

Induction of cellular immunity by immunization with novel hybrid peptides complexed to heat shock protein 70

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Heat shock proteins 70 (hsp70) derived from tissues and cells can elicit cytotoxic T lymphocyte (CTL) responses against peptides bound to hsp70. However, peptides can markedly differ in their affinity for hsp, and this potentially limits the repertoire of peptides available to induce CTL by the hsp immunization. Hybrid peptides consisting of a high-affinity ligand for the peptide-binding site of hsp70 joined to T cell epitopes by a glycine–serine–glycine linker were constructed. Immunization with hybrid peptides complexed to mouse hsp70 effectively primed specific CTL responses in mice and were more potent than T cell peptide epitopes alone with hsp70. *In vivo* immunization with hsp70 and hybrid peptides led to rejection of tumors expressing antigen with greater efficacy than immunization with peptide epitope plus hsp70. Induction of CTL responses occurred independently of CD4⁺ T cells, suggesting that immunization directly primed antigen-presenting cells to elicit CD8⁺ cytotoxic T cell responses without T cell help. Both peptide/hsp70 complexes and mouse hsp70 alone were able to induce cultures of mouse bone marrow-derived dendritic cells (DC) to release cytokines, including DC from endotoxin-resistant C57BL/10Sc mice. Thus, hsp70/hybrid peptide complexes can activate DC for cytokine release, providing a potential adjuvant effect that could bypass T cell help.

Heat shock proteins 70 (hsp70) are a family of highly conserved molecules with ATPase activity and relative molecular masses around 70,000 kDa. They are found in all prokaryotes and in most compartments of eukaryotic cells. Hsp70 proteins play essential roles in protein metabolism under both stress and nonstress conditions, including functions in *de novo* protein folding and membrane translocation and the degradation of misfolded proteins (1, 2). Hsp protein preparations, including hsp70 and grp94/gp96 derived from tumor cells and virus-infected cells, are capable of eliciting cellular immunity (3–5). The immunogenicity of hsp protein preparations has been attributed to peptides bound to hsp proteins (6). Preparations of hsp are able to induce cross-priming of T cells, where antigens expressed by one cell can prime the immune system through antigen presentation by a distinct cell that does not endogenously express the antigen (7). These observations suggest that hsp proteins chaperone antigenic peptides into antigen-presenting cells, potentially allowing peptides to enter the MHC class I pathway for loading onto MHC class I molecules, where they can be presented to cytotoxic CD8⁺ T cells. In turn, this provides a strategy for immunization against infectious organisms and cancer by using hsp proteins and bound peptides that are isolated from infected tissues or tumors.

Antigenic peptides have been identified in infectious organisms and tumors (8–13). However, only a subset of peptides may bind hsp with relevant affinities (14). Potential motifs of peptides that are associated with hsp binding have been identified, including motifs for binding to BiP, an hsp70 family member located in the endoplasmic reticulum (14, 15). Based on these observations, we constructed hybrid peptides that contained T

cell epitopes linked to a peptide domain with high affinity for hsp70 proteins.

Materials and Methods

Mice and Cell Lines. Female C57BL/6, C57BL/10Sc, and CD4^{-/-} (H-2^b) mice were obtained from the National Cancer Institute Animal Resource Center (Frederick, MD), The Jackson Laboratory, or Taconic Farms, respectively. Mice were used between 6 and 12 weeks of age. All mouse experiments were performed under protocols approved by the Institutional Animal Care and Utilization Committee of Memorial Sloan–Kettering Cancer Center. Mouse thymoma cell line EL-4 and its E.G7 derivative [EL-4 transfected with cDNA encoding ovalbumin (OVA)] were provided by Janko Nikolic-Zugic, Memorial Sloan–Kettering Cancer Center (16). MO4 (H-2^b, mouse melanoma cell line B16 transfected with OVA gene) has been described (17).

Peptides and Recombinant Mouse hsp70. Peptides were synthesized by using the standard fluorenylmethoxycarbonyl method, followed by HPLC purification and MS analysis. Peptides were >96% pure as evaluated by these methods. Peptide epitopes included OVA_{257–264} (SIINFEKL, single-letter amino acid code) from OVA and HSV_{498–505} (SSIEFARL) from herpes simplex virus (HSV) glycoprotein B. Both peptides are known to bind class I MHC molecule H-2K^b. Table 1 shows the panel of peptides that were synthesized, including hybrid peptides. Hybrid peptides were constructed with a glycine–serine–glycine linker. The HWDFAWPW peptide domain binds to hsp70 proteins, and the designation for this peptide domain was shortened to BiP.

