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ORIGINAL ARTICLE

Michael Graner · Amy Raymond
Emmanuel Akporiaye · Emmanuel Katsanis

Tumor-derived multiple chaperone enrichment by free-solution isoelectric focusing yields potent antitumor vaccines

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Abstract We have utilized a free-solution/isoelectric focusing technique (FS-IEF) to obtain fractions rich in multiple chaperone proteins from clarified A20 tumor lysates. Vaccines prepared from chaperone-rich fractions are capable of providing protective immunity in mice subsequently challenged intravenously with the same A20 B cell leukemia cells. This protection is at least equal to that provided by purified, tumor-derived heat-shock protein 70, which was the best chaperone immunogen in our hands against this aggressive murine leukemia model. Dosage escalation studies, however, revealed that increasing vaccine dosages actually abrogated the protective effects. The physical nature of the enriched chaperones indicates that they are associated in complexes, which may have implications for their function. FS-IEF is relatively simple, rapid, and efficient, thus making combined multi-chaperone therapy feasible.

Key words Isoelectric focusing · Chaperones · Complexes · Antitumor immunity

Introduction

Tumor-derived chaperone proteins are unique mediators of specific antitumor immunity when purified from tumor tissue and used to vaccinate animals (reviewed in [32, 31, 13, 26, 35, 14, 30]). We [12] and others [39–41, 2, 43, 4, 25] have demonstrated that the individual purified chaperone proteins calreticulin, heat-shock proteins 70 and 90 (hsp70, hsp90), and glucose-regulated protein 94/glycoprotein 96 (grp94/gp96) are each capable of generating immune responses against their tumors of origin. The immunogenic potential of these proteins appears to lie not in the intrinsic characteristics of the chaperones themselves, but rather in the repertoire of antigenic, tumor-derived peptides that are carried by the chaperones [39, 34, 36, 2, 15, 25].

Chaperone proteins and their cohorts are typically involved in the births, lives, and deaths of cellular proteins. While chaperone proteins were originally recognized for their protective roles during cellular stress, chaperones fold, unfold, refold, stabilize, oligomerize, salvage, and discard cellular proteins during the routine events of intracellular activities (reviewed in [19, 11, 7, 21, 16, 9]). The chaperone proteins perform these intracellular functions as multi-protein complexes consisting of chaperones, co-chaperones, substrate molecules, etc. In contrast, vaccination studies indicate that these proteins, once purified away from their normal cellular environment, can have antitumor activity even when isolated from their usual cohorts. A remaining question has been whether or not multi-chaperone/co-chaperone vaccines would be more effective as a combined therapy than the single-component vaccines.

The potential to use multiple chaperone proteins as a combined vaccine led us to purify all four of the aforementioned chaperone proteins from a single tumor source [12]. This proved to be laborious and time-consuming, so we sought to streamline the purification procedure of the chaperones by free-solution isoelectric focusing (FS-IEF) of clarified murine A20 B cell tumor homogenate. All four of the chaperones with known immunogenic properties were enriched in a number of the resulting fractions. Without further purification, these fractions were used to immunize BALB/c mice, and these vaccines provided statistically significant

Michael Graner and Amy Raymond contributed equally to this work.

M. Graner · A. Raymond · E. Katsanis (✉)
Department of Pediatrics,
Steele Memorial Children's Research Center,
University of Arizona, 1501 N. Campbell Ave,
P.O. Box 245073, Tucson, Arizona 85724-5073, USA
e-mail: katsanis@peds.arizona.edu
Tel.: +1-520-626-6527; Fax: +1-520-626-4220

E. Akporiaye
Department of Microbiology and Immunology,
University of Arizona,
P.O. Box 245049, Tucson, Arizona 85724-5049, USA

immunological protection against lethal intravenous tumor challenges. The level of protection was equivalent to or better than that provided by purified A20-derived hsp70, which in our hands was the single most effective chaperone vaccine in the A20 B cell leukemia model [12]. The data herein provide evidence that vaccination with multiple FS-IEF-enriched chaperones is more effective than vaccination with an equivalent total quantity of any of the purified chaperones individually (calreticulin, hsp70, hsp90, grp94/gp96).

FS-IEF is a relatively simple and rapid procedure that utilizes small amounts of starting materials to yield potent tumor-derived chaperone protein anticancer vaccines. The data presented here demonstrate the utility and effectiveness of this technique to provide an immune protection against an aggressive murine leukemia.

