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Peptides chaperoned by heat-shock proteins are a necessary and sufficient source of antigen in the cross-priming of CD8+ T cells

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The form in which antigens are transferred from cancer cells or infected cells to antigen-presenting cells as a part of the process of priming CD8+ T cells has been a longstanding unresolved issue. Intact proteins or protein fragments in the form of free peptides or peptides chaperoned by heat-shock protein are possible sources of antigen. We address this here using β-galactosidase and ovalbumin. Immunization with cell lysates containing intact proteins and heat-shock protein–peptide complexes or with cell lysates depleted of either component demonstrated that protein fragments chaperoned by heat-shock protein and not intact protein were the necessary and sufficient source of antigen transferred to antigen-presenting cells for priming CD8+ T cell responses.

Antigens derived from cancers or infected cells are captured by local antigen-presenting cells (APCs). The APCs process these antigens and re-present antigenic epitopes derived from them on their major histocompatibility complex (MHC) class I molecules, a process known as cross-presentation. Concurrently, the APCs undergo maturation and migrate to the draining lymph nodes, where they can stimulate naive T cells[1]. The form in which antigens are captured by the APCs has been a long-standing unresolved issue in immunology. The possible forms of antigen in epithelial cells include free intact proteins, intact proteins in complex with other molecules, fragments of protein, or protein-derived peptides presented by MHC class I molecules or chaperoned by heat-shock proteins (Hsps).

Some studies have approached this issue using ovalbumin (OVA) protein as a model antigen and have reached divergent conclusions. Cross-presentation has been examined *in vitro* using cells engineered to express intact secreted OVA, cytosolic OVA or the precise H-2K^b-presented SIINFEKL epitope derived from OVA as antigen donors[2]. That study also examined the function of the proteasomes in generating the antigenic moieties required for cross-presentation and noted that neither the intact antigen nor the precise SIINFEKL epitope is the main form of transferred antigen[2]. Instead, a form of antigen longer than the precise epitope and shorter than the intact antigen is favored. In addition, proteasomal activity is required for generation of the antigenic moieties necessary for cross-presentation. A separate approach to this issue has biochemically tested the ability of subcellular fractions containing various forms of OVA to immunize and elicit H-2K^b–SIINFEKL–specific cytotoxic T lymphocytes (CTLs)[3]. Intact OVA and not the peptide fragments derived from it was found to be responsible for cross-priming *in vivo*. By deduction, there is no involvement of proteasomal activity in generating the antigenic moieties required for cross-presentation. Another study showing that the antigen transfer is mediated by a pre-proteasomal entity and not by proteasomal products also supports the idea that intact OVA is the form of antigen transferred during cross-presentation[4].

Here we have approached this issue using two independent antigen systems: β-galactosidase (β-gal) and OVA. Our data show that cross-presentation and priming required Hsp-peptide complexes and not intact antigen. Our conclusions are in apparent conflict with some of the studies discussed and in agreement with others. We show how some of the apparent inconsistencies in the literature stem from experimental lacunae that, when resolved, lead to a clear answer to the question.

RESULTS

Minimum immunogenic dose of free antigen

We first determined the minimum amount of free, soluble β-gal or OVA required to immunize mice so as to prime an antigen-specific, MHC class I–restricted CD8+ T cell responses to the H-2K^b-restricted epitopes 'embedded' in the two proteins (DAPIYTNV for β-gal; SIINFEKL for OVA)[5,6]. We immunized naive C57BL/6 mice once subcutaneously with titrated amounts (1 μg to 1 mg) of intact soluble β-gal or OVA. Because commercial preparations of these antigens are heavily contaminated with insoluble aggregates and bacterial lipopolysaccharide (LPS), we centrifuged the antigen preparation used for immunization at 100,000*g* for 90 min to remove aggregates and treated it to render it free of LPS. We then cultured spleen cells

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from immunized mice *in vitro* with DAPIYTNV-pulsed (for β-gal-immunized mice) or SIINFEKL-pulsed (for OVA-immunized mice) irradiated EL.4 cells to increase the frequency of antigen-specific T cells. After 5 d, we tested the cultures for cytotoxicity against cognate peptide–pulsed EL.4 cells. There was cytotoxic activity in cultures of spleen cells from mice immunized with a minimum of 5–10 μg β-gal or 75–100 μg OVA (**Fig. 1a**).

The OVA system provides an independent pentamer-based method for measuring the frequency of H-2K^b–SIINFEKL–specific T cells. We stained spleen cells from immunized mice *ex vivo* without stimulation *in vitro* using pentamers directed against H-2K^b-SIINFEKL–specific T cells and monoclonal antibody to CD8 and analyzed the cells by flow cytometry. Mice had to be immunized with a minimum of 50 μg OVA to develop a detectable H-2K^b–SIINFEKL–specific T cell response (**Fig. 1b** and **Supplementary Fig. 1** online). The response reached a plateau at a dose of 100 μg. Thus, the CTL and the pentamer assays yielded similar results in the system in which both assays could be used. These experiments show that the minimum immunogenic dose for free OVA and β-gal is in the range of 10–50 μg.