Purified mouse cytosolic hsp70 was prepared from *Escherichia coli* DH5 α cells transformed with the pMS236 vector encoding mouse cytosolic hsp70 (18). A lysate was prepared and centrifuged at low speed followed by 30 min at 100,000 \times g. The cleared lysate was applied to a DEAE-Sephacel column. The eluate was applied to an ATP-agarose column (Sigma) and eluted with 1 mM MgATP preadjusted to pH 7.0. EDTA was added to the eluate to a final concentration of 2 mM. The eluate, which

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Abbreviations: hsp, heat shock protein(s); DC, dendritic cells; LPS, lipopolysaccharide; TNF, tumor necrosis factor; CTL, cytotoxic T lymphocyte; HSV, herpes simplex virus; OVA, ovalbumin.

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Table 1. Peptides used in this study

| Peptide | Amino acid sequence |
|----------|---------------------|
| OVA | SIINFEKL |
| OVA-BiP | SIINFEKLgsgHWDFAWPW |
| BiP-OVA | HWDFAWPWgsgSIINFEKL |
| mBiP-OVA | HDWISYPNgsgSIINFEKL |
| mOVA-BiP | SIIDFEKLgsgHWDFAWPW |
| HSV | SSIEFARL |
| HSV-BiP | SSIEFARLgsgHWDFAWPW |

contained essentially pure hsp70, was precipitated by addition of $(\text{NH}_4)_2\text{SO}_4$ to 80% saturation. The purified hsp70 contained ≤ 3 pg endotoxin/ μg hsp70.

Binding Assay for Hybrid Peptide and hsp70. ^{14}C -labeled OVA-BiP peptide was prepared by alkylation of OVA-BiP with ^{14}C -formaldehyde. OVA-BiP (0.9 mg) in 300 μl of 10% DMSO/ dH_2O was added to 175 μl of ^{14}C -formaldehyde (250 μCi), and, immediately, 50 μl of fresh 200 mM NaCNBH₃ was added. The reaction was mixed and left at 25°C for 3 h. The labeled peptide was repurified by reverse-phase HPLC on a C-4 column in 15 min by using a 0–100% acetonitrile (0.1% trifluoroacetic acid) gradient. The ability of the OVA-BiP peptide to bind hsp was measured by incubating 100 mM (5 μg) ^{14}C -labeled OVA-BiP with 50 μg of hsp70 in final volume of 20 μl of binding buffer (20 mM Mops, pH 7.2/200 mM NaCl/5 mM magnesium acetate) at 37°C for 30 min in the presence or absence of 2 mM ADP or 2 mM ATP. The samples were loaded onto Sephacryl S-300 column equilibrated in binding buffer, and 50 μl of each ≈ 200 - μl fraction was counted in scintillation liquid. Ten microliters of each fraction was run on 12% SDS/PAGE reducing gel.

Immunization, ^{51}Cr Release Assay, and Dendritic Cell (DC) Assays. For immunization with hsp70 coupled with hybrid peptides, complexes of hsp70 and hybrid peptide were obtained after 30-min incubation with 1.5 mg/ml hsp70 and either 1.2 mg/ml hybrid peptide (unless indicated otherwise) or 0.5 mg/ml epitope peptide in 20 mM Hepes, pH 7.0/150 mM KCl/10 mM $(\text{NH}_4)_2\text{SO}_4$ /2 mM MgCl_2 /2 mM ADP. For peptide/TiterMax immunizations, peptides were emulsified in TiterMax (CytRx, Norcross, GA) according to the manufacturer's instructions at a concentration of 0.5 mg/ml peptide (19). Ten microliters of hsp + hybrid peptide or TiterMax/peptide mixture was injected intradermally into flank skin. Six days later, spleen cells from immunized mice were restimulated at 2 – 3×10^7 responder cells per flask in the presence of 1×10^7 peptide-pulsed, irradiated (30 Gy), normal syngeneic spleen cells. Peptide pulsing was performed by incubation of spleen cells with 10 $\mu\text{g}/\text{ml}$ peptide for 30 min at room temperature. After 5 days of incubation, cytotoxic activity of restimulated cells was determined in a standard, 4-h ^{51}Cr release assay. EL4 target cells were labeled with 100 mCi (3.7 MBq) of sodium ^{51}Cr chromate and incubated with or without 1 $\mu\text{g}/\text{ml}$ peptide (unless indicated otherwise) for 1 h and used as target cells. In some experiments, E.G7 cells were used as target cells. Specific lysis was determined as follows: percent specific release = $100 \times (\text{release by effector cells} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release})$. Spontaneous release was $< 20\%$ of maximum release in all experiments.