Materials and methods

Tumor generation

All tissue/cell culture reagents were purchased from Gibco/BRL (Gaithersburg, Md. USA). A20 murine leukemia/lymphoma cells were cultured at 37 °C and in 5% CO_2 in RPMI medium containing 10% heat-inactivated fetal calf serum and supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate, 0.025 μg/ml amphotericin B, 0.5 × minimal essential medium non-essential amino acids, 1 mM sodium pyruvate, and 50 μM 2-mercaptoethanol. Cells were prepared for injection by washing and resuspending them in Hanks' balanced salt solution. The cells were then counted and brought to a concentration of 5×10^6 cells/ml. Female BALB/c (H-2^d) mice (Jackson Laboratories, Bar Harbor, Me., USA) 8–12 weeks old, were injected with 0.2 ml (10^6 cells) subcutaneously in both flanks and were monitored for tumor development. Tumors greater than 1 cm in diameter were surgically harvested after the mice had been humanely killed. In vivo passaging of tumors involved harvesting and mincing the tumor to produce a cell suspension. The cell suspension was spun through a Nitex filter to remove debris and the cell pellet was resuspended, washed, counted, and injected as described above. Mice were housed in a dedicated facility and all animal experimentation was conducted under protocols approved by the University of Arizona Institutional Animal Care and Use Committee. *Principles of laboratory animal care* (NIH publication 85-23, revised 1985) were followed accordingly.

FS-IEF for chaperone enrichment/conventional purification of hsp70

Tumor tissue grown in vivo was homogenized at 4 °C in a motor-driven glass/Teflon homogenizer; the buffer was 10 mM Tris/Cl (pH 7.4)/10 mM NaCl, 0.1% Triton X-100/0.1% Triton X-114/0.1% Igepal CA-630 (equivalent to Nonidet P-40), with the following protease inhibitors (Roche Molecular Biochemicals, Indianapolis, Ind., USA): leupeptin (2 μg/ml), pepstatin A (1 μg/ml), phenylmethylsulfonyl fluoride (0.5 mM) and a Complete protease inhibitor cocktail tablet. This buffer was chosen for its low ionic strength and ability to solubilize membranes. The homogenate was centrifuged at 10 000*g* for 30 min at 4 °C, and the supernatant was collected. The "low-speed" supernatant was centrifuged at 100 000*g* for 90 min at 4 °C to obtain a "high-speed" supernatant. This supernatant was dialyzed against 5 mM Tris/Cl (pH 7.4)/5 mM NaCl, 0.05% Triton X-100/0.05% Triton X-114/0.05% Igepal CA-630. The dialysate was apportioned and frozen into 5-ml aliquots. One aliquot (approximately 40–50 mg protein) was filtered through a 0.8-μm filter and prepared for isoelectric focusing by adding urea to 6 M, the detergents Triton X-100, Triton X-114, and Igepal each to 0.5%, ampholytes (2 parts pH 5–8, 1 part pH 3–10; Sigma, St. Louis, Mo., USA) to 5%, and water to a total volume of 60 ml. The high concentrations of detergents and ampholytes were necessary to maintain protein solubility during isoelectric focusing, as proteins often tend to precipitate at or near their pI values. FS-IEF was carried out in a Rotofor device (Bio Rad Laboratories, Hercules, Calif., USA). Isoelectric focusing was conducted for 4 h at 15 W constant power while the apparatus was cooled with recirculating water at 4 °C; the anode compartment contained 0.1 M H_3PO_4, while the cathode compartment contained 0.1 M NaOH. Twenty fractions were harvested; the pH of each fraction was determined with a standard pH meter, and the protein content was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting as described in [12]. Purification of A20-derived hsp70 was done via conventional and nucleotide-affinity chromatography as described in [12].

Size-exclusion chromatography (SEC) of FS-IEF fractions

Chaperone-rich FS-IEF fractions were chosen and pooled, and a 100-μl sample was taken for SEC. SEC was performed on a Waters Alliance 2690 separations module equipped with a PDA 996 photodiode array detector (Waters, Milford, Mass., USA) using a YMC-Pack Diol S5 300Å column, 6 × 300 mm (Wilmington, N.C., USA). The column was equilibrated and developed in 6 M urea, 0.4 mM Tris/0.4 mM NaCl, pH 6.0 at a flow rate of 0.5 ml/min. Fractions (1 ml) were collected, and chromatograms were extracted at 214 nm. Size standards (high- and low-molecular-mass kits; Amersham Pharmacia, Piscataway, N.J., USA) included thyroglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), albumin (67.0 kDa), ovalbumin (43.0 kDa), chymotrypsinogen A (23.5 kDa), and ribonuclease A (13.7 kDa). Blue dextran 2000 (2000 kDa) was used to determine the void volume.

Retention times for proteins were converted into elution volumes, which were then used to produce K_{av} values [$K_{av} = (V_e - V_o)(V_t - V_o)$, where V_t = total bed volume, V_o = void volume, and V_e = elution volume of the substance of interest]. K_{av} values were plotted against log (molecular mass) values of standards to generate a standard curve. Molecular mass determinations of chromatographic peaks in the FS-IEF sample were obtained from the equation of the standard curve. Collected fractions were concentrated with Centricon 10 devices and analyzed for chaperone content by SDS-PAGE and Western blotting as described above. The FS-IEF pooled fractions were also dialyzed into 0.1 M phosphate/0.2 M NaCl, pH 7.0, and chromatographed over the column in the same buffer. Additional controls included chromatography of ampholytes (pH 3–10) under conditions identical to those used for proteins.