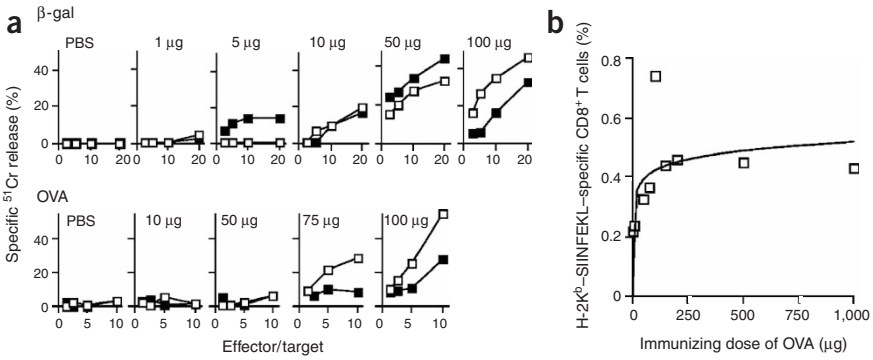

Figure 1 Amount of free antigen required to prime a CD8$^+$ T cell response. (**a**) Mice were immunized subcutaneously with aggregate-free and endotoxin-free β-gal or OVA (dose, above graph). Spleen cells were removed after a week and were cultured for 5 d with peptide (DAPIYTNV or SIINFEKL for β-gal or OVA, respectively), pulsed and irradiated EL.4 cells and were tested in a cytotoxicity assay against peptide-pulsed or unpulsed target cells. Two mice per group were used. Symbols (■ and □) represent the specific lysis of (peptide-pulsed minus unpulsed) targets by spleen cells from one individual mouse. (**b**) Percentage of H-2K^b–SIINFEKL–specific CD8$^+$ T cells in response to the immunizing dose of OVA, determined by flow cytometry. Positive staining above background staining (0 μg) was obtained when mice were immunized with 50 μg of OVA or more. Data represent the mean percentage of two mice in each group and are representative of three independent experiments.

Minimum immunogenic dose of cellular antigen

We next determined the minimum number of lysed, β-gal-expressing P13.4 cells (P815 cells transfected with the gene encoding β-gal[7]) or OVA-expressing E.G7 cells (EL.4 cells transfected with the gene encoding OVA[8]) as immunogens that were required to elicit H-2K^b–DAPIYTNV–specific or H-2K^b–SIINFEKL–specific T cells. We use cell lysates as immunogens because many viruses such as influenza and smallpox induce lytic cell death and necrotic cell lysis is nearly universal in growing tumors. Thus, there is considerable evidence that necrotic lysis is a potent immunogenic event for viruses and cancers[9,10]. We made a deliberate effort to prepare cell lysates with the minimum amount of manipulation *in vitro*.

We prepared lysates from titrated doses of P13.4 or E.G7 cells and used these to immunize mice. We detected CTL responses in mice immunized with lysates from a minimum of 1×10^5 P13.4 cells or 5×10^4 E.G7 cells or higher doses (**Fig. 2a**). Notably, higher doses of lysates did not lead to higher or even sustained CTL responses in either system. Immunization with P13.4 lysate from 1×10^8 cell equivalents or E.G7 lysate from 5×10^8 cell equivalents elicited significantly weaker response than immunization with lysates from 1×10^5 to 1×10^7 cell equivalents in either system ($P < 0.01$, 1×10^5 or 5×10^5 dose versus 1×10^8 or 5×10^8 dose in both systems). This 'dose window' of immunization with lysates was consistent and reproducible. In contrast, immunization with free intact antigen did

