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Sensitization of MHC class I-restricted T cells to exogenous proteins: evidence for an alternative class I-restricted antigen presentation pathway

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SUMMARY

Immunization with exogenous proteins usually fails to immunize $CD8^+$ T cells *in vivo*. Here we report that chicken ovalbumin (OVA) denatured by heat or sodium dodecyl sulphate (SDS) effectively induced $CD8^+$ cytolytic T cells *in vivo*. The cytolytic T-lymphocyte (CTL) population generated recognized syngeneic target cells pulsed with the immunodominant OVA peptide (257–264) or transfected with the OVA protein-encoding gene. To analyse the mechanisms of how denatured OVA enters the class I-restricted pathway of antigen presentation, we took advantage of the fact that denatured OVA sensitizes target cells *in vitro* for lysis by OVA-specific CTL. We found that neither inhibition of protein synthesis (by cycloheximide) nor blocking of transport via the Golgi apparatus (by brefeldin A) interfered with the class I-restricted presentation of denatured OVA *in vitro*. In addition, transporter associated with antigen presentation (TAP)-dependent transport into the endoplasmic reticulum (ER) was not required for effective presentation, as TAP-deficient cells (RMA-S) could be sensitized effectively by denatured OVA for recognition by class I-restricted CTL. In contrast, class I-restricted presentation of denatured OVA was sensitive to lysosomotropic agents (NH_4Cl, vinblastine and leupeptin), indicating that endosomal-like compartments are involved in the presentation of denatured OVA. Sensitization was inhibited at low temperature, yet took place in the presence of sucrose and in the absence of K^+, indicating that denatured OVA enters the cell via fluid-phase endocytosis. Hence the results provide further evidence for an alternative class I-restricted pathway of antigen presentation for exogenous proteins. As that pathway seems to be effective *in vivo*, it offers a new and effective way of vaccination of $CD8^+$ CTL.

INTRODUCTION

$CD8^+$ cytolytic T lymphocytes (CTL) recognize short peptides presented at the surface of antigen-presenting cells (APC) in association with major histocompatibility complex (MHC) class I molecules.[1] These peptides are derived from intracellular proteins (antigens) that are degraded by proteasomes in the cytosol[2] and then transported into the lumen of the endoplasmic reticulum (ER) by a membrane-associated transporter heterodimer (TAP).[3] In the ER the peptides form a heterotrimeric complex with newly synthesized MHC class I molecules and β_2-microglobulin, which is then transported via Golgi cisterns to the cell surface.[4] Consequently, only proteins synthesized within the cell, as a result of infection or pathogen gene expression, enter the endogenous MHC class I-specific antigen presentation pathway.[1]

Exogenous proteins usually fail to enter the endogenous pathway of antigen presentation and thus fail to sensitize MHC class I-restricted T cells *in vivo* and *in vitro*.[5] Exogenous proteins are known to be processed and presented via the MHC class II-restricted antigen-presentation pathway,[1] which is characterized by endocytosis, lysosomal degradation of the antigen, and binding of the processed peptides to MHC class II molecules within the endosomal compartment.[1] Peptide-loaded MHC class II molecules are then transported to the cell surface and recognized by MHC class II-restricted $CD4^+$ T cells.

These two pathways of antigen presentation may not be mutually exclusive. It has been shown that chemically modified exogenous proteins sensitize $CD8^+$ cytolytic T cells *in vivo*, indicating that under certain instances exogenous proteins[6] or even peptides[7] may enter the class I pathway of antigen presentation. Modification includes attachment of lipophilic side groups[8,9] or entrapment of proteins into specialized

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Abbreviations: BFA, brefeldin A; CHX, cycloheximide; LY, luciferin yellow; OVA, ovalbumin; OVA_{hd}, heat-denatured ovalbumin; OVA_{SDS}, SDS-denatured ovalbumin; P5, ovalbumin peptide 5 (257–264); $P5_{hd}$, heat-denatured P5; SDS, sodium dodecyl sulphate; VSV-peptide, VSV NP-peptide (52–59) (vesiculo stomatitis virus nucleoprotein).

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vehicles, such as immune-stimulating complexes (ISCOM)[10–12] or liposomes.[13,14] However, it is still unclear at which stage of antigen presentation modified exogenous antigens enter the class I pathway, and whether class I and class II pathways are interconnected.

