

## Cutting Edge: Tumor Secreted Heat Shock-Fusion Protein Elicits CD8 Cells for Rejection<sup>1</sup>

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**The endoplasmic reticulum resident heat shock protein gp96 chaperons peptides, including those derived from tumor Ags, on their way to presentation by MHC class I. Replacement of the endoplasmic reticulum retention signal of gp96 with the Fc portion of murine IgG1 generated a secretory form of gp96, gp96-Ig. Tumor cells secreting gp96-Ig exhibited decreased tumorigenicity and increased immunogenicity in vivo and were rejected after initial growth. Rejection required CD8 T cells during the priming and effector phase. CD4 T cells were not required for rejection in either phase. Carrageenan, a compound known to inactivate macrophages in vivo, did not diminish CD8-mediated tumor rejection. Therefore, immunization with tumors secreting gp96-Ig generates efficient tumor-rejecting CD8 CTL without requirement for CD4 or macrophage help. In contrast, immunization with purified, tumor-derived gp96 or with irradiated tumor cells requires both. *The Journal of Immunology*, 1999, 163: 5178–5182.**

**T**he heat shock protein (hsp)<sup>3</sup> gp96, localized in the endoplasmic reticulum (ER), is thought to serve as a chaperon for peptides on their way to MHC class I and II molecules (1–4). Gp96-chaperoned peptides comprise the entire spectrum of peptides and larger protein fragments generated in cells and transported into the ER (5–9). Gp96 obtained from tumor cells and used as a vaccine induces specific tumor immunity (3, 10–13), presumably through the transport of tumor-specific peptides to APCs.

We developed a secretory form of gp96, gp96-Ig, and tested it in tumor models. Transfection of tumor cells with the cDNA for gp96-Ig resulted in gp96-Ig secretion. As shown in this publica-

tion, gp96-Ig-secreting tumor cells caused powerful immunization and tumor rejection in vivo dependent exclusively on CD8 cells.

### Materials and Methods

#### Cell lines

All cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured in medium with 10% FCS. Human small cell lung carcinoma (SCLC) cell lines (SCLC-2 and SCLC-7) were established as described (14). Chicken OVA cloned into the expression vector, apc-NEO-OVA, was kindly provided by Dr. M. Bevan (Seattle, WA) (15) and used to transfect Lewis lung carcinoma (LLC).

#### Construction of gp96-Ig

To generate the gp96-Ig fusion protein, the KDEL sequence was deleted and replaced with the hinge, CH2 and CH3 domains of murine IgG1 (16–23); double-stranded cDNA was prepared from Jurkat DNA (24) with the GeneAmp RNA PCR Kit (Perkin-Elmer Cetus, Norwalk, CT) and amplified by PCR. The PCR primers were 5'-ATTACTCGAGGCGCCGACGC CATGAGGG-3' and 5'-GCCCGGATCCTTCAGCTGTAGATTCCTT TGC-3' (18, 19). The PCR primers included an *XhoI* site (forward primer) and a *BamHI* site (reverse primer). The hinge, CH2 and CH3 domains of murine IgG1, was amplified by using murine IgG1 cDNA as a template and mutating the three cysteines of the hinge portion to serines (21, 25). The PCR primers were 5'-GCGAGGATCCGTGCCAGGGATTCTGGTT CTAAG-3' and 5'-CTAAGCGGCCGCAAGGACACTGGGATCATTT ACCAGG-3'. The PCR primers included a *BamHI* site (forward primer) and *NotI* site (reverse primer). Gp96 was inserted into *XhoI* and *BamHI* sites of the eukaryotic expression vector, pBCMGSNeo and pBCMHis (26–29), and transfected into SCLC-2, SCLC-7, B16F10, MC57, LLC NIH3T3, EL4, E.G7, and P815. Transfected cells were selected with 1 mg/ml of G418 or 2.5–10 mM of L-Histidinol (Sigma, St. Louis, MO).

#### ELISA

This was conducted using Abs to the Ig tag. Gp96-Ig-producing cells were plated at 10<sup>6</sup>/ml in AIMV or IMDM with 10% FCS, and culture supernatants were harvested at different time points. For analysis of intracellular expression of gp96-Ig, cells were lysed by three freeze-thaw cycles and centrifuged 60 min at 13,000 × g (30).