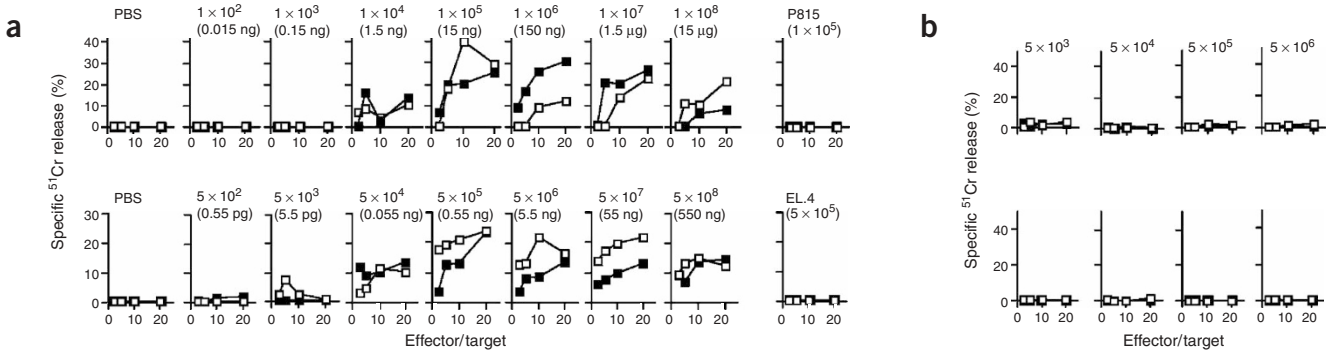

Figure 2 Amount of cell-associated antigen required to prime a CD8$^+$ T cell response. (**a**) Mice were immunized subcutaneously with P13.4 (top row) or E.G7 (bottom row) cell lysate. Above graphs, cell equivalents of lysate (in parentheses, intact β-gal or OVA in that quantity of lysate). Spleen cells were removed a week after immunization and were cultured with DAPIYTNV or SIINFEKL peptide for P13.4 or E.G7, respectively, and were analyzed by cytotoxicity assay 5 d later. The specific lysis of targets by spleen cells from individual mice is plotted as described in **Figure 1a**. Mice were also immunized with P815 or EL.4 lysate (far right) or PBS (far left) as specificity controls ($P < 0.01$, 1×10^5 or 5×10^5 dose versus 1×10^8 or 5×10^8 dose in both systems). Symbols represent the results from two individual mice (■ and □). (**b**) EL.4 lysate in titrated doses (above graphs) was spiked with 10 μg OVA (top row) or was left unspiked (bottom row) and mice were immunized with each preparation. After immunization, spleen cell cultures were generated as described in **a**. In other experiments, higher and titrated quantities of OVA were used to spike EL.4 lysates, whose immunogenicity was tested. Data from two mice in each group are presented and are representative of three independent experiments. Each line represents the specific lysis of (peptide-pulsed minus unpulsed) targets by spleen cells from one individual mouse.

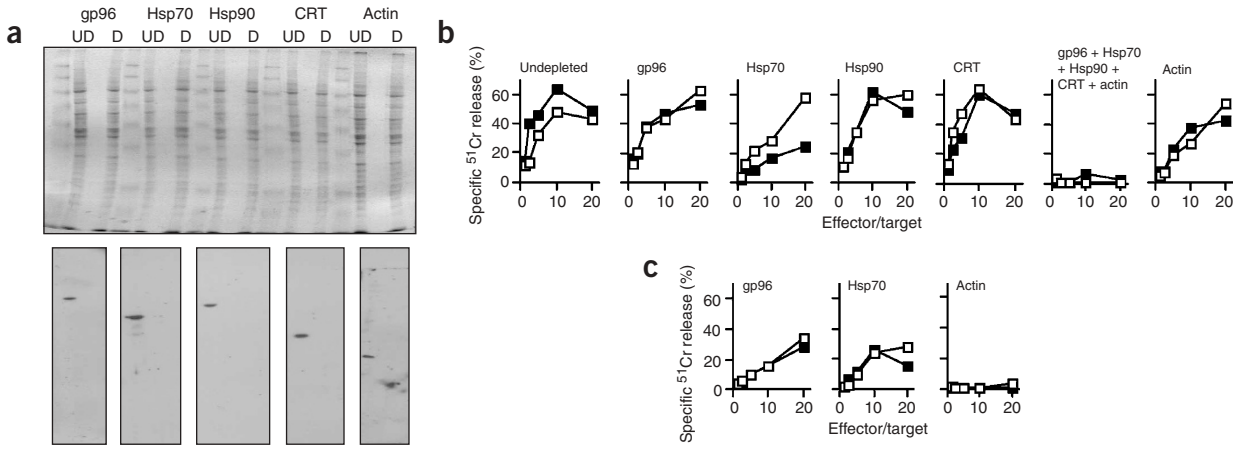

Figure 3 Hsps in lysates are necessary for priming of immune responses. (**a**) Cell lysates from P13.4 cells were depleted of various Hsps (above lanes) with antibodies specific for each Hsp. Silver-stained gel (top) and immunoblots (bottom) are of depleted (D) or undepleted (UD) lysates. The β-gal content of each lysate, depleted or undepleted, is comparable at 150 ng per 1×10^6 cell equivalents (data not shown). (**b**) Mice were immunized with lysate from 1×10^6 cell equivalents left undepleted (far left) or depleted of Hsps or actin (control; above graphs). Spleen cells were cultured and tested for cytotoxicity as described in **Figure 1a**. Symbols represent the specific lysis of (peptide-pulsed minus unpulsed) targets by spleen cells from one individual mouse (■ and □). (**c**) Lysates depleted of all four major Hsps and that had lost cross-priming activity were reconstituted with P13.4-derived gp96 or Hsp70 or actin. Each reconstituted lysate was used to immunize mice as described in **b**. Spleen cells were cultured and tested for cytotoxicity as described in **Figure 1a**. Each line represents the specific lysis of targets by spleen cells from individual mice. Data are representative of two independent experiments (■ and □).

not show 'dose restriction' (**Fig. 1a**,**b**). We noted the classical 'high dose tolerance' (data not shown) only with an immunizing dose of more than 1 mg of free soluble antigen. We detected no responses in mice immunized with lysates from fewer cell equivalents or with phosphate-buffered saline. We detected no H-2K^b–DAPIYTNV–specific or H-2K^b–SIINFEKL–specific responses in mice immunized with lysates of the parental P815 or EL.4 cells at any dose (**Fig. 2a** and data not shown). We determined the absolute quantity of intact antigen in the cell lysates in each titrated dose tested by enzymatic assay and immunoblot for β-gal and by enzyme-linked immunosorbent assay (ELISA) for OVA, as described[3] (**Fig. 2a**). The quantities of OVA present in E.G7 cells were similar to the quantities used before in OVA transfectants[3].