Bone marrow-derived DC were generated as described (20). Pooled bone marrow cells from 3–5 mice (C57BL/6 or C57BL/10ScN) were incubated with a mixture of rabbit complement (Pel-Freez Biologicals) and mAbs (anti-CD4 GK1.5, anti-CD8 3.155, anti-I-A^{b,d,q} M5/114.15.2, and anti-B220 RA3–3A1/6.1 from the American Type Culture Collection, Manassas, VA) to

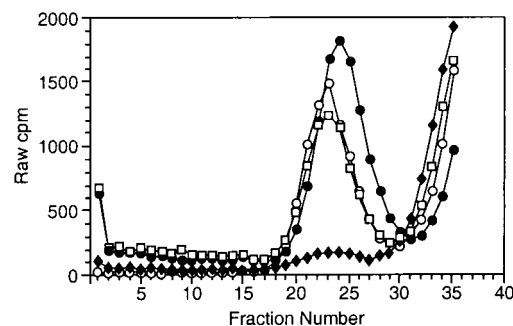


Fig. 1. Hybrid peptide binding to hsp70. The ability of OVA-BiP hybrid peptide to bind hsp70 was measured by incubating 72 μM ^{14}C -labeled OVA-BiP with 50 μg hsp70 at 37°C for 30 min in the presence or absence of 2 mM ADP, 2 mM ATP, or 416 μM OVA. The samples were loaded onto a Sephacryl S-300 column, and fractions were counted in scintillation liquid. The binding assays were performed in the absence (\circ) or presence of ADP (\bullet), ATP (\blacklozenge), or unlabeled OVA peptide (\square).

deplete lymphocytes and Ia-positive cells. Nonlymphoid Ia⁺ cells were cultured with 10% supernatant of J558L cells transfected with mouse granulocyte macrophage–colony-stimulating factor (mGM-CSF) (a kind gift of R. M. Steinman, The Rockefeller University, New York). Medium containing mGM-CSF was replaced every other day, thereby removing most of the developing granulocytes without dislodging clusters of developing DC. Nonadherent DC were harvested at day 6 and used for cytokine stimulation assays. The DC populations were $\approx 70\%$ positive for DEC205, CD11c, and I-A^b. DC were plated in 24-well tissue culture plates at a cell density of 1×10^6 cells/ml and were exposed to hsp70 with or without hybrid peptide or lipopolysaccharide (LPS) (serotype 0127:B8; Sigma). Polymyxin B (Sigma) was added to selected cultures at 20 mg/ml. After 24 h, the medium supernatants were analyzed for cytokine release. The concentrations of tumor necrosis factor α (TNF- α), IL-1 β , IL-6, and IL-12 released into the supernatant by cultured DC were measured with ELISA kits according to the manufacturer's instructions (R & D Systems).

Tumor Challenge Experiments. MO4 cells (1×10^5 or 1×10^6) or 4×10^6 E.G7 cells were injected s.c. into the right flank of mice immunized in the left flank 7 days previously. These doses of tumor cells are at least 10- to 400-fold higher than the minimal tumorigenic dose. Tumor growth was monitored at least three times a week, and results were expressed as the mean diameter (mm) calculated from the longest and its perpendicular diameter of tumor. Survival of different groups of mice was compared by using the generalized Wilcoxon test.

Results

Binding of Hybrid Peptides to hsp70. Hybrid peptides were constructed that contained a T cell epitope, a linker (GSG in single-letter amino acid code), and an octapeptide sequence (HWDFAWPW), which is known to bind with high affinity to BiP, the endoplasmic reticulum-resident hsp70 homologue (Table 1). This hybrid peptide containing a BiP (hsp70)-binding sequence is termed a hybrid peptide. A prototype is the OVA-BiP hybrid peptide, which contains the H-2K^b-restricted T cell epitope OVA (SIINFEKL) followed by a carboxyl BiP-binding domain (designated BiP in the hybrid peptide). ^{14}C -labeled OVA-BiP hybrid peptide was assessed for its ability to bind to hsp70 in the presence or absence of ADP or ATP after 30-min incubation at 37°C followed by size-exclusion chromatography to resolve hsp70-bound peptide from free peptide. Fig. 1 shows elution of OVA-BiP hybrid peptides bound to hsp70 as the major

peak between fractions 19 and 30. Elution of hsp70 was determined by SDS/PAGE (data not shown).

Hsp70 will bind peptide in the absence of nucleotide, but has a higher affinity for peptide in the ADP-bound state whereas ATP binding and hydrolysis induce peptide release (2, 14). These characteristics were observed for OVA-BiP hybrid peptide binding to hsp70. In the presence of ADP, binding of OVA-BiP hybrid peptide to hsp70 increased, whereas binding was abrogated in the presence of ATP. Furthermore, binding of OVA-BiP hybrid peptide to hsp70 was not competed by a 6-fold molar excess of OVA peptide, showing that OVA peptide has a significantly lower binding affinity to hsp70 or binds to a distinct site on hsp70. Finally, analysis of the OVA-BiP hybrid peptide bound to the hsp70 eluting from the gel filtration column indicated that $\approx 20\%$ of the hsp70 had been saturated with the peptide (results not shown). Thus, the hybrid peptide has increased loading of hsp70 compared with peptide without the BiP-binding domain, and binding of hybrid peptide is specific.