#### Purification of gp96-Ig fusion protein

Gp96-Ig was purified by affinity chromatography on a protein A column using standard procedures (Bio-Rad, Hercules, CA) (31). The concentration of gp96-Ig was determined by the Micro BCA protein assay reagent kit (Pierce, Rockford, IL). SDS-PAGE and Western blotting were done using a standard procedure.

#### FACS analysis

For membrane staining of gp96-Ig-transfected SCLC, cells were stained with goat anti-mouse IgG-FITC or goat anti-rabbit IgG-FITC as a control for 15 min at 4°C and analyzed by a Becton Dickinson FACScan flow cytometer (San Diego, CA). For intracellular staining, cells were fixed with 4% paraformaldehyde and permeabilized with 1% saponin followed by staining with goat anti-mouse IgG-FITC, goat anti-mouse IgG-PE, goat

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Received for publication August 12, 1999. Accepted for publication September 15, 1999.

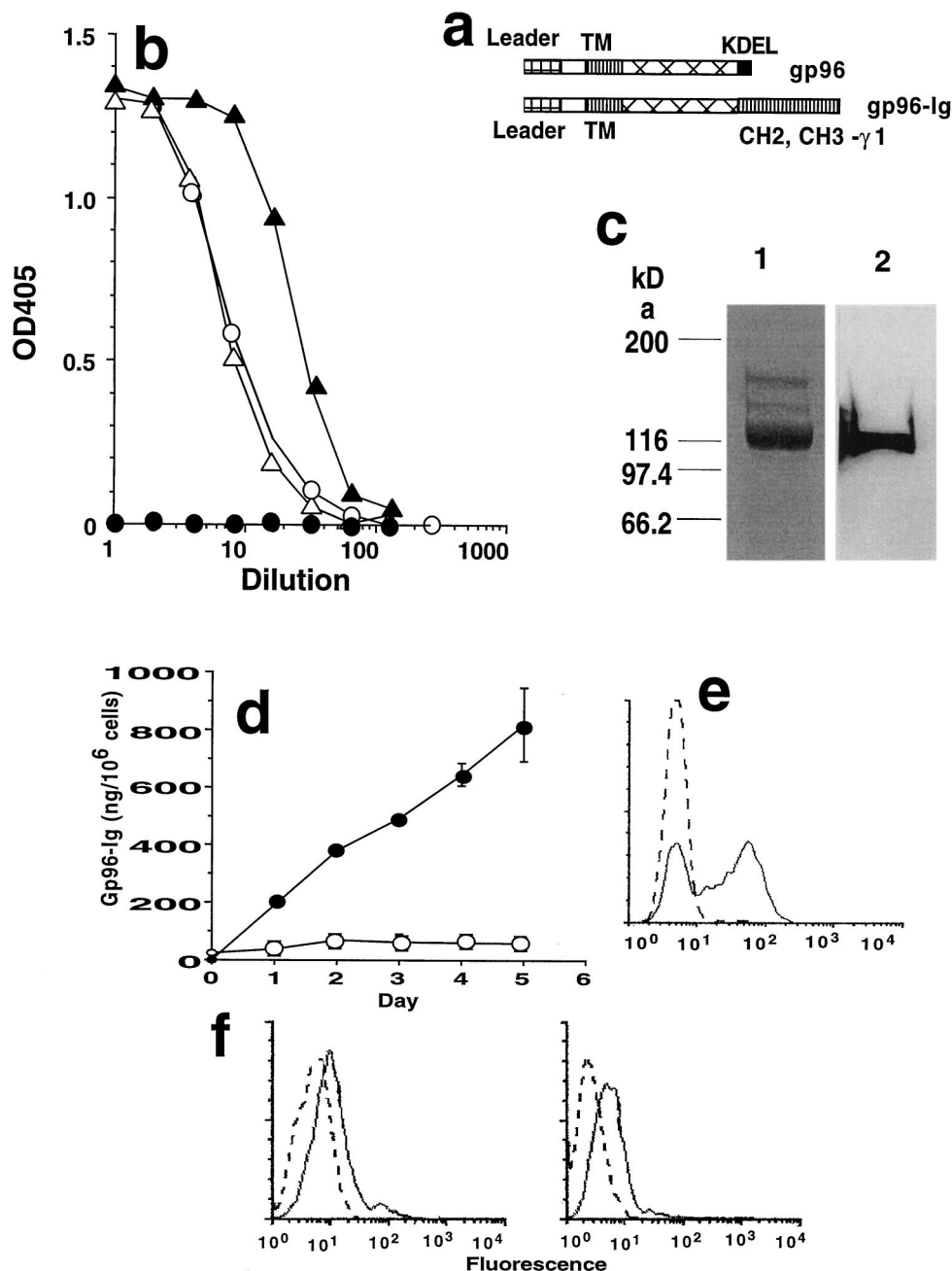
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<sup>1</sup> This work was supported by National Institutes of Health Grants CA590351, CA39201, and CA80228 and Department of Defense Grant DAMDS17-98-1-8317.