Comparison of the data in **Figs. 1** and **2a** demonstrates a notable anomaly. A minimum of about 10 μg of β-gal as immunogen was required to elicit the H-2K^b–DAPIYTNV–specific CTLs, whereas lysates of P13.4 cells containing as little as 15 ng β-gal elicited a comparable response. Thus, free β-gal was 0.1% as efficient as cell-associated β-gal in eliciting specific CTLs to the same epitope. Similarly, a minimum of 75 μg of OVA as immunogen was required to elicit H-2K^b–SIINFEKL–specific CTLs, whereas lysates of E.G7 cells containing as little as 550 pg OVA elicited a comparable response. Thus, free OVA was 0.001% as efficient as cell-associated OVA in eliciting specific CTLs to the same epitope.

To understand the anomaly between the minimum immunogenic doses of free versus cellular antigen, we used the OVA system to address the possibility that moieties present in cell lysates known to be endogenous adjuvants, such as Hsps[11], DNA[12] or uric acid[13], may have been responsible for the greatly enhanced immunogenicity of cells. We used EL.4 lysates titrated to different amounts of cell equivalents and 'spiked' these with a subimmunogenic amount of free OVA (10 μg, a quantity that failed to prime a response (**Fig. 1a**) but that was well above the quantity of cell-associated OVA successful in immunizing (**Fig. 2a**)), then immunized mice with the 'unspiked' or spiked lysates and tested them for H-2K^b–SIINFEKL–specific CTLs. None of the groups generated a CTL response (**Fig. 2b**), whereas mice immunized with E.G7 lysate (5×10^5 cell equivalents) as a positive control did so (**Fig. 2a**). In other experiments, we used larger and titrated quantities of OVA to spike EL.4 lysates and tested their immunogenicity as in **Figure 2**. Even with quantities of OVA up to 50 μg, we could detect no adjuvanticity of EL.4 lysates (**Supplementary Fig. 2** online). Hence, the orders of magnitude of difference between the immunogenicities of free OVA and E.G7 cell lysates cannot be explained by the 'adjuvanticity' of cell lysates.

Hsp-peptide complexes necessary for priming T cells

To determine if depleting P13.4 cell lysates of the major peptide-binding chaperones (glycoprotein 96 (gp96), Hsp90, Hsp70 and calreticulin) had an influence on the ability of the lysate to prime CTLs, we immunized C57BL/6 mice (H-2K^b haplotype) with undepleted lysates of 1×10^6 P13.4 cells (H-2K^d haplotype) or with lysates depleted individually of gp96, Hsp90, Hsp70 or calreticulin or of all four chaperones together or, as a negative control, of actin. We monitored depletion of individual chaperones from lysates by SDS-PAGE and immunoblot with antibodies to the individual chaperones (**Fig. 3a**); depletion was complete and did not alter the overall protein profile of the lysate in any detectable way. Furthermore, depleting the lysates of chaperones had no influence on the β-gal content of the lysates, which remained comparable in all samples at 150 ng per lysate of 1×10^6 cells. The undepleted or individually depleted lysates were equally capable of eliciting strong CTL responses that could be titrated. The differences between the undepleted lysate and each individually depleted lysate were not statistically significant ($P > 0.25$; **Fig. 3b**). However, P13.4 lysates depleted of all the major peptide-binding chaperones were completely devoid of the ability to elicit CTL responses, even though they contained the same quantity of β-gal (150 ng) as the other lysates ($P < 0.001$, compared with undepleted lysate). Furthermore, when these 'pan-depleted' lysates were reconstituted with gp96 or Hsp70 purified from P13.4 cells, they regained immunogenicity (**Fig. 3c**). Reconstitution with actin did not restore immunogenicity ($P < 0.001$, for gp96 or Hsp70

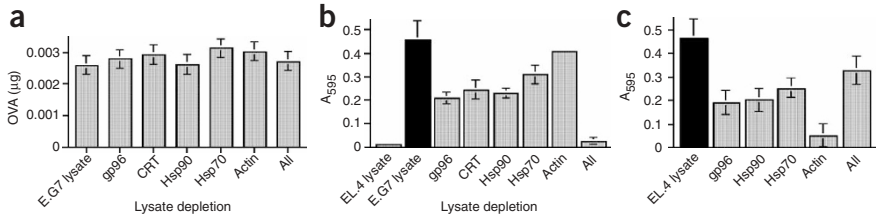

Figure 4 Hsps in lysates are necessary for presentation. (**a**) Soluble supernatants of E.G7 cell lysates were depleted of each individual Hsp or actin or a combination of Hsps (All). The OVA in each lysate was measured by ELISA. Data represent mean ± s.d. of two independent experiments done in triplicate. (**b**) Mice were immunized with E.G7 lysates from 1×10^6 cell equivalents containing or depleted of Hsps or actin (horizontal axis). Lymph node cells were removed 12 h after immunization and CD11c$^+$ cells were purified and used to stimulate B3Z cells. Stimulated B3Z cells were stained with X-gal and blue color was measured as absorbance at 595 nm (A_{595}). (**c**) Lysates depleted of all four major Hsps and that had lost antigenic activity were reconstituted with E.G7-derived gp96, Hsp90 or Hsp70 or all three (All) or with actin. Each reconstituted lysate was used to immunize mice; draining lymph nodes were removed and cross-presentation was measured as described in **b**. Data are representative of three independent experiments. Data in **b**,**c** represent mean ± s.d. from six individual mice.

reconstitution versus actin reconstitution). These results demonstrate that endogenous Hsp-peptide complexes as a group are essential for immunogenicity of P13.4 lysates, even though individual Hsps fulfill redundant functions.