Cytotoxic T Cell Responses Induced by Immunization with Hybrid Peptide Complexed to hsp70. To determine the ability of hybrid peptide/hsp70 complexes to elicit CTL responses *in vivo*, OVA-BiP hybrid peptide/hsp70 complexes were injected intradermally into the flank skin of C57BL/6 mice without any additional immune adjuvant. After a single immunization, followed by *in vitro* stimulation, splenic T cells from immunized mice were capable of lysing H-2K^b EL4 target cells pulsed with OVA peptide (Fig. 2a). The CTL response from mice immunized with OVA-BiP hybrid peptide/hsp70 was at least as strong as that from mice immunized with OVA peptide mixed with TiterMax, an optimal adjuvant for inducing CTL responses (19). Immunization with OVA peptide mixed with hsp70 consistently induced weaker CTL responses. No CTL responses were detected after immunization with either OVA peptide alone or hsp70 alone. Surprisingly, immunization with OVA-BiP hybrid peptide alone was capable of generating weak but reproducible CTL lysis against target cells pulsed with OVA peptide, perhaps related to binding to hsp70 in tissues (Fig. 2a).

The responding CTL cells induced by OVA-BiP hybrid peptide/hsp70 immunization were characterized further. They were CD8⁺ T cells and restricted by the H-2K^b molecule because specific killing was inhibited completely by the addition of anti-CD8 antibody or anti-H-2K^b antibody (data not shown). CTL were able to recognize endogenously processed OVA peptide because they lysed E.G7 cells, which are an EL4 variant transfected with the OVA gene (data not shown).

The immunogenicity of hybrid peptide constructs with different orientations of T cell epitopes was compared by using hybrid peptides that carried T cell epitopes at either the carboxyl terminus (OVA-BiP) or amino terminus (BiP-OVA) (Table 1 and Fig. 2b). Both hybrid peptides were capable of inducing strong CTL responses with comparable potency.

We investigated the possibility that hybrid peptides do not require hsp70 binding for inducing cytotoxic T cell responses, but that the hsp70-binding domain provides effects that are independent of hsp70 binding. A shuffled hybrid peptide (mBiP-OVA in Table 1) that bound poorly to hsp70 was used as an immunogen. Fig. 2b shows that immunization with hsp70 plus mBiP-OVA peptide lost the capacity to elicit CTL and was even less potent than immunization with OVA-BiP peptide alone without hsp70. These results also support the notion that weak CTL responses elicited by OVA-BiP hybrid peptide required binding to hsp70 released in tissues. Specificity of induction of CTL responses also was confirmed by altering 1 aa in the T cell epitope sequence of OVA-BiP, SIINF₃EKL to SIIDFEKL (mOVA-BiP). Immunization with mOVA-BiP combined with hsp70 did not generate any detectable CTL response to OVA peptide (Fig. 2b).