Preparation of chaperone-enriched vaccines and in vivo immunoprotection experiments

Fractions from FS-IEF that contained substantial amounts of four chaperone proteins (hsp70, hsp90, grp94/gp96, and calreticulin), as determined by SDS-PAGE and Western blotting, were dialyzed stepwise out of urea and detergents (starting in 0.1 × phosphate-buffered saline (PBS), 4 M urea, and 0.25% detergents, ending with 0.1 × PBS). Fractions were then concentrated by vacuum centrifugation and reconstituted in PBS. Protein concentrations were determined by the BCA (bicinchoninic acid) method (Pierce, Rockford, Ill., USA, using bovine serum albumin as a standard), and each concentrated fraction was diluted to 20–200 μg/200 μl in sterile PBS. A20-derived hsp70 was concentrated and prepared for vaccine use in the same fashion.

BALB/c mice were immunized s.c. on the flank with 20- to 200-μg samples of chosen fractions in 200 μl sterile PBS on days −14 and −7, followed by intravenous challenge on day 0 with 10^4 or 10^6 viable, A20 leukemia cells grown in vivo. For dosage escalation

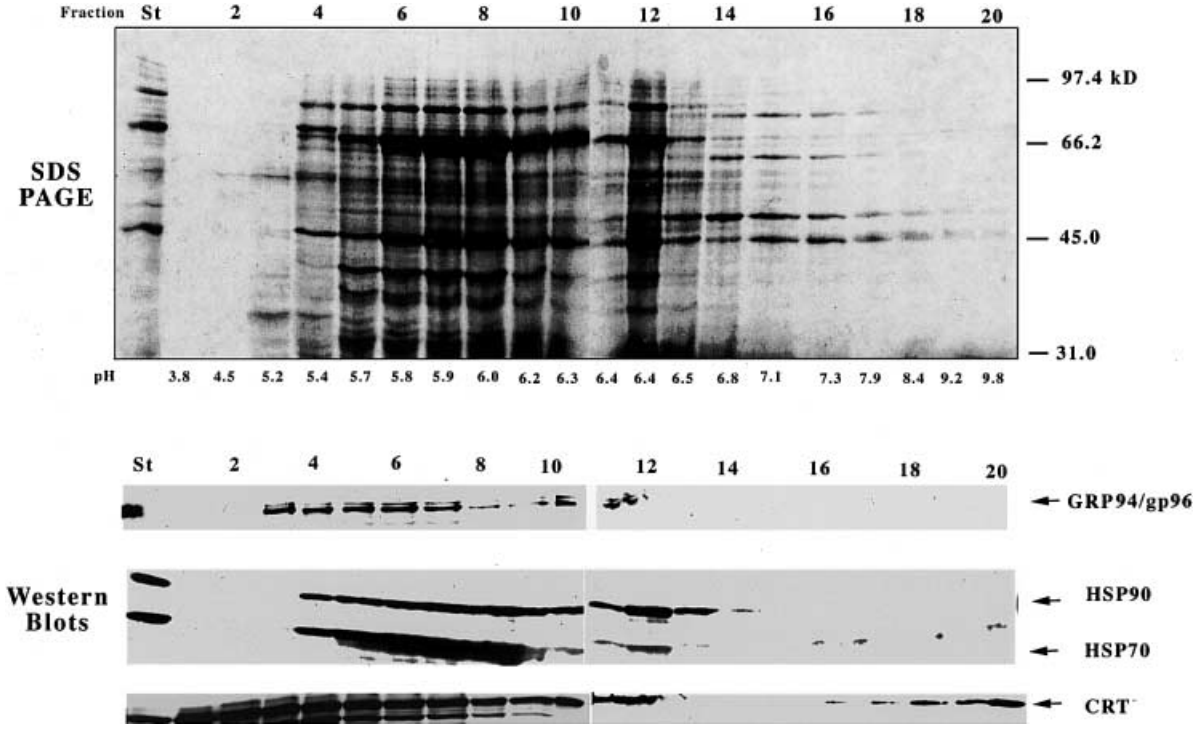
studies, 20, 50, 75, 100, or 200 μg FS-IEF fractions/200 μl were used for vaccination. Control vaccines consisted of PBS, A20 tumor lysate, A20-derived hsp70, or A20-derived FS-IEF fractions that contained none of the chaperones of interest (as determined by Western blotting). Survival was thereafter monitored; for statistical analyses, the Kaplan-Meier product-limit was used to assess survival [17]. The log-rank statistic was used to test differences between treatment groups [27].