Hsp-peptide complexes necessary for cross-presentation of OVA

We also tested the requirement for chaperone proteins in eliciting CTL responses in the OVA system, which allows for measurement of the intermediate step of antigen presentation rather than T cell priming. This measurement uses the B3Z hybridoma that synthesizes a reporter enzyme when its T cell receptor engages the H-2K^b–SIINFEKL complex[14]. Immunization of C57BL/6 mice with E.G7 lysates of 1×10^6 cells led to appearance in the draining lymph nodes of CD11c$^+$ cells that could stimulate B3Z. The antigenic activity resided entirely in the soluble rather than the membrane fraction of the lysate, thus eliminating the possibility of MHC class I–presented SIINFEKL as the source of antigen (data not shown). We depleted E.G7 lysates of the main peptide-binding Hsps individually or collectively as described before. All lysates, depleted or not, had identical amounts of OVA, as measured by ELISA (about 2.5 ng per lysate from 1×10^6 cell equivalents; **Fig. 4a**). Stimulation of the B3Z cells by CD11c$^+$ cells from mice immunized with undepleted E.G7 lysate elicited robust antigenic activity, whereas EL.4 lysate of the same number of cells did not (**Fig. 4b**). Depletion of any of the Hsps (gp96, Hsp90, Hsp70 or calreticulin) caused a loss of 30–50% the initial activity of the E.G7 lysate, whereas depletion of actin caused a loss of activity of about 10% of the undepleted lysate (**Fig. 4b**). Depletion of all four Hsps led to a depletion of the total activity of the undepleted lysate, even though this lysate contained the same quantity of OVA protein as the undepleted lysate (about 2.5 ng, as in **Fig. 4a**). Using lysate depleted of all four Hsps and containing no or baseline activity, we reconstituted it individually with gp96, Hsp90 or Hsp70 (in quantities isolated from the initial lysate) and tested each reconstituted lysate (**Fig. 4c**). Lysate reconstituted individually with gp96, Hsp90 or Hsp70 but not actin regained the ability to cross-present SIINFEKL. When we reconstituted the depleted lysate with a combination of gp96, Hsp90 and Hsp70, nearly 80% of the initial antigenic activity was recovered.

Hsp-peptide complexes are sufficient for priming

We also tested the involvement of intact antigen in cross-priming. In contrast to the experiments in **Figures 3** and **4**, we depleted cell lysates of the antigen rather than Hsps and tested the immunogenicity of the depleted lysate. We depleted E.G7 lysate of OVA using reagents and methodology that have been described[3]. The undepleted E.G7 lysate had about 2.5 ng OVA, whereas the depleted lysate did not have detectable OVA protein (**Fig. 5a**). Given the 'dose-restricted' nature of immunogenicity of lysates (**Fig. 2a**), we used the OVA-depleted and 'mock-depleted' E.G7 lysates to immunize mice with titrated doses (**Fig. 5b**). Mock-depleted E.G7 lysates were subjected to the depletion procedure in the same fashion as the OVA-depleted lysates but with isotype control antibodies. There was again a 'dose restriction' of activity (that is, the bell-shaped curve of immunogenicity in **Fig. 2a**), although the absolute numbers were somewhat skewed, presumably because of experimental variation. Depletion of OVA had no effect on the ability of the E.G7 lysate to elicit H-2K^b–SIINFEKL–specific T cells. The mock-depleted and OVA-depleted lysates had identical activity at all doses. We obtained identical results when we tested β-gal-depleted extracts for immunogenicity (**Fig. 5c**,**d**). Extracts depleted of β-gal had full immunogenicity within but not outside a 'dose-restricted window' between 1×10^5 and 1×10^7 cells. These

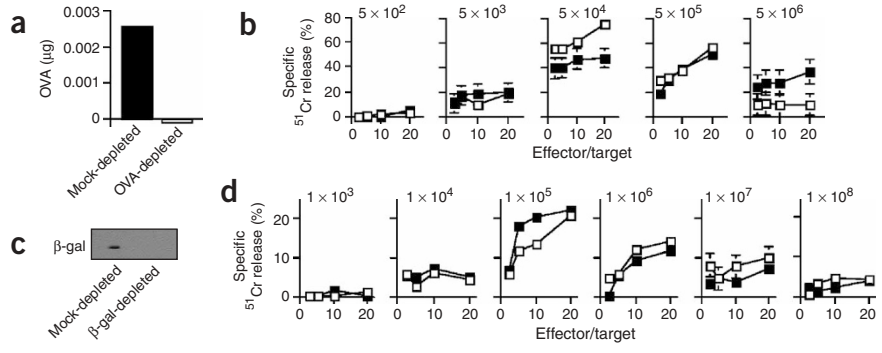

Figure 5 Cell-associated intact antigen is not required for priming of immune responses. (**a**) E.G7 lysates were mock-depleted or were depleted of OVA with anti-OVA; depletion was measured by ELISA. (**b**) Mice were immunized with titrated doses of mock-depleted (■) or OVA-depleted (□) E.G7 lysates. Spleen cell cultures were incubated with SIINFEKL peptide and were tested for cytotoxicity 5 d later. Each line represents the mean specific lysis of targets by spleen cells from two mice. Specific responses were monitored in a 'dose window' between 5×10^4 and 5×10^5 cell equivalents. (**c**) P13.4 lysates were mock-depleted or were depleted of β-gal with anti-β-gal; depletion was measured by immunoblot with anti-β-gal. (**d**) Mice were immunized with titrated doses of mock-depleted (■) or β-gal-depleted (□) P13.4 lysates. Spleen cell cultures were incubated with DAPIYTNV peptide and were tested for cytotoxicity 5 d later. Each line represents the mean specific lysis of targets by spleen cells from two mice. Data are representative of three independent experiments.