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<sup>3</sup> Abbreviations used in this paper: hsp, heat shock protein; ER, endoplasmic reticulum; gp96-Ig, secretory form of gp96; SCLC, small cell lung carcinoma; LLC, Lewis lung carcinoma.

**FIGURE 1.** Characterization of gp96-Ig. *a*, Schematic representation of the gp96-Ig construct. *b*, ELISA for gp96-Ig of supernatants from gp96-Ig cDNA-transfected and untransfected SCLC-7; cells were plated at  $10^6$ /ml, and supernatants were harvested on day 3 and day 6; purified mouse IgG (500 ng/ml) served as standard.  $\circ$ , Murine IgG standard (500 ng/ml);  $\triangle$ , SCLC-7 gp96-Ig on day 3;  $\blacktriangle$ , SCLC-7 gp96-Ig on day 6. *c*, SDS-PAGE of protein A-purified gp96-Ig. Lane 1, Coomassie blue stain (1  $\mu$ g protein); lane 2, Western blot with monoclonal anti-gp96-Ig (anto-Grp94, 9G10) (100 ng protein). *d*, Accumulation of gp96-Ig.  $\bullet$ , Gp96-Ig in the culture supernatant;  $\circ$ , Gp96-Ig in cell lysates. Gp96-Ig was quantitated by ELISA; SCLC-gp96-Ig was plated at  $10^6$ /ml. *e*, Intracellular staining of gp96-Ig by FACS analysis of permeabilized SCLC-gp96-Ig; dashed line, goat anti-rabbit IgG-FITC (negative control); solid line, goat anti-mouse IgG-PE. *f*, SCLC surface stain and SCLC-gp96-Ig surface stain. Absence of surface stain for gp96-Ig on unpermeabilized SCLC; *left*, untransfected; *right*, gp96-Ig-transfected SCLC. Dashed line in both panels is goat anti-rabbit IgG-FITC; solid line, goat anti-mouse IgG-FITC.



anti-rabbit IgG-FITC, or goat anti-syrian hamster IgG-FITC for 15 min at 4°C and analyzed by a flow cytometer.

#### Tumor inoculation and vaccination

Tumorigenicity *in vivo* was determined by s.c. injection of live tumor cells in 200  $\mu$ l PBS into the flanks of mice. The size of tumors was measured in two dimensions twice weekly for at least 2 mo. When mean tumor growth exceeded 10 mm diameter, the mice were sacrificed.

Mice were immunized by s.c. injection of  $10^6$  live E.G7-gp96-Ig or irradiated E.G7 as a control (in 200  $\mu$ l PBS), given in the right flank. Two immunizations at 2-wk intervals were given. Two weeks later, mice were challenged by s.c. injections of the indicated number of live tumor cells (EL4, E.G7, LLC, or LLC-OVA in 200  $\mu$ l PBS) into the left flank.

#### Depletion of T cells or macrophages *in vivo*

A total of 100  $\mu$ g of GK1.5 (anti CD4) or 2.43 (anti CD8) in 200  $\mu$ l PBS was administered by i.p. injection (32, 33). Depletion of CD4 and CD8 cells was verified by FACS analysis. CD4 or CD8 levels remained low (>95% depletion) for >2 wk following Ab injection (data not shown). For

functional inhibition of macrophages, 1 mg of Carrageenan (type II; Sigma) in 200  $\mu$ l PBS was administered by i.p. injection (32, 34).