Results

FS-IEF yields fractions that are enriched in multiple chaperone proteins

FS-IEF separates soluble protein components of a mixture by their isoelectric points (pI). The separation conditions we chose included nonionic detergents and 6 M urea as a mild chaotropic agent to reduce protein precipitation. Using a Bio Rad Rotofor device, proteins were separated into 20 fractions covering a pH gradient determined by ampholyte choice. The isoelectric points, determined by two-dimensional gel electrophoresis of the murine chaperone proteins from various tissues, are as follows: calreticulin, pH 4.41–4.52; hsp70, pH 5.25–5.30; hsp90, pH 4.99–5.04; and grp4/gp96, pH 4.86–4.91 [1, 29]. These values are sufficiently distinct to allow for adequate separation of each of these proteins via isoelectric focusing. However, SDS-PAGE (Fig. 1, top) and Western blot (Fig. 1, bottom) results indicated that, following FS-IEF, several fractions ranging from pH 5.4 to pH 6.4 (lanes 4–11) contained all four of the aforementioned chaperone proteins. Other chaperone protein members found in those fractions (as indicated by Western blotting with specific antibodies, data not shown) include the ER hsp70 paralog BiP/grp78, grp75/mt hsp70 (mitochondrial hsp70), small amounts of hsp72 (the murine stress-inducible form of hsp70), hsp60, and hsp40, in addition to other unidentified proteins. The isoelectric points of this enrichment of chaperone proteins did not strictly match those predicted or published. Altering the ampholyte ratios during FS-IEF did not enhance separations, and only served to move the overall protein pattern up or down the pH gradient (data not shown). The nature of this enrichment implied that the chaperone proteins and cohorts were separating as complexes rather than with the characteristics of individual proteins.

Fig. 1 Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) (*top*) and Western blot (*bottom*) analyses of A20-tumor-derived free-solution isoelectric focusing (FS-IEF) fractions. A20 tumor was homogenized in lysis buffer and a 100 000*g* supernatant was obtained. The high-speed supernatant was dialyzed into a low-ionic-strength buffer; this preparation was brought to 6 M urea, 5% in ampholytes (pH 3–10 and pH 5–8) and 0.5% in detergents. The sample was subjected to FS-IEF in a Bio Rad Rotofor cell for 4 h at 15 W constant power. Twenty fractions were harvested, the pH was determined for each fraction, and samples of each fraction were taken for SDS-PAGE and Western blotting. *Top* Coomassie-blue-stained gels of the starting material prior to isofocusing (*St*) and the resultant 20 fractions following isofocusing. The pH of each fraction is listed beneath the respective gel lane. Molecular mass markers are indicated at the *right* and are as follows (*from top to bottom*): phosphorylase *b*, serum albumin, ovalbumin, and carbonic anhydrase. Following SDS-PAGE, gels were electrotransferred to nitrocellulose and probed with specific antibodies for the chaperones grp94/gp96, hsp90, hsp70, and calreticulin (*CRT*) (*bottom*)



The multiple chaperone proteins found in FS-IEF fractions are associated in complexes

If the chaperone proteins were indeed separating as complexes during FS-IEF, rather than as individual proteins, the differences in size between chaperone proteins in the complex and individual chaperone proteins should be evident if molecular sieving techniques are applied. We therefore performed SEC on A20-derived FS-IEF samples that has been enriched for chaperone proteins. The column was run in essentially the same buffer as was used for FS-IEF. As shown in the chromatogram in Fig. 2, approximately 50% of the protein/peptide content in the FS-IEF samples eluted with retention times (t_R) between 7 min and 13 min. The bulk of that material eluted with t_R values less than 10, with a peak at approximately 8 min. This peak corresponded to molecular masses above 300 kDa, with shoulder fractions of over 500 kDa, as determined by generating K_{av} values for those peaks and comparing these values with those of standards of known size (see inset, Fig. 2). The standards are denoted by arrowheads placed at their approximate t_R in the chromatogram. It is worth noting that the elution volume of the initial peaks approached that of the void volume (V_o, indicated by the blue dextran arrowhead; $t_R \approx 7$ min). Such molecular masses are clearly severalfold larger than those of any single chaperone protein represented here. While the exact composition of the complexes remains unknown, Western blot analyses showed that all four of the immunogenic chaperones (calreticulin, hsp70, hsp90, and grp94/gp96) were present in the SEC fractions under the very high-molecular-mass peak (fractions 1 and 2, Fig. 2, bottom). Carrier ampholytes, which are structurally similar to amino acids, almost exclusively contributed to the late-eluting, large peak ($t_R > 14$ min, data not shown). Following dialysis of the FS-IEF pooled sample into 0.1 M phosphate/0.2 M NaCl, pH 7.0, SEC of that sample, performed in that buffer, resulted in peaks of very similar t_R (data not shown). Thus, even in a relatively dissociating buffer such as 6 M urea, the immunogenic chaperones apparently maintained their associations in high-molecular-mass complexes.

The chaperone complexes from tumor-derived FS-IEF fractions provide protective immunity when used as prophylactic vaccines

FS-IEF compared to A20 lysate

Following FS-IEF of A20 tumor lysate, we identified fractions of interest by SDS-PAGE and Western blotting. The fractions selected were those that contained all four of the previously described immunogenic chaperones, grp94/gp96, hsp90, hsp70, and calreticulin. These samples were desalted, concentrated, and injected s.c. into mice as vaccines on days −14 and −7. Viable A20 cells, grown in vivo, were then given i.v. on day 0, and survival was monitored thereafter. Analysis of survival curves showed that FS-IEF vaccination resulted in statistically significantly longer survival than did mock-vaccinated (PBS) controls (Fig. 3A). In addition, immunization of animals with A20 lysate generated no protection. These data are important since they indicate that unfractionated A20 lysate itself was not a suitable immunogen, and that the FS-IEF steps employed were necessary to enhance the immunogenicity.