data demonstrate that the intact OVA or β-gal present within cells are not required for cross-priming and that endogenous Hsp-peptide complexes that contain the antigenic epitopes derived from these proteins are sufficient for cross-priming.

DISCUSSION

Here we sought to understand the nature of the antigen transferred from a 'nonprofessional' APC such as a cancer cell or a virus-infected cell to a 'professional' APC such as the dendritic cell. We approached this issue using a simple and comprehensive analytical tool. There are only three possible sources of antigen in a cell: intact protein; less-than-intact protein, such as defective ribosomal products[15]; and antigenic peptides generated from either of those. There are compelling data suggesting that the antigenic peptides are not free but are chaperoned by the peptide-binding Hsps[16–18]. The MHC-chaperoned peptides are excluded from discussion, because in a cross-priming system they are not candidates as antigen donors. We depleted cell lysates of intact antigen or Hsp-peptide complexes and determined which maneuver, if any, led to depletion of the transferable antigen. Four outcomes were possible. If depletion of the intact antigen led to loss of activity, intact antigen or less-than-intact antigen would represent the active principle. Abrogation of activity by depletion of Hsp-peptide complexes, in contrast, would indicate the antigenic peptides derived from the antigen, intact or otherwise. Diminished activity by depletion of either component would suggest a combined contribution of protein and peptides. Abrogation of activity by neither of the maneuvers would be unlikely and would call into question the assumptions about possible sources of antigen. The data we obtained are firmly consistent with the second outcome; that is, depletion of selected peptide-binding chaperones led to complete abrogation of antigenic activity in the two independent antigen systems we used here. Conversely, depletion of intact antigen had no effect on antigenic activity. From those observations, it can be deduced that chaperone-peptide complexes constitute the form of antigen transferred to APCs during cross-presentation and cross-priming by lysed cells.

A published study whose design, antigen system, reagents and 'readout' was very similar to ours here noted that depletion of intact OVA abrogates priming ability[3]. That study did not use depletion of chaperone-peptide complexes and concluded that the intact antigen was the source of cross-priming antigen[3]. This apparent discrepancy can be completely attributed to the 'dose restriction' of cross-priming activity of cell lysates: that study did not provide quantitative data on the amount of lysates used to immunize beyond a general comment that lysates from 2×10^6 to 5×10^6 cells were used to immunize mice. That dose range of cell lysate straddles the transition zone of dose restriction, as shown by our work here. The 'lability' of activity at this dose range was exemplified by a minor difference in immunogenicity as a consequence of OVA depletion at the highest effector/target ratio in the group of mice immunized with the largest number of lysate cell equivalents. Although the difference achieved significance at a single effector/target ratio of 20:1, it did not do so for the overall titration curve ($P > 0.5$). 'Dose titration' of the OVA-depleted extracts showed potent immunogenic activity in the depleted extracts. That observation is consistent with the known 'dose restriction' of immunogenicity of Hsp-peptide complexes[19–21] and further reinforces the hypothesis that Hsp-peptide complexes represent the active cross-priming entity. That study[3] also argues that membrane fractions from cells that express OVA solely as a membrane protein can cross-prime even though they do not contain Hsps. Its demonstration that membrane fractions are devoid of Hsps is possibly a consequence of the techniques used for detection, as a considerable body of literature over the years has indicated membrane localization of many Hsps, including gp96, Hsp90, Hsp70 and calreticulin[19,22–28].

Our data presented here have shown that the source of cross-priming antigen contains a tremendous amount of redundancy. Depletion of any single chaperone-peptide complex did not abrogate cross-priming even though it may have attenuated it to a degree; only when all the main chaperone-peptide complexes were depleted did the deficit become apparent. Thus, our data support the conclusion that gp96 itself alone is not responsible for cross-priming of OVA[29]. An apparent discrepancy in the results of **Figures 3** and **4** needs clarification. In **Figure 4**, the depletion experiments suggest that gp96, calreticulin and Hsp90 each carry approximately 50% of the available antigenic material. This clear effect of depletion of individual Hsps is in contrast to the data in **Figure 3**, in which depletion of individual Hsps had no consequence at all. This apparent discrepancy is resolved by the distinct end points measured by the assays in **Figures 3** and **4**: cross-priming and cross-presentation, respectively. Cross-presentation is the more proximal endpoint, and indeed there was a diminution in this parameter (quantity of H-2K^b–SIINFEKL on dendritic cells) when samples were depleted of individual Hsps. Cross-priming, in contrast is 'distal' to cross-presentation and involves an amplification step. Such amplification provides a 'leveling' effect, such that the influence of depletion of individual Hsps is not seen. The lack of effect of depletion of single Hsps in **Figure 3** was also because only a small number of MHC-peptide complexes on the dendritic cells were needed for activation of T cells.