## Results

The ER-resident hsp gp96 purified from tumor cells can provide tumor-specific immunity (35). The C-terminal sequence KDEL of gp96 serves as ER retention signal. Deletion of this sequence resulted in the secretion of gp96 together with bound peptides from transfected tumor cells and may render tumors more immunogenic to allow tumor rejection by the immune system.

#### Characterization of secreted hsp gp96-Ig

Replacing the KDEL sequence of gp96 with the hinge, CH2 and CH3 domain of murine IgG1 (Fig. 1a), an Ig isotype inefficient in Fc receptor binding, and transfection of the cDNA into tumor cells resulted in the secretion of gp96-Ig into the culture supernatant,

Table I. Secretion of gp96-Ig into culture supernatants<sup>a</sup>

Cell Lines	Gp96-Ig/10 <sup>6</sup> Cells × 24 h (ng)
SCLC-2	140
SCLC-7	500
NIH3T3	500
EL4	160
E.G7	60
P815	<5
LLC	70
B16F10	312.5 <sup>b</sup>
MC57	3,300

<sup>a</sup> Gp96-Ig cDNA was expressed in a bovine papilloma virus-derived episomal vector under the CMV or metallothioneine promoter.

<sup>b</sup> Metallothioneine promoter.

where it was quantitated by ELISA (Fig. 1*b*). Protein A purified gp96-Ig upon SDS-PAGE migrated with a major band of the predicted molecular mass of 120 kDa for the fusion protein and two minor, higher molecular bands previously reported also for unmodified gp96 (Fig. 1*c*) (10). Western blotting with a mAb specific for gp96 confirmed the identity of the fusion protein. Only the major band is stained, suggesting that the minor bands are glycosylation variants of gp96 not recognized by the Ab.

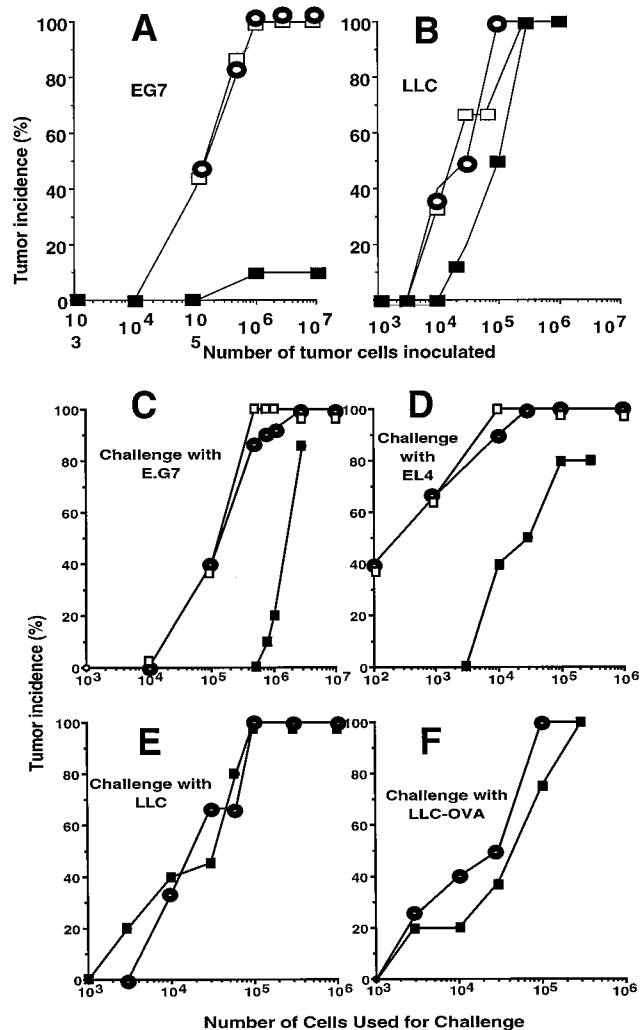
Secretion of gp96-Ig resulted in its time-dependent, linear accumulation in the supernatant (Fig. 1*d*). Intracellular gp96-Ig was detected at a low and constant steady-state level in lysates of transfected cells, indicating that it does not accumulate in the cell. FACS analysis of membrane-intact, transfected tumor cells revealed no staining with anti-mouse IgG above background, indicating that the Ig moiety of the fusion protein is not displayed on the outer leaflet of the plasma membrane (Fig. 1*f*). In contrast, upon permeabilization of the membrane, gp96-Ig is detected intracellularly with a goat anti-mouse IgG Ab, but not by control goat anti-rabbit IgG Abs (Fig. 1*e*). The transmembrane domain of gp96 does not interfere with the secretion of gp96-Ig and does not lead to intracellular accumulation. These data are consistent with previous reports suggesting that the transmembrane domain is not used for anchoring of gp96 in the membrane and that gp96 is not an integral membrane protein (36).