Individual FS-IEF fractions compared to each other

The survival curves shown in Fig. 3A were generated following vaccination of mice with pooled FS-IEF fractions that contained the appropriate chaperones. By breaking down the pool into its individual members, one may determine whether any particular fraction is especially immunodominant. We chose two fractions distinct in overall protein profile but still containing all four of the known immunogenic chaperones (e.g., fractions 4 and 10 from Fig. 1) to be used as vaccines in side-by-side comparisons. As shown in Fig. 3B, vaccination with tumor-derived FS-IEF fractions, designated FS-IEF$_1$ and FS-IEF$_2$, caused mice to survive significantly longer than PBS-treated controls. The FS-IEF$_1$ and FS-IEF$_2$ curves are essentially identical, and are similar to results obtained in mice vaccinated with FS-IEF pooled fractions. It is important to note that irrelevant FS-IEF fractions (i.e., containing little or no detectable chaperone protein, curve FS-IEF$_0$) provided no protective benefit. Thus, no particular FS-IEF fractions contributing to the vaccine pool were immunodominant, and these results were not an artifact of the isofocusing procedure.

FS-IEF compared to A20-derived hsp70

We have previously reported that purified, A20-derived hsp70 was the single most effective immunogen of the four individual chaperone proteins used in these experiments with A20 murine leukemia [12]. In a direct comparison, survival of mice vaccinated with pure A20-derived hsp70 was comparable to that of mice vaccinated with an A20-derived FS-IEF fraction (Fig. 3C). Equal total amounts of protein were used for these vaccinations (20 μg for each of two vaccinations). There was clearly more hsp70 in the single-protein vaccine, because hsp70 made up a minor portion of the 20 μg of the multiprotein vaccine (5%–10%, data not shown). The comparable survival data from the two types of vaccines implied that there was a synergistic effect when multiple chaperone proteins were present in the vaccine.

FS-IEF dosage escalation

All of the experiments described above involved vaccination of animals with two 20-μg FS-IEF fractions,