Antigenic peptides are associated with chaperones, including gp96, Hsp90, Hsp70 and calreticulin, which were depleted here. We did not investigate additional peptide-binding chaperones such as Hsp110, grp170 and TriC[18,30]. Hsp110 and grp170 have been used to immunize against the chaperoned peptides[18,30]; similar studies using TriC are in progress. Lysates depleted of gp96, Hsp90, Hsp70 and calreticulin still contain the other chaperone-peptide complexes. We attribute the lack of priming activity in those lysates, despite the presence of the other chaperone-peptide complexes, to the fact that gp96, Hsp90, Hsp70 and calreticulin constitute the overwhelming bulk (more than 90%) of the peptide-binding chaperones in the cells.

Neither intact antigen nor precise antigenic peptides are responsible for cross-priming activity; instead, a form of antigen that is a product of proteasomal degradation but is larger than the precise peptide is the active entity[2]. Hsp-chaperoned peptides fit in this category perfectly. The peptides chaperoned by gp96 and Hsp90 are larger precursors of the precise peptide[17,31]. The gp96-peptide complexes derived from cells are able to cross-prime against antigens expressed by those cells[32,33]. Those results also unambiguously demonstrate that the Hsps do not chaperone only the precise MHC class I–bound peptides, although they may indeed do so. However, another study has claimed that cross-priming is not dependent on proteasomal activity[4]. The centerpiece of that argument was the demonstration that treatment of cells with lactacystin followed by infection with a virus expressing OVA did not inhibit the cross-priming ability of the cells. Although inhibition of proteasome activity using a reporter construct was demonstrated, the studies failed to test if, at the concentrations used, lactacystin inhibited loading of MHC class I molecules with peptides derived from proteasomal cleavage. In the absence of such a demonstration, those conclusions may be premature.

In summary, it is apparent that cell-associated antigen is 'log orders' more efficient than soluble antigen in priming CTLs[34] and that this difference cannot be attributed to the nonspecific adjuvanticity of the cells due to endogenous and nonspecific adjuvants present in cells. It seems that cells simply do not have anywhere near the amount of intact

antigen sufficient to prime CTLs. The answer to this paradox lies in use of receptors[35]. Receptors are the tool most often exploited to obtain economies. The use of chaperone-peptide complexes and their uptake by dendritic cells through Hsp receptors such as CD91 (refs. 36,37), LOX1 (ref. 38) and perhaps other receptors provides a highly efficient and economical means of achieving indirect presentation in conditions of antigen limitation, which represent perhaps the overwhelmingly prevalent physiological condition[15]. We have addressed this issue using necrotic cell lysates; exploration of this issue using apoptotic cells might indicate involvement of uptake of apoptotic cells through receptors on dendritic cells[39] and downstream channeling of Hsp-peptide complexes into the antigen presentation pathway. In conditions in which the quantity of antigen is not limited, involvement of a receptor would be unnecessary, and in such rare circumstances, intact antigen may indeed be the vehicle of cross-priming.

METHODS

Cell lines. The B3Z hybridoma was obtained from N. Shastri (University of California at Berkley, Berkley, California) and was used as described[14]. P815 cells transfected with β-gal (P13.4) and EL.4 cells transfected with OVA (E.G7) were maintained in complete medium (RPMI medium supplemented with 5% FCS and 1% each of essential amino acids, glutamine and pyruvate) with geneticin (Gibco-BRL). Untransfected cells were maintained in complete medium.

Preparation of cell lysates. We considered many methods of cell lysis, including 'freeze-thaw', polytron homogenization, nitrogen cavitation, French press, glass-bead lysis, sonication and Dounce homogenization. After experimenting with each of these methods, we determined that gentle lysis by Dounce homogenization at a high cell/buffer ratio with a low-molarity, volatile buffer (such as bicarbonate, a physiological buffer) was the most reproducible and the least obtrusive way to lyse cells. Thus, cells were swollen at 4 °C in three volumes of 30 mM sodium bicarbonate, pH 7.4, and were homogenized in a medium-clearance Dounce homogenizer using the minimum number of strokes necessary for complete cell lysis. Lysis was considered complete when no live cells could be detected by low-power magnification. The homogenate was used without further manipulation for the experiments in **Figure 2**. For the experiments in **Figures 3–5**, cell lysates were made by solubilization with 0.05% (volume/volume) Nonidet-P40 and then the lysate preparations were diluted in PBS to a final concentration of 0.0005% Nonidet-P40 before immunization. No further procedures were used to remove the detergent before immunization. Such minute quantities do not alter the immunogenicity of the lysate or have any effect on viability of cells in culture. The results obtained with lysate prepared in the absence (**Fig. 2**) or presence (**Fig. 3**) of 0.0005% Nonidet-P40 were comparable. Further, Nonidet-P40 at a comparable concentration was added to both positive and negative controls.