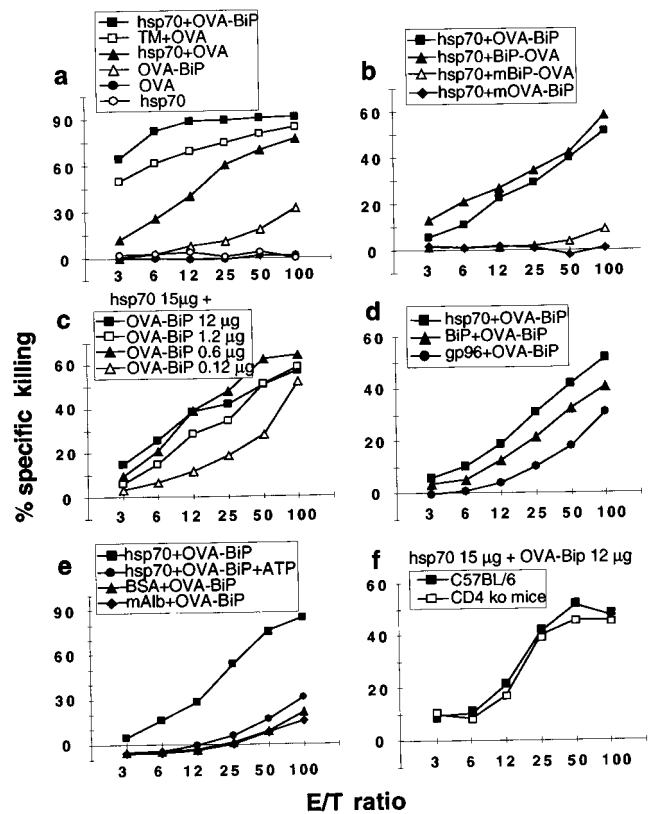


Fig. 2. Immunization with OVA-BiP/hsp70 complex elicits specific CTL responses in C57BL/6 mice. (a) Mice were injected once intradermally, and 7 days later splenocytes from immunized mice were restimulated *in vitro* as described in *Materials and Methods*. Results are shown as ⁵¹Cr release assays of splenocytes from mice immunized with OVA-BiP hybrid peptide/hsp70 complex, OVA peptide plus TiterMax (TM) adjuvant, OVA peptide plus hsp70, OVA-BiP hybrid peptide alone, OVA peptide alone, or hsp70 alone. Results are representative of more than 20 experiments. (b) CTL induction after immunization with OVA-BiP, BiP-OVA, mBiP-OVA, or mOVA-BiP plus hsp70. Results are representative of three experiments. (c) CTL induction after immunization with 15 μg (213 pmol) of hsp70 coupled with 12 μg (5.2 nmol), 1.2 μg (0.52 nmol), 0.6 μg (0.26 nmol), or 0.12 μg (53 pmol) of OVA-BiP peptide. Results are representative of two experiments. (d) CTL induction after immunization with 1.2 μg of OVA-BiP peptide complexed with 15 μg of hsp70, BiP, or gp96. Results are representative of two experiments. (e) CTL induction after immunization with hsp70 + OVA-BiP, BSA + OVA-BiP, mouse albumin (mAlb) + OVA-BiP, or hsp70 + OVA-BiP prepared by the incubation with 2 mM ATP instead of 2 mM ADP. The experiment were repeated three times with similar results. (f) CTL induction after immunization with hsp70 + OVA-BiP in wild-type C57BL/6 mice and in CD4^{-/-} mice. Results represent mean of two mice. Results are representative of two experiments.

To optimize immunization with OVA-BiP hybrid peptide/hsp70 complexes, we compared CTL responses after a single immunization with different doses of OVA-BiP hybrid peptide complexed to the same amount of hsp70 [15 μg (213 pmol)/dose]. Immunogenicities of hsp70 complexes containing 12, 1.2, or 0.6 μg (5.2, 0.52, or 0.26 nmol, respectively) of OVA-BiP hybrid peptides were comparable (Fig. 2c). Even a dose of 0.12 μg (52 pmol) of OVA-BiP and 15 μg of hsp70, in which the molar ratio of OVA-BiP hybrid peptide and hsp70 was approximately 4:1, still induced substantial CTL responses *in vivo*. For subsequent experiments we used complexes containing 1.2 μg of hybrid peptide and 15 μg of hsp70.

The immunogenicity of hybrid peptides with other hsp molecules, BiP (in the hsp70 family) or grp94/gp96 (in the hsp90 family), was assessed. Both BiP and grp94/gp96 are located in

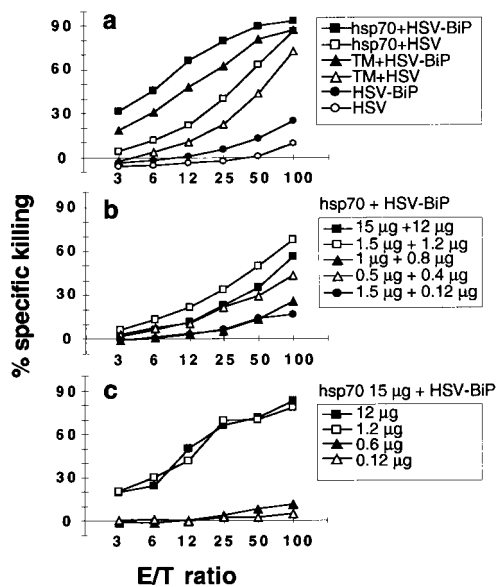


Fig. 3. OVA-BiP hybrid peptide/hsp70 complex induces strong CTL response against a HSV antigen in mice. (a) CTL responses after immunization with hsp70 + HSV-BiP hybrid peptide complex, hsp70 + HSV, TiterMax (TM) + HSV-BiP hybrid peptide, TiterMax + HSV, HSV-BiP hybrid peptide alone, or HSV alone. Results are representative of five experiments. (b) CTL induction in a dose-response evaluation keeping a fixed ratio of hsp70/HSV-BiP hybrid peptide. Results are representative of two experiments. (c) CTL responses in a dose-response evaluation by using a constant amount of hsp70 and decreasing amounts of HSV-BiP hybrid peptide. Results are representative of two experiments.

the lumen of the endoplasmic reticulum, and hsp70 exists in the cytosol. Complexes of OVA-BiP hybrid peptide and either BiP or gp96 were made by incubation for 30 min at 37°C by using the same conditions described for complexes with hsp70. Immunization with OVA-BiP hybrid peptide/BiP complexes induced strong CTL responses comparable to, although perhaps weaker than, the efficacy of OVA-BiP hybrid peptide/hsp70 complexes (Fig. 2d). Priming with OVA-BiP hybrid peptide/gp96 complexes also elicited a CTL response against OVA, although weaker. These results indicated that these other hsp proteins could provide immunological adjuvant effects with hybrid peptides.

Incubation of OVA-BiP peptide with hsp70 in the presence of ATP markedly decreased CTL responses after immunization, with only minimal levels of cytotoxicity equal to those observed after immunization with hybrid OVA-BiP peptide alone (Fig. 2e). Also, immunization with mixtures of OVA-BiP plus BSA or mouse albumin elicited only weak CTL responses (Fig. 2e). These findings showed that potent CTL responses required the binding of hybrid peptide to hsp70. Finally, CTL responses were generated independently of CD4⁺ T cells in CD4^{-/-} mice (Fig. 2f) and in mice depleted of CD4⁺ cells with mAb GK1.5 (data not shown).