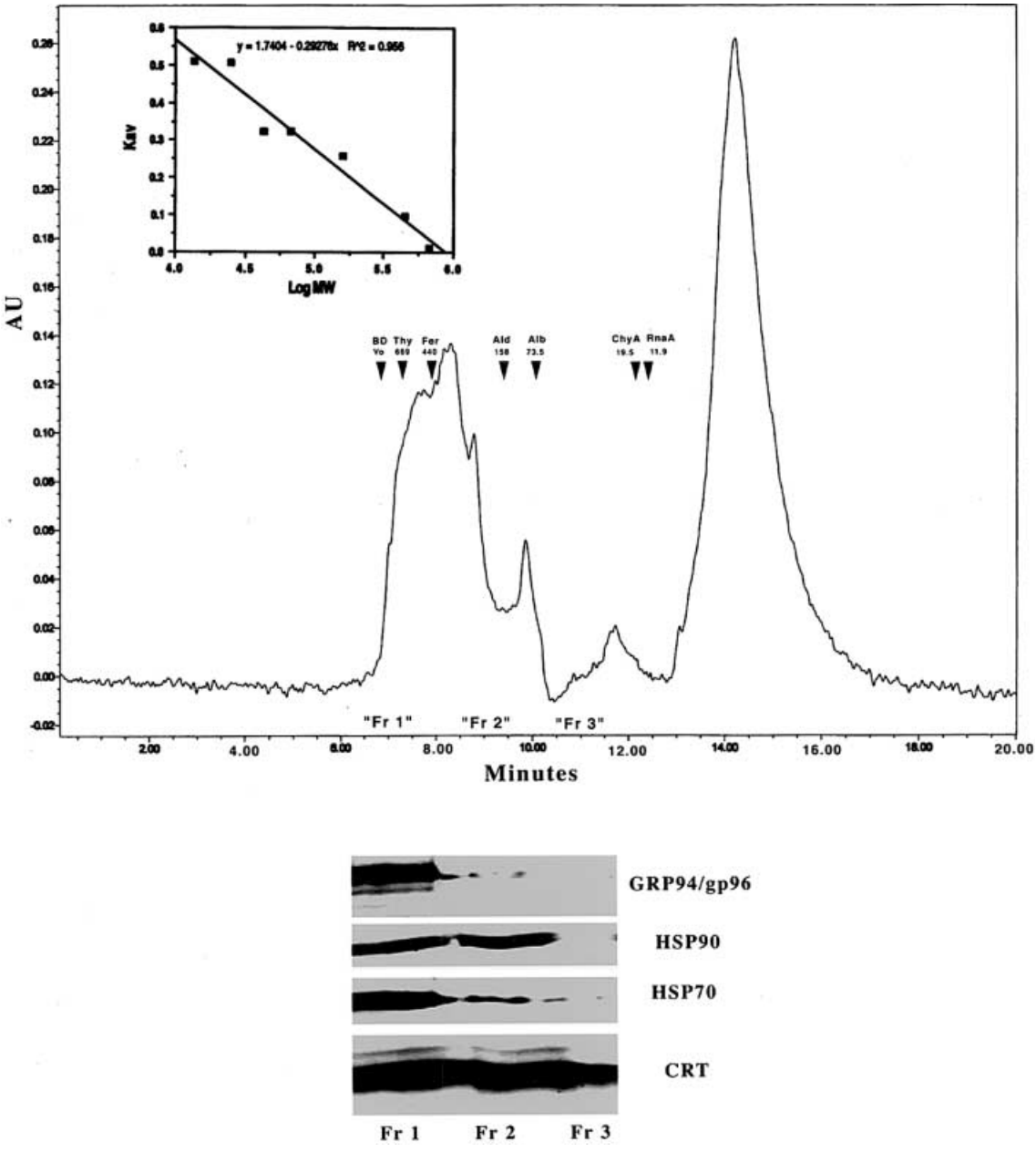


Fig. 2 Size-exclusion chromatography (SEC) of A20-tumor-derived FS-IEF vaccine pool. SEC was performed on a 100-µl vaccine sample prepared by FS-IEF (prior to dialysis) or on molecular mass standards, using a Waters Alliance HPLC system and a YMC-Pack Diol column (6 × 300 mm) with photodiode array detection (extracted at 214 nm). The buffer was 6 M urea in 0.4 mM Tris/0.4 mM NaCl, pH 6.0; the column flow rate was 0.5 ml/min. *Top* The resulting chromatogram; molecular mass standards were used to generate the standard curve (*inset*); retention times of standards are indicated on the chromatogram as arrowheads with molecular masses (in kDa) shown beneath the arrowheads. Standards included blue dextran 2000 (BD), thyroglobulin (*Thy*), ferritin (*Fer*), aldolase (*Ald*), albumin (*Alb*), chymotrypsinogen A (*ChyA*), and ribonuclease A (*RnaA*). During chromatography of the FS-IEF sample, fractions were collected, concentrated, and analyzed by SDS-PAGE followed by Western blotting with the specific antibodies (*bottom*). The positions of fractions 1, 2 and 3 (*Fr 1*, *Fr 2*, *Fr 3*) physically and spatially correspond to peaks in retention time frames of 6–8 min, 8–10 min, and 10–12 min respectively

hsp70, or A20 tumor lysate. These quantities were based on a dosage of tumor-derived hsp70 found to be effective in generating protective immunity against A20 tumor challenges [12]. Dosage escalation studies were initiated using 20, 50, 75, 100, and 200 µg FS-IEF vaccines. Mice in each dosage group received two vaccinations of the particular assigned dosage (i.e., 20, 50, 75 µg, etc.) on days −14 and −7, followed by i.v. tumor challenge on day 0. Survival curves shown in Fig. 3D demonstrated that dosages in excess of 20 µg actually abrogated immune protection in the face of tumor challenge (for clarity, data are shown only for dosages of 20, 50, and 100 µg; nearly identical results were obtained for 75-µg and 200-µg injections). Dosages of 50 µg or more are statistically equivalent to saline only. While the nature of this loss of immune protection is not clear, it is obvious