We show here that an exogenous protein (ovalbumin; OVA) denatured by heat or sodium dodecyl sulphate (SDS) effectively vaccinates class I MHC-restricted $CD8^+$ T cells *in vivo*. Class I-restricted presentation of denatured OVA was found to be independent of the classical route of endogenous antigen presentation. We provide evidence that the endosomal compartment is involved in this alternative pathway of class I-restricted antigen presentation.

MATERIALS AND METHODS

Reagents

α-phosphatidylcholine, L-α-phosphatidylcholine-glycerol, cholesterol, chicken egg OVA, vinblastine, chloroquine, leupeptin, brefeldin A (BFA), cycloheximide (CHX), luciferin yellow (LY), fluorescein isothiocyanate (FITC), sucrose and dimethyl sulphoxide (DMSO) were purchased from Sigma (Munich, Germany). Quil A (Spikoside) was obtained from Isotec Production AB (Lulea, Sweden). The chicken OVA peptide 4 (176–183; NAIVFKGL; P4), the immunodominant OVA peptide 5 (257–264; SIINFEKL; P5), and the vesiculo stomatitis virus (VSV) nucleoprotein-derived peptide (52–59; RGYVYQGL) were custom synthesized by Neosystem Laboratoire (Strasbourg, France). The other OVA peptide, P2 (55–62, KVVRFDKL), was synthesized by the solid-phase method with an automated peptide synthesizer (430A, Applied Biosystems, Foster City, CA) in the laboratory of U. Koszinowski (University of Ulm, Germany). Human recombinant interleukin-2 (rIL-2) was donated by Eurocetus (Amsterdam, the Netherlands). OVA was suspended in phosphate-buffered saline (PBS) at 10 mg/ml, filtered through a 0·2 μm filter, diluted as indicated, and then centrifuged to pellet and remove aggregates. The supernatant was harvested and used in subsequent experiments as native OVA. Two methods were used to denature OVA. First, 10 mg/ml of native OVA was solubilized in PBS or culture medium, sterilized by filtration and then denatured for 3 min at 95° (OVA_{hd}). The stock solution was diluted with PBS or culture medium to the working concentrations. Second, to solutions of PBS containing either 1·0%, 0·5% or 0·1% SDS, 10 mg/ml native OVA was added, filter sterilized and diluted with the corresponding PBS/SDS solution (OVA_{SDS}). The stock solutions of native and heat-denatured OVA P5 were prepared in the same way as the protein solutions described above. Coupling of FITC to OVA was performed according to standard methods.

Mice

Female C57BL/6 ($H\text{-}2^b$) mice were obtained from Bomholtgard Breeding and Research Center Ltd (Bomholtvej, Denmark). Animals were used at 8 to 12 weeks of age.

Cell lines and hybridomas

EL4 ($H\text{-}2^b$) thymoma cells and P815 ($H\text{-}2^d$) were from American Type Culture Collection (ATCC, Rockville, MD). EG-7, OVA cDNA-transfected EL4 cells, originally described by Dr M. Bevan (Seattle, WA) were kindly donated by Dr H. G. Rammensee (Tübingen, Germany). The RMA-S cell line was a kind gift from Dr K. Kärre (Stockholm, Sweden). Hybridoma 53672 producing anti-CD8 monoclonal antibody (mAb) came from ATCC. Hybridomas were grown in tissue cultures and mAb were purified using protein G columns.

Liposome formation

The peptide–liposomes were manufactured by a rehydration method according to R. R. C. New, modified by Dr G. B. Lipford.[14] The liposomes entrapping the whole OVA protein were produced by the same technique. Briefly, in a 100-ml round-bottomed flask 18 mg phosphatidylcholine, 2 mg phophatidylglycerol and 5 mg cholesterol were suspended in 5 ml chloroform and then rotary evaporated under reduced pressure until a thin lipid film was formed on the flask wall. Residual chloroform was removed by vacuum desiccation. Three milligrams of peptide or of the whole OVA protein was solubilized in 100 μl of water and diluted to 1 ml with PBS containing 0·4 mg/ml Quil A. The solution was added to the dried lipid and shaken by hand until the lipids were resuspended. After equilibrating for 30 min at room temperature, the suspension was filtered through a 0·22 μm Anotop 10 Plus (Whatman, Maidstone, UK) and stored at 4° until use.