Immunization with a HSV Peptide and hsp70. The ability of hsp70 to provide adjuvant effects with a second hybrid peptide was assessed. An H-2^b-restricted peptide epitope (SSIEFARL) from HSV was used to construct HSV-BiP hybrid peptide. A single immunization with HSV-BiP hybrid peptide/hsp70 elicited strong CTL responses that were comparable to CTL elicited by immunization with TiterMax adjuvant and HSV peptide (Fig. 3a). A complex of hsp70 and HSV peptide alone generated weaker CTL responses. Quite weak but reproducible CTL responses were observed after immunization with HSV-BiP

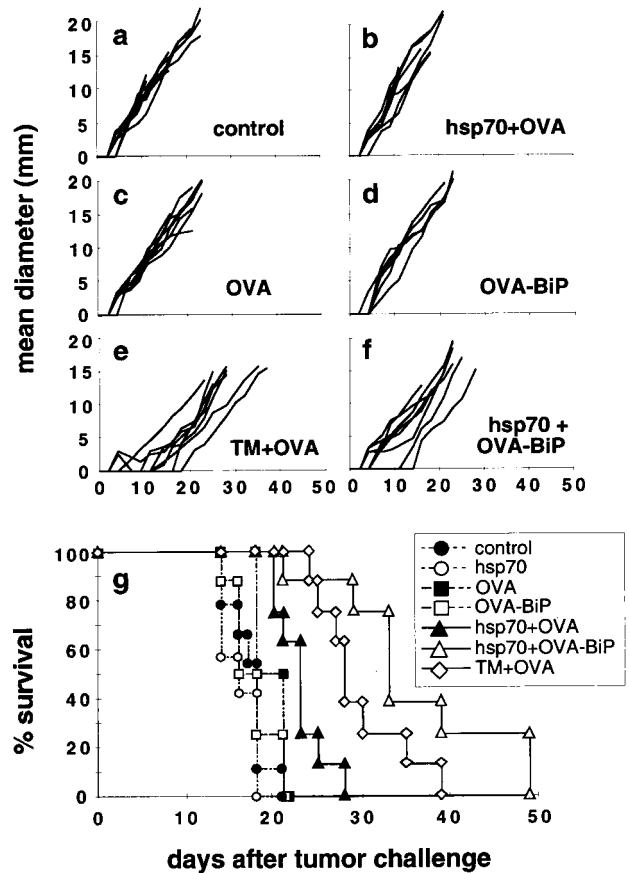


Fig. 4. OVA-BiP hybrid peptide/hsp70 complex induces significant protection against challenge with tumor cells. Mice, 8–10 per group, were challenged intradermally with 10⁶ MO4 cells after no treatment (a) or 1 week after a single immunization with hsp70 and OVA peptide (b), OVA peptide alone (c), OVA-BiP hybrid peptide alone (d), TiterMax (TM) + OVA (e), or hsp70 + OVA (f). Results are shown for individual tumor growth of each mouse as mean tumor diameter. The proportion of surviving mice in each group is shown after tumor challenge (g).

hybrid peptide alone whereas there was no detectable response after immunization with HSV peptide alone. When different doses of HSV-BiP hybrid peptide and hsp70 were used, keeping the ratio constant, CTL induction was detected even after immunization with 0.5 µg of hsp70 and 0.4 µg of HSV-BiP hybrid peptide (Fig. 3b). Interestingly, CTL responses after immunization with HSV-BiP hybrid peptide complexed to hsp70 did not depend on the dose of HSV-BiP hybrid peptide in a linear manner, but disappeared below an HSV-BiP hybrid peptide dose of 1.2 µg, suggesting a threshold effect (Fig. 3c). These results confirm that immunization with HSV-BiP hybrid peptide/hsp70 complexes can prime CTL responses.

Tumor Challenge Experiments. The ability of immunization with hybrid peptide/hsp70 complexes to induce immunity was assessed *in vivo* by using tumor challenge models. In a preliminary experiment, groups of five mice were immunized once with OVA-BiP hybrid peptide/hsp70 complexes, OVA peptide plus TiterMax adjuvant, or OVA peptide alone. Seven days later mice were challenged cutaneously with the syngeneic melanoma line MO4 transfected to express OVA. All mice in the untreated group and treated with OVA peptide alone developed tumors by day 26 (data not shown). On the other hand, tumor growth in mice immunized with OVA-BiP hybrid peptide/hsp70 was delayed, with tumors first appearing after 45 days and two of five

