem plausible in our experimental setting.

The concept of using chaperone proteins purified from tumors as vaccines against those tumors suggests that a multivalent response against the full repertoire of potentially antigenic peptides in a tumor may be important in generating effective immunoprotection. We suggest that to induce the maximally effective antitumor response, it may be necessary to use several types of chaperones that could be complexed with a wide variety of tumor-derived peptides. Our purification scheme enables one to purify a variety of such chaperones from a single tumor source; thus isolated, one may quantify the effects of each chaperone in terms of generating an antitumor response, and perhaps, one may determine the mechanisms behind that response. Ongoing studies in our laboratory are presently examining the benefits of multiple chaperone protein vaccines from the same tumor used alone or in combination. An understanding of how tumor-derived chaperones may differ in eliciting tumor-specific T-cell responses will undoubtedly prove useful in designing effective combinations of chaperones for anticancer treatments.

ACKNOWLEDGMENTS

We thank Dr. Gary Lee for advice, encouragement, and patience. We also wish to thank Marcy Owens, Nicole Davis, and Hector Sandoval for technical assistance.

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