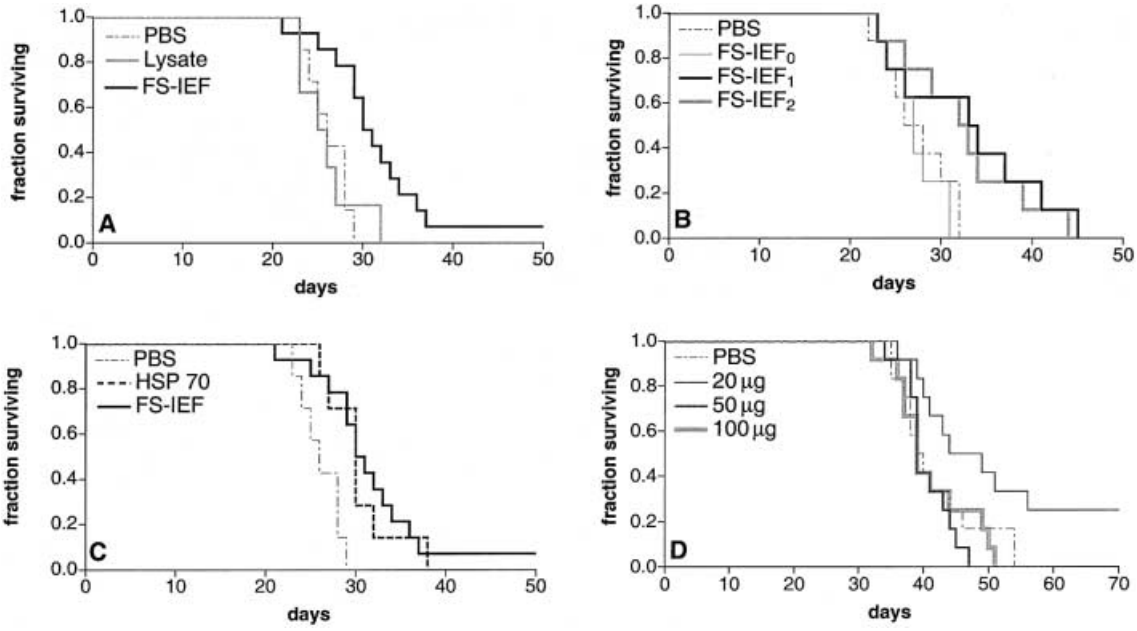


Fig. 3A–D Kaplan-Meier plots for mice immunized with tumor-derived chaperone. Mice were immunized subcutaneously with the indicated vaccines on day −14 and day −7 and received intravenous tumor challenge on day 0. **A**, **B**, **C** Mice received 10^6 viable A20 cells grown in vivo; **D** mice were challenged with 10^4 cells. **A** Mice (n = 6–14/group) were immunized with 20 µg tumor-derived FS-IEF material, or with 20 µg A20 tumor lysate, or with saline. P values: saline versus lysate, NS; versus FS-IEF, $P < 0.002$; lysate versus FS-IEF, $P < 0.02$. **B** Mice (n = 8/group) were immunized with 20 µg A20-derived FS-IEF fractions with differential protein content, or with saline. *FS-IEF*$_1$, *FS-IEF*$_2$ individual fractions from isoelectric focusing that are part of the vaccine pool (e.g., such as that used in **A**). *FS-IEF*$_0$ a fraction from the same isofocusing run that is devoid of the four known immunogenic chaperones. P values: saline versus *FS-IEF*$_0$, NS; versus *FS-IEF*$_1$, $P < 0.03$; versus *FS-IEF*$_2$, $P < 0.02$. *FS-IEF*$_0$ versus *FS-IEF*$_1$, $P < 0.05$; *FS-IEF*$_0$ versus *FS-IEF*$_2$, $P < 0.04$. *FS-IEF*$_1$ versus *FS-IEF*$_2$, NS. **C** Mice (n = 7–14/group) were immunized with 20 µg tumor-derived FS-IEF material, or with 20 µg tumor-derived, purified hsp70, or with saline. P values: saline versus FS-IEF, $P < 0.002$; versus hsp70, $P < 0.002$; FS-IEF versus hsp70, NS. **D** Mice (n = 12/group) were immunized with increasing quantities (20 µg–200 µg) of A20-derived FS-IEF material, or with saline. For clarity, only data from immunizations with 20, 50, and 100 µg vaccine are shown. P values: saline versus 20 µg FS-IEF, $P < 0.04$; versus 50 µg or 100 µg FS-IEF, NS. P values for saline versus 75 µg or 200 µg FS-IEF vaccinations were also NS (data not shown)

that "more is not better" in the case of the vaccine produced by FS-IEF.

Discussion

We have demonstrated that vaccination of mice with A20-leukemia-derived multiple chaperone proteins, prepared by an isoelectric focusing technique (FS-IEF), provides statistically significant immunological protection against an autologous leukemia challenge. This immunoprotective effect was at least equivalent to that provided by A20-derived hsp70, despite the relative paucity of hsp70 in the multichaperone vaccine. The various chaperone-rich FS-IEF fractions were independently capable of providing equivalent protection, but fractions that contained no chaperone proteins or unfractionated A20 tumor lysate failed to provide any protective immunity. Curiously, increasing the vaccine dosage actually nullified the original protective effect. Concerning the physical nature of the chaperone proteins, they do not separate strictly according to published isoelectric points during FS-IEF, and their mobility on size-exclusion chromatography suggests that the proteins are migrating as complexes.

While vaccination against tumor challenge with multiple tumor-derived chaperone proteins has been a goal of ours, we were never able to obtain sufficient quantities of the chaperones by conventional chromatographic methods. Using FS-IEF to produce chaperone-enriched fractions has enabled us to overcome this lack of material. Starting from as little as 1–2 g tumor tissue, we have been able to obtain milligram quantities of enriched chaperone proteins; the potential clinical utility of this is obvious. In addition, the procedure is rapid and relatively uncomplicated, allowing one to generate a vaccine from tumor in 1 day.

The immune response generated following vaccination with tumor-derived FS-IEF samples may be due, in part, to an extended multivalency of antigenic peptides escorted by the various chaperone proteins enriched by FS-IEF. If different chaperone protein family members preferentially escort different peptides, those preferences may be exploited by harvesting all of the chaperones of interest, as opposed to enriching for one chaperone at the expense of the others. The result would be an expanded antigenic repertoire. At the level of the antigen-presenting cells, having multiple chaperone proteins present in the vaccine should lead to the occupancy of multiple putative chaperone protein receptors presumed

to be on the surfaces of antigen-presenting cells [6, 3, 42]. In addition, there are reports of cytokine-releasing effects of chaperone proteins on antigen-presenting cells [5, 38]; thus, having multiple chaperones present may result in a greater stimulus for these cells.