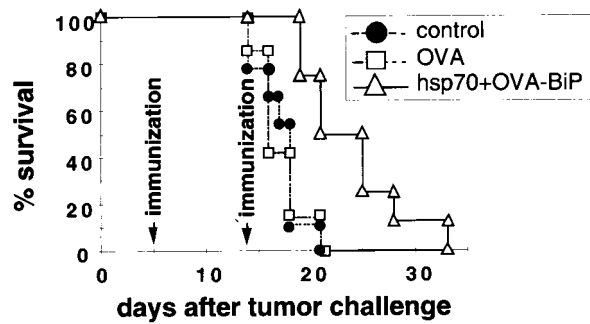


Fig. 5. Therapeutic immunization with OVA-BiP hybrid peptide/hsp70 complexes of mice with 5-day established tumors. Mice were injected intradermally with 5×10^5 MO4 cells, and then 5 and 14 days later, as tumors were becoming palpable, they were immunized with OVA-BiP hybrid peptide/hsp70 complex or OVA peptide alone or were not immunized (control).

mice in this group remaining tumor-free more than 100 days after tumor challenge (data not shown). This experiment was repeated with groups of 8–10 mice challenged with a 10-fold-higher dose of MO4 tumor cells (Fig. 4). Delay in appearance of MO4 tumors was observed in mice immunized with OVA-BiP hybrid peptide/hsp70 (Fig. 4f). No significant protection or improvement in survival was observed in mice injected with hsp70 alone, OVA peptide alone, OVA-BiP hybrid peptide alone, or OVA peptide plus hsp70 compared with an untreated control group (Fig. 4). Immunization with OVA-BiP hybrid peptide/hsp70 showed significant tumor protection ($P < 0.01$, generalized Wilcoxon test) (Fig. 4). Although mice immunized with OVA-BiP hybrid peptide/hsp70 had a trend toward longer survival compared with mice immunized with OVA plus Titer-Max adjuvant, this did not reach significance ($P > 0.10$).

Tumor protection was confirmed in a second tumor model by using E.G7, a mouse T cell lymphoma transfected with OVA gene. Delay in growth of E.G7 tumors was observed in mice immunized with OVA-BiP/hsp70 ($P < 0.01$), but no significant effects were observed with OVA alone or OVA-BiP peptide alone (data not shown). We then asked whether therapeutic immunization with OVA-BiP peptide/hsp70 would have anti-tumor effects if immunization were started after tumor challenge. When immunization was started 5 days after challenge with MO4 tumor cells, a delay in outgrowth of tumors was observed, which was associated with a significant improvement in survival ($P < 0.01$) (Fig. 5).

Activation of Cytokine Secretion from Cultures of Bone Marrow-Derived DC by hsp Preparations. Hybrid peptide/hsp70 elicited CTL responses in the absence of $CD4^+$ T cells. This result suggested that this immunization strategy bypasses T cell help, perhaps by directly activating antigen-presenting cells that can prime CTL responses. To explore possible roles for hsp70 in activating antigen-presenting cells to prime T cell responses, we investigated whether hsp70 and hybrid peptide/hsp70 complexes might directly activate bone marrow-derived DC. Hsp70 or hybrid peptide/hsp70 complexes were coincubated with cultured bone marrow-derived DC for 24 h, and secretion of cytokines was monitored. Hybrid peptide/hsp70 complexes as well as hsp70 alone were inducers of inflammatory cytokines, including TNF- α , IL-1 β , IL-6, and IL-12 (Fig. 6). Hybrid peptide alone and medium were not active (Fig. 6 and data not shown). Because hsp70 was purified from bacteria, we investigated whether DC activation could occur independently of signaling by endotoxin. Cytokine induction was not inhibited in the presence of polymyxin B sulfate, which is an antibiotic that binds soluble LPS and that completely blocked the activation of DC by LPS (Fig. 6 and

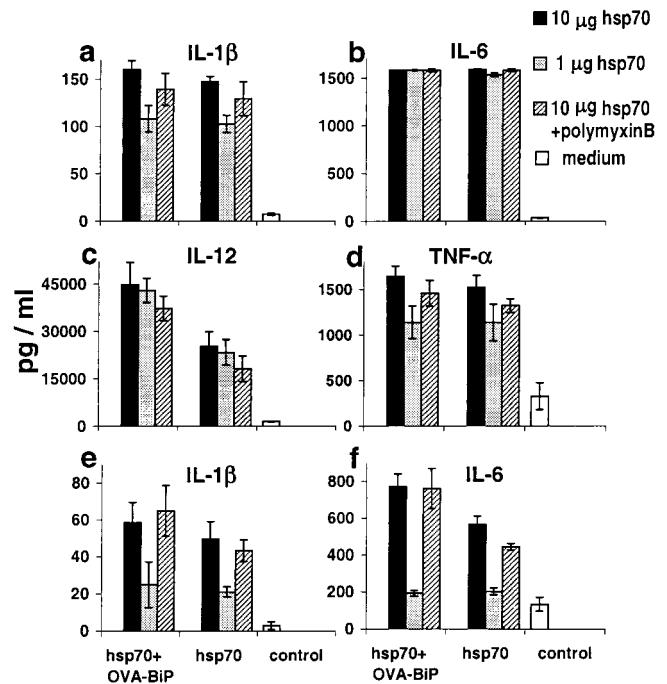


Fig. 6. Cytokine secretion by cultured DC incubated with OVA-BiP hybrid peptide/hsp70 or hsp70. Bone marrow-derived DC from C57BL/6 mice (a–d) or C57BL/10ScN mice (e and f) were cultured for 6 days as described in *Materials and Methods* and then incubated for 24 h with hsp70 plus or minus OVA-BiP hybrid peptide, with or without polymyxin B, or with tissue culture medium alone. Secretion of IL-1 β , IL-6, IL-12, or TNF- α was measured. Hsp70 complexed with OVA-BiP hybrid peptide gave the same results as with hsp70 alone. OVA-BiP hybrid peptide alone induced no significant secretion of cytokines (data not shown).