Depletion and reconstitution of cell lysates. Protein G beads (Sigma) were blocked, saturated with antibody for the protein to be depleted and washed to remove excess antibody. The antibodies used were rabbit antibody to gp96 (anti-gp96; courtesy of Antigenics), rabbit anti-Hsp70 (StressGen), rabbit anti-Hsp90 (NeoMarkers), rabbit anti-calreticulin (StressGen) and mouse anti-actin (SIGMA). Antibody-coated protein G beads were placed in cell lysates (prepared by Nonidet-P40 lysis) and samples were incubated for 8 h. Protein G beads were replaced three times. Depletion of each Hsp was monitored by immunoblot. For reconstitution of depleted lysates, gp96, Hsp70 and Hsp90 were purified from antigen-expressing cells[40–42]. Anti-β-gal (Oncogene) was used in immunoblots to monitor β-gal during this depletion process. The ELISA described below was used to monitor OVA during the depletion of Hsps. Depletion of OVA was achieved by incubation of cell lysates with protein G beads bound to a mixture of four OVA antibodies (HYB 99-01, 99-02 and 99-09; AntibodyShop; and polyclonal rabbit anti-OVA). Depletion was monitored by the ELISA described below. Samples were depleted of β-gal by incubation of P13.4 cell lysates with protein G beads bound to polyclonal anti-β-gal (Cortex Biochem). Depletion was monitored by immunoblot (**Fig. 5c**) and by enzymatic digestion of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal; data not shown).

ELISA. OVA in cell lysates was measured as described[3]. First, 96-well plates were coated with goat polyclonal anti-OVA (ICN). Lysates from cells or purified OVA were then placed in the plates in titrated amounts, and samples were incubated for 1 h and then washed and blocked with 5% milk. Wells were then incubated with polyclonal rabbit anti-OVA (ICN) followed by anti-rabbit immunoglobulin–horseradish peroxidase. Plates were developed with a peroxidase substrate and the absorbance at 405 nm was measured. There is a possible concern regarding the use of this ELISA for absolute quantification of OVA in cell lysates. Cell lysates might contain fragments that contain the T cell epitope but do not at least one epitope for each antibody. Such fragments are apparently not counted in our assay. To minimize this possibility, we used polyclonal antibodies. To the extent that this possibility remains, the following consideration provides a satisfactory answer. Once a particular fragment of a given molecule has been 'counted', in effect the molecule and all its fragments are 'counted', as the molar content of the fragments cannot exceed that of the intact molecules. Thus, each molecule of intact protein is counted at least once. Some molecules may be counted more than once, depending on their fragmentation pattern and antibody epitope distribution. This will result in an overestimation of the amount of OVA in the cell lysate. As the data presented here indicate that cells do not contain sufficient intact antigen or antigenic fragments to cross-prime, the overestimatation of antigen in cell lysates enhances the validity of that argument.

Flow cytometry. Spleen cells were doubly labeled with anti-CD8–fluorescein isothiocyanate (BD Biosciences) and H-2K^b–SIINFEKL–phycoerythrin pentamers. The Pro5 MHC Pentamers contain five fluorescence-labeled MHC-peptide complexes that are made multimeric by a self-assembling coiled-coil domain and are held facing the same direction for maximum interaction with T cell receptors (ProImmune). Labeling was done according to the recommended protocols supplied with the reagents. A FACSCalibur (BD) was used for flow cytometry.

Mice and reagents. Female C57BL/6 mice were obtained from Jackson Laboratories and were maintained in the Center for Laboratory Animal Care facilities at the University of Connecticut Health Center (Farmington, Connecticut). OVA was purchased from Sigma; β-gal was obtained from Calbiochem. LPS was removed from β-gal and OVA by incubation of solubilized protein with endotoxin-removal Sepharose beads (Pierce) for 12 h, followed by incubation with fresh beads for an extra hour. Beads were removed by centrifugation and the LPS content was subsequently determined to be lower than can be detected (0.01 enzymatic units) by the limulus amebocyte lysate assay (BioWhittaker). Proteins were centrifuged at 100,000*g* to remove aggregates. LPS-free and aggregate-free proteins were used in all studies. Peptides (DAPIYTNV and SIINFEKL) were synthesized at Genemed Synthesis to a purity of more than 95%. Animal work was done with permission from the Institutional Animal Care and Use Committee of the University of Connecticut School of Medicine (Farmington, Connecticut) and was in compliance with its guidelines.

Priming of CTLs. Mice were immunized in the nape of the neck with cell lysate or protein. Then, 1 week, later spleens were removed and crushed and splenocytes were incubated in complete medium with peptide-pulsed and irradiated EL.4 cells. On day 5, cytotoxicity was measured with a chromium-release assay as described[21]. Peptide-pulsed or unpulsed target cells were loaded with ^{51}Cr and were incubated with titrated amounts of T cells. The ^{51}Cr released was measured in a scintillation counter and the percent specific release was calculated as described[43]. The percent lysis of unpulsed cells (less than 2% for all mice) was subtracted from percent lysis of peptide-pulsed cells.

***In vivo* representation.** Mice were immunized with 1×10^6 cell equivalents of lysate. Two mice per group were used. Draining inguinal lymph nodes were removed after 12 h, and CD11c$^+$ cells were isolated and were incubated with B3Z cells as reported[14]. B3Z cells were washed and fixed and were 'developed' with X-gal. Blue color was measured as absorbance at a wavelength of 595.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Immunology* website for details).

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