All murine and human cell lines transfected with gp96-Ig secreted the fusion protein (Table I). Mock-transfected cells did not secrete gp96-Ig. E.G7 is an OVA transfectant of the EL4 lymphoma forming lethal tumors in syngeneic C57BL/6 mice. Gp96-Ig transfection of E.G7 allows the determination whether E.G7-gp96-Ig immunizes against the EL4 parent tumor in addition to E.G7, the OVA surrogate Ag-transfected tumor. As second tumor, LLC transfected with gp96-Ig or with OVA was used because, in contrast to E.G7, it is a nonhemopoietic, low-immunogenic tumor. Both cell lines secrete comparable amounts of gp96-Ig (Table I).

#### Secreted gp96-Ig is responsible for decreased tumorigenicity

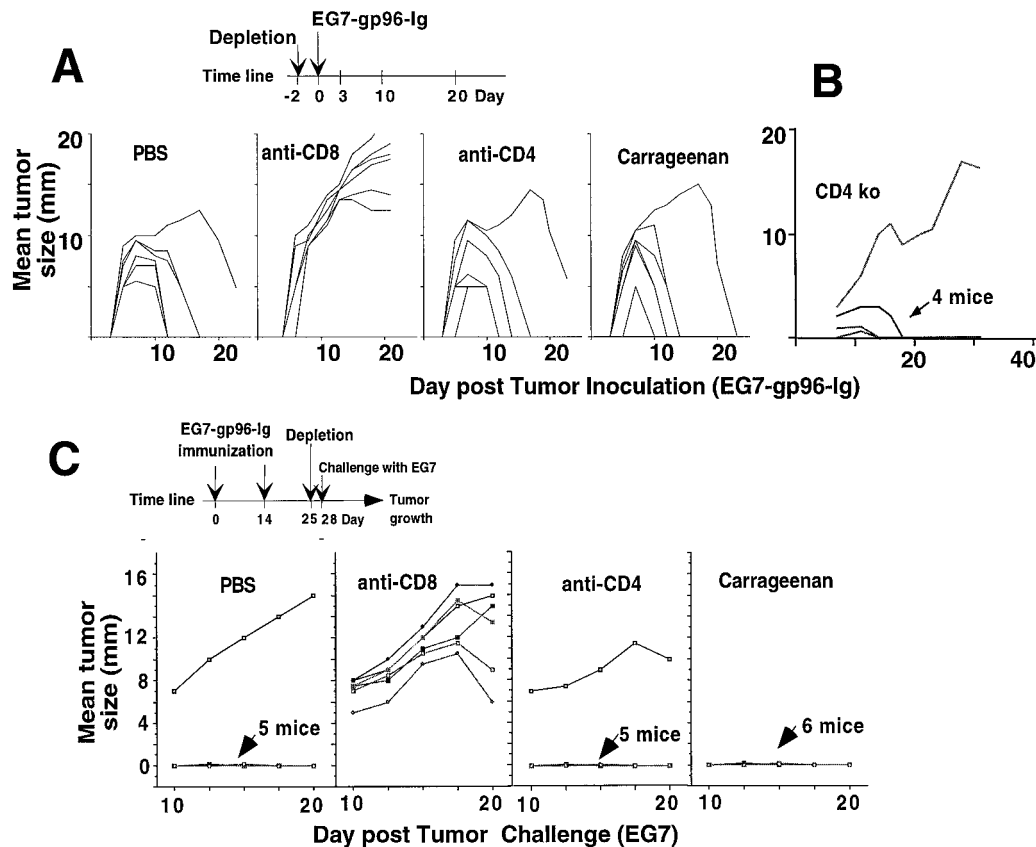
Secretion of gp96-Ig decreases the tumorigenicity of E.G7 in C57BL/6 mice by >100-fold when compared with mock-transfected or untransfected E.G7. Subcutaneous inoculation of 10 million hsp-secreting tumor cells caused tumors in only 10% of the inoculated mice (Fig. 2*A*). A similar reduction of tumorigenicity by gp96-Ig secretion was observed with transfected EL4 (data not shown). Gp96-Ig secretion by LLC resulted in a more moderate, ~5-fold, decrease of tumorigenicity (Fig. 2*B*).