results not shown). More importantly, hsp70 and hybrid peptide/hsp70 complexes were able to induce cytokine release in LPS-resistant DC cultured from C57BL/10ScN mice that do not express toll-like receptor 4, which mediates LPS signaling (Fig. 6). Incubation with equimolar concentrations of bovine serum albumin and four purified recombinant proteins made in *E. coli* did not induce specific cytokine release (data not shown). DC from C57BL/10ScN are not absolutely resistant to LPS; they do respond to purified LPS by cytokine release, but require ≥ 100 ng LPS, which is $>2,000$ -fold more than any possible LPS contamination in purified hsp70 (data not shown). These results show that hsp70 induces cytokine release by DC.

Discussion

Immune adjuvants are a crucial part of vaccine construction, particularly those that are designed to elicit T cell responses (19). At this point, no potent immune adjuvants that induce cellular immunity are broadly approved for human use. Therefore, there is a need for well-tolerated, immune adjuvants that induce T cell responses. hsp proteins are candidates for immune adjuvants that induce T cell responses. Srivastava and his coworkers as well as others (3–7) have demonstrated that hsp70 or gp96 preparations isolated from tumor cells are able to induce specific CTL responses and tumor protection. Other reports have shown that immunization with recombinant hsp70 and a peptide epitope from lymphocytic choriomeningitis virus induced specific CTL responses and virus protection (21).

However, there are limitations to immunization strategies when using hsp proteins and bound peptides isolated from cells or tissues of individuals. First, potentially immunogenic peptides may not bind to hsp proteins, yet still bind MHC class I molecules

and stimulate T cells. Second, the isolation and purification of hsp with bound peptides from human tissues and tumors are relatively complicated. Third, the yield of purified hsp-peptide complexes can be low, and, therefore, substantial amounts or special handling of tissues and tumors may be required. Fourth, hsp preparations must be made for each patient, and only patients with sufficient tissue or tumor are candidates for this treatment. Furthermore, the tissue or tumor must be accessible to surgical excision.

We demonstrate that hybrid peptides containing an antigenic peptide with an hsp70-binding domain complexed to hsp70 can enhance the potency of CTL induction and *in vivo* immunity. The increased efficacy of hybrid peptides is due to their ability to bind with higher affinity to hsp70 and perhaps other hsp. We even detected weak CTL responses after immunization with hybrid peptides alone, without recombinant hsp70, presumably because of binding of these peptides to cellular hsp induced or released during immunization. CTL responses also were detected in the absence of CD4⁺ T cells, suggesting that this strategy could be used in diseases in which helper T cells are deficient, such as HIV-related diseases. The hybrid peptide strategy can be applied broadly to other antigenic peptides, particularly peptides that are recognized by T cells but do not efficiently bind to hsp70.

The priming of CTL is likely due, at least in part, to the capacity of hsp70 to deliver peptides to MHC class I pathways of antigen-presenting cells. However, strong induction of CTL by peptide/hsp70 complexes may require mechanisms other than simply increasing efficacy of antigen presentation through delivery of peptide to the MHC class I pathway. An additional and

potentially synergistic mechanism that can be considered is based on the observation that effective priming of CD8⁺ CTL requires activation of antigen-presenting cells (22–24). For DC, this is known to happen through interactions between CD40 ligand on CD4⁺ T cells and CD40 on DC or through activation of DC by bacterial LPS or virus infection. Bacterial hsp60 (groEL) has been shown to activate monocytic cell lines (25), suggesting that hsp can activate antigen-presenting cells for competence to prime CTL responses.

The hypothesis that hsp70 can provide signals to antigen-presenting cells inducing competence for priming T cells is attractive and is one potential explanation for its adjuvant effects. Tissue stress or damage could provide sources of hsp70 that alert antigen-presenting cells for potential pathogens, directly linking tissue damage to acquired immunity. Our findings show that hsp70 induces activation of DC measured by cytokine secretion, although it remains to be determined whether hsp70 could induce maturation of DC. These findings lead to the hypothesis that hsp proteins released during tissue damage, or injected by immunization as we have done, activate resident or recruited DC. Activated DC presenting cognate peptides then efficiently prime or activate CD8⁺ T cells. One prediction of this model is that CTL priming can be independent of CD4⁺ T cells, a prediction that was confirmed.

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