Their separation during isoelectric focusing and their mobility during SEC raise the possibility that the tumor-derived, FS-IEF-enriched chaperone proteins are acting in complexes. The implications of this phenomenon are not entirely clear, but it is possible that it results from a "native-state" preservation of chaperone interactions with FS-IEF. These chaperone complexes may be acting as "danger signals" [22, 23, 38, 10] for the immune system in ways that individual purified proteins cannot. Dying cells, whether necrotic or apoptotic, presumably do not release individual, purified chaperone proteins during their demise; rather, cellular contents are likely spilled en masse during necrosis or partitioned into apoptotic bodies during apoptosis. Thus, chaperones in a complex may more accurately re-create the danger signal to which the antigen-presenting cells of the immune system are primed to respond.

In the aforementioned scenario, however, the tumor cell lysate seems to be the most appropriate mimic, since it, too, has all of the chaperones, co-chaperones and cohorts together. Unlike FS-IEF fractions, however, A20 tumor lysate provides no protective benefit in tumor-rejection experiments. This lack of protective immunity may be due to an insufficient concentration of chaperone proteins in the lysate, or to the presence of "tumor-enhancing activities" [33], or of immune inhibitory substances found in lysate that are removed during FS-IEF. On the other hand, increasing dosages of FS-IEF vaccines over the level of 20 μg/injection lead to abrogation of the protective effect of the vaccines. Thus, it would appear that tumor-promoting or immune-inhibiting factors are present in the FS-IEF fractions, but they may be at sub-threshold levels in our typical vaccination scheme. These factors are likely to be partially removed during FS-IEF (but are at full strength in the tumor lysate) and reach "effective" levels when larger quantities of FS-IEF vaccines are used. In addition, injection of large quantities of the tumor-derived chaperone grp94/gp96 has been shown to down-regulate the antitumor response usually associated with vaccination by that protein [8]. Such an effect, either from grp94/gp96 or from other proteins in the vaccine, could be part of the reason for the loss of immune response with high-dose FS-IEF vaccinations. An additional mechanism for the loss of immune response might be the induction of tolerance or of anergy following vaccination with FS-IEF-derived proteins beyond the optimum, possibly resulting from a hyperabundance of antigen in the vaccines. The nature of this immune unresponsiveness following vaccination with larger quantities of FS-IEF material is currently under study in our laboratory. One may clearly see the need to understand such details in order to proceed appropriately into a clinical setting.

In prior work [12] we found that A20-derived, purified hsp70 was the best immunogen of the four chaperones in the A20 leukemia model. We have shown in this report that equal amounts of total protein (20 μg) of FS-IEF vaccine and purified, tumor-derived hsp70 are equally immunoprotective. The earlier report [12] stated that other individual, purified chaperone proteins (grp94/gp96, hsp90, calreticulin) did not protect animals as well as hsp70; thus, it does not seem inherently obvious that adding all four of the chaperone proteins together (in greatly reduced quantities) would improve the vaccine's effectiveness. However, that seems to be the case for FS-IEF vaccines, since they contain all four of the known immunogenic chaperones in smaller amounts than the overall 20-μg quantity of protein. From the perspective of those four chaperones, the whole is greater than the sum of its parts. The inclusion of co-chaperones and other potentially important but unidentified proteins may also contribute to the apparent synergism between the chaperones.

In general, the level of protection provided by A20-tumor-derived chaperone proteins against intravenous tumor challenge is somewhat modest when compared to similar vaccinations using other tumor models, albeit with different measures of effectiveness. In previous work [12] we have mentioned that A20-derived hsp70 vaccination prolongs survival in mice following tumor challenge, an effect equivalent to a 100-fold reduction in tumor burden. FS-IEF-generated vaccines provide at least this level of protection, as well. In light of the aggressive nature of the A20 leukemia and its metastatic potential once introduced into the bloodstream, such protection is substantial for this particular tumor model. While our results are promising, there is clearly room for improvement.

This work is certainly not the first to utilize isoelectric focusing to purify tumor antigens (e.g. [28, 18, 20, 37]). In previous experiments, investigators sought previously unknown or uncharacterized antigens by laboriously assaying proteins for biological function (i.e., tumor rejection). Unlike those studies, in ours we at least knew the identity of the proteins we initially sought. However, tumor-derived, FS-IEF vaccines may have enhanced immunogenicity because other uncharacterized but still useful proteins are not discarded via "overpurification" of the sample. The roles for such proteins may include co-chaperone functions or outright escorting of antigenic peptides; definition of these roles awaits further characterization of these proteins. There has been a recent report on the purification of multiple heat-shock proteins from a single tumor source [24]. Using those methods it may be feasible and informative to compare the efficacy of the FS-IEF vaccine, with all of its characterized and uncharacterized proteins, to a vaccine consisting of a pre-chosen combination of purified heat-shock/chaperone proteins. Thus, the effective roles for the various components of chaperone-based anticancer vaccines could be elucidated.

In conclusion, we have developed a technique that provides for the enrichment of chaperone proteins from clarified cell lysates. If the chaperone proteins are derived from tumor tissue, those chaperone-enriched fractions may be used as vaccines that are capable of providing protective immunity to animals that are subsequently challenged from that same tumor type. The procedure is relatively simple, rapid, and efficient, and takes advantage of the increasingly well-documented use of tumor-derived chaperone proteins as agents of antitumor immune responses.

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