Induction of OVA-specific CTL populations

Mice were immunized in the hind footpad with 50 μl per foot of peptide/protein liposomes or 50 μl PBS per foot containing different concentrations of OVA or P5 in a denatured or native form. After 4 days the draining popliteal lymph nodes (LN) were removed and a single-cell suspension was prepared.[11,14]

Cell culture

Cell culture medium was Click's-RPMI (Biochrom, Berlin, Germany) supplemented with 5% fetal calf serum (FCS; Sigma, Munich, Germany), 5×10^{-5} 2-mercaptoethanol, 10 mM L-glutamine, 10 mM HEPES, 50 μg/ml indomethacine and antibiotics. For some experiments serum-free culture medium (AEM V; Gibco, Eggenstein, Germany) was used. LN cells were cultured at 3×10^6/ml in 24-well culture plates (Nunc, Wiesbaden, Germany) in the presence of 10 U/ml rIL-2. After 4 days *in vitro* culture, the blast cells were harvested by centrifugation over a Ficoll gradient ($d = 1{\cdot}077$ kg/l). Blast cells were tested for lytic activity or used as lytic effector cells in the sensitization experiments *in vitro*.

^{51}Cr-release assay

The target cells EG-7, EL4 and RMA-S were labelled with $Na_2{}^{51}CrO_4$ for 90 min at 37° and then washed twice. The ^{51}Cr-labelled EL4 target cells were then incubated for 150 min at 37° with peptide or various concentrations of protein solutions. Cells were finally washed in order to remove free peptide or protein. To replicate serial dilutions of effector CTL in 100 μl, the same volume of target cells (10^4/ml) was added. Effector CTL and target cells were pelleted (50 *g*, 2 min) and then incubated for 4 hr at 37°. Supernatants were removed and γ-irradiation was measured.

Cytometrical analyses

LY (6·6 mg/ml) or FITC-conjugated OVA_{hd} (1 mg/ml) was added to 10^6 EL4 cells for 90 min. When indicated, hyperosmotic sucrose solution was added 10 min prior to LY or

OVA_{hd}. Cells were then washed three times with ice-cold PBS, subsequently fixed with 1% paraformaldehyde and analysed on a Profile Cytometer (Coulter, Hialeh, FL). Cells were gated on forward and side scatter; 2×10^4 gated events were recorded and the mean fluorescence intensity was determined.

K^+ depletion

Cells were incubated for 5 min at 37° with hypotonic medium (Click's RPMI and H_2O, 1:1) followed by incubation with buffer A (50 mM HEPES, 100 mM NaCl, pH 7·4) for 10 min at 37°. Cells were then transferred into isotonic K^+-free buffer B (50 mM HEPES, 100 mM NaCl, 1 mM $CaCl_2$, 10% FCS, pH 7·4) and incubated with 1 mg/ml OVA_{hd} or 1 μM P5 for 120 min at 37°. The cells were then washed four times.

Blockade experiments for class I presentation

NH_4Cl (10 mM or 100 mM), vinblastine (1 μM or 10 μM) or leupeptin (1 μg/ml or 10 μg/ml) were added to the labelled EL4 target cells together with P5 or OVA_{hd} for 150 min Cells were then washed and used as target cells. During the cytotoxicity assay the inhibitory agents were present at the following concentrations: NH_4Cl, 10 mM;[15] vinblastine, 1 μM or 5 μM;[16] leupeptin, 1 μg/ml or 5 μg/ml.[17] To analyse the effect of BFA and cycloheximide, ^{51}Cr-labelled target cells EL4 were pre-incubated for 30 min at 37° with BFA (10 μg/ml) or cycloheximide (10 μg/ml).[18]

RESULTS

Induction of cytotoxic T cells by exogenous denatured OVA *in vivo*

Immunization of mice with exogenous proteins or peptides usually fails to sensitize MHC class I-restricted $CD8^+$ cytotoxic T cells. To bypass this failure, proteins or peptides have to be entrapped in specialized vehicles, such as ISCOM[10–12] or liposomes,[13,14] which permit effective vaccination of $CD8^+$ CTL. However, in our experience some batches of OVA allow priming of $CD8^+$ CTL *in vivo* without the use of adjuvant (data not shown). We speculated that denaturation or aggregation might confer immunogenicity for $CD8^+$ T cells to OVA. To test this hypothesis, OVA was denatured by heat (95°, 3 min) (OVA_{hd}) and then injected at varying concentrations into B6 mice. After 4 days the draining popliteal lymph nodes were removed, and the lymphocytes prepared and cultured for an additional 4 days *in vitro* in the presence of rIL-2. These lymphocyte populations were tested for lytic activity against the OVA-expressing target cell EG-7 (Fig. 1a). While OVA in its native form failed to induce CTL *in vivo* at 300 mg/injection (Fig. 1a), injection of OVA_{hd} efficiently generated OVA-specific CTL in a dose-dependent manner (Fig. 1a). The efficacy of OVA_{hd} inoculation was less than that of an optimal immunization protocol using OVA entrapped into Quil A-containing liposomes (Fig. 1a). Interestingly, heat-denaturation of the immunodominant OVA peptide (P5) failed to confer immunogenicity to P5 (Fig. 1a), although P5 entrapped in Quil A-containing liposomes is an efficient inoculum.[14] The control EL4 cells were not lysed by any of the CTL populations analysed (data not shown).