To determine immunogenicity and immune memory responses, C57BL/6 mice were immunized twice at 2-wk intervals with a



**FIGURE 2.** Decreased tumorigenicity of gp96-Ig-transfected E.G7 (A) and LLC (B). ■, Gp96-Ig transfected; ○, mock transfected; and □, untransfected cells. Groups of six mice were used per dose of inoculated cells. C–F, Secretory gp96-Ig vaccination generates tumor-specific memory. C57BL/6 mice were immunized twice in biweekly intervals with 10<sup>6</sup> gp96-Ig-transfected E.G7 (■ in all panels), with 10<sup>6</sup> irradiated E.G7 (□), or not immunized (●). Two weeks later, mice were challenged (six mice per group) with the number of tumor cells indicated in the panels. Mice not developing tumors were observed for 3 mo and then judged tumor free.

dose of nonirradiated E.G7-gp96-Ig (10<sup>6</sup>) that was rejected. Subsequently, they were challenged with untransfected or mock-transfected E.G7, parental EL4, untransfected LLC, and OVA-transfected LLC (Fig. 2, C–F). Mice immunized with irradiated E.G7 or unvaccinated mice served as controls. E.G7-gp96-Ig-immunized mice resisted a 10-fold higher tumor challenge by E.G7 than mice vaccinated with irradiated cells or unimmunized mice (Fig. 2C). Tumor growth in vaccinated mice was frequently delayed. The effect of immunization was even more pronounced when challenged with EL4, allowing a fifty-fold dose increase of EL4 challenge compared with the controls (Fig. 2D). As expected, E.G7-gp96-Ig immunization offered no protection against challenge with untransfected or vector-transfected LLC (Fig. 2E), while a moderate, ~3-fold, increase in protection was observed when OVA-transfected LLC were used as challenge (Fig. 2F). The strong protection of mice immunized with E.G7-gp96-Ig against EL4 challenge may be due to multiple tumor Ags shared by E.G7 and



**FIGURE 3.** A, Effect of depletion of immunocompetent cells on the rejection of  $10^6$  E.G7-gp96-Ig during the priming phase; controls received PBS. Tumor growth curves of individual mice are shown. The depletion schedule is shown schematically on top. Depletion of immunocompetent cells was done with anti-CD8, anti-CD4, or Carrageenan 2 days prior to inoculation of  $10^6$  E.G7-gp96-Ig. B, CD4-deficient mice can reject E.G7-gp96-Ig. Five CD4-deficient mice were challenged with unirradiated  $10^6$  E.G7-gp96-Ig s.c. Tumor growth was recorded, and the mean tumor diameter is reported. C, Effect of depletion of immunocompetent cells on the effector phase of E.G7 rejection. The schedule of immunization and immunodepletion is shown schematically on the top. For immunization,  $10^6$  unirradiated E.G7-gp96-Ig were inoculated twice s.c. into groups of six mice. Three days prior to challenge with  $10^6$  E.G7, immune cells were depleted as above; controls received PBS. Tumor growth was recorded and is reported as mean tumor diameter.

EL4. The weak protection against challenge with LLC-OVA depends on T cells recognizing a single or limited number of epitopes derived from the OVA surrogate Ag for T cell recognition.

#### CD8 cells are required in the priming and effector phase

The involvement of immune mechanisms in the rejection of E.G7-gp96-Ig was further examined by *in vivo* depletion/inactivation of immunocompetent cells. It has been reported that Meth A tumor-derived gp96 requires CD4 cells, CD8 cells, and macrophages for effective immunization, while immunization with irradiated Meth A tumor cells required CD4 and CD8 cells but no macrophages (3).

For priming one million unirradiated, live E.G7-secreting gp96-Ig were inoculated s.c. This dose is sufficient to establish tumors that grow to a mean diameter of about 8 mm, subsequently shrink, and are rejected. Tumor rejection is blocked in mice treated with the anti-CD8 Ab 2.43, either 2 days before (Fig. 3A) or up to 3 days after tumor inoculation (not shown). The anti-CD4 Ab GK1.5 had no effect on tumor rejection (Fig. 3A) regardless of time of injection, even though it completely depleted CD4 cells for >14 days (data not shown). CD4-deficient mice were able to reject E.G7-gp96-Ig (Fig. 3B), supporting the importance of CD8 cells. E.G7 not secreting gp96-Ig forms tumors in untreated and immune-depleted mice. Carrageenan, known to inactivate macrophages *in vivo* (34), had no effect on tumor rejection. However, because the effect of Carrageenan is difficult to assess, these ex-

periments cannot rule out a role for APCs in the generation of CD8 CTL.

To study the effector phase of tumor rejection, mice were immunized twice at 14-day intervals with live E.G7-gp96-Ig. Eleven days later (day 25), immune cells were depleted, and after 3 days the mice were challenged with untransfected E.G7. Only CD8 cells are required in the effector phase; depletion of CD4 cells or Carrageenan inactivation of macrophages had no influence on E.G7 rejection in the effector phase (Fig. 3C).

#### Discussion

Deletion of the endoplasmic retention signal of gp96 and replacement with the Fc portion of IgG1 readily results in the secretion of gp96-Ig, which appears to be dimerized through the IgG1 H chain. E.G7-secreted gp96 is able to provide long-lasting specific immunity, suggesting that it chaperons tumor peptides. In contrast, irradiated or mock-transfected E.G7 are not able to provide protective immunity. *Corynebacterium parvum* also failed to serve as adjuvant for E.G7 immunization (37). Secreted gp96-Ig provides immunologic specificity for both the surrogate Ag OVA and other EL4 Ags, but does not cross-immunize to LLC-derived tumor Ags.

The data are consistent with the explanation that peptides associated with secreted gp96-Ig are transferred to and presented by class I MHC and stimulate a tumor-specific CD8<sup>+</sup> CTL response



causing tumor rejection. The CD8 response appears to be independent of CD4 help and does not require macrophages. Whether the cellular requirements are due to gp96-Ig dimerization is not known.

It is instructive to compare the mechanisms of immunization by purified tumor-derived gp96 and by tumor-secreted gp96-Ig. Udono et al. (32), using gp96 purified from Meth A tumor cells for immunization, reported a requirement for CD8 cells and macrophages in the priming phase and a requirement for CD4 and CD8 cells as well as macrophages in the effector phase of tumor rejection of Meth A tumors. Immunization with irradiated Meth A tumors required CD4 cells in the priming phase, and both CD4 and CD8 cells in the effector phase. Irradiated EG7 do not produce immunity against subsequent challenge. The dramatic effect of tumor-secreted gp96-Ig is entirely dependent on CD8 cells without CD4 help. CD8 cells are required in the priming and effector phase of the CTL response to the tumor. Macrophages appear not to be needed. The role of dendritic cells or other APCs in the presentation of gp96-chaperoned peptides to CD8 cells is not known, but remains a possibility. It is also possible that gp96-Ig-secreting EG7 stimulate CD8 cells directly.

Regardless of the underlying mechanism, tumor-secreted gp96-Ig is a powerful vaccine for some tumors. The precise mechanism CD8 CTL activation by tumor-secreted gp96-Ig and its action in different tumors remains to be determined.

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