MHC class I-restricted peptide-specific $CD8^+$ T cells are induced by denatured OVA *in vivo*

To establish that CTL induced by exogenous OVA_{hd} were MHC class I-restricted peptide-specific $CD8^+$ T cells, we first tested whether CTL generated by OVA_{hd} *in vivo* would recognize the immunodominant OVA P5 or an unrelated third-party peptide from VSV nucleoprotein, on syngeneic EL4 ($H\text{-}2^b$) or allogeneic P815 ($H\text{-}2^d$) target cells (Fig. 1b). The data showed that the CTL recognized OVA peptide but not VSV-derived peptide on syngeneic but not on allogeneic target cells (Fig. 1b). Thus the CTL were MHC restricted and peptide specific (Fig. 1b). When lymphocyte populations induced *in vivo* by OVA_{hd} were depleted by magnetic cell separation for $CD8^+$ T cells and then tested for cytolytic activity, lysis of EG-7 was completely abolished (Fig. 1c), demonstrating that the effector T cells induced by OVA_{hd} were $CD8^+$. We next analysed whether CTL induced by OVA_{hd} would recognize the minor determinants P2 (55–62) and P4 (176–183), as well as the immunodominant $H\text{-}2K^b$-restricted OVA

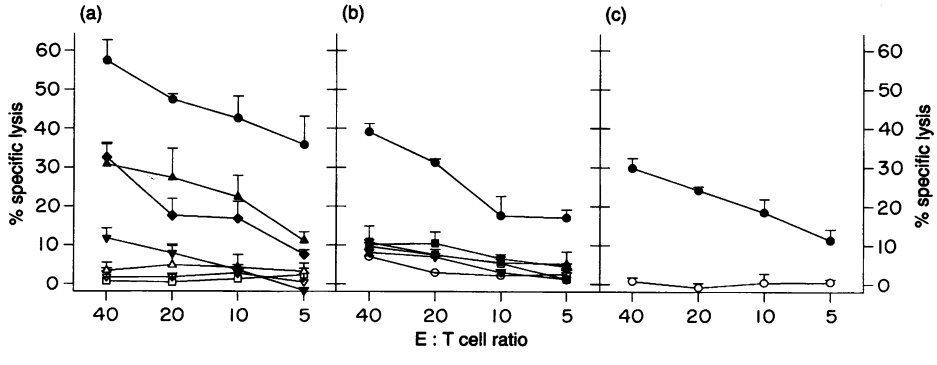

Figure 1. Heat-denatured OVA induces specific $CD8^+$ cytotoxic T cells *in vivo*. (a) B6 mice were primed with 300 μg native OVA (□) or 300 μg (◆), 30 μg (▲) or 3 μg (▼) heat-denatured OVA (OVA_{hd}) or the immunodominant OVA P5 in native (P5) (▽) or heat-denatured form ($P5_{hd}$) (△), or OVA entrapped in liposomes (●). The *in vivo* induced CTL were then tested for OVA-specific lytic activity against EG-7 and EL4 (data not shown). (b) CTL induced with 30 μg OVA_{hd} were tested for lytic activity against EL4 (○), EL4 pulsed with P5 (●), EL4 pulsed with VSV peptide (▲), P815 (□), P815 pulsed with P5 (■) or P815 pulsed with VSV peptide (▼). (c) CTL sensitized *in vivo* with 30 μg OVA_{hd} were depleted for $CD8^+$ T lymphocytes using the Dynabeads system. The remaining cells were then tested for lytic activity against EG-7 and EL4 (data not shown). (●), T cells; (○) T cells depleted for $CD8^+$. E:T, effector:target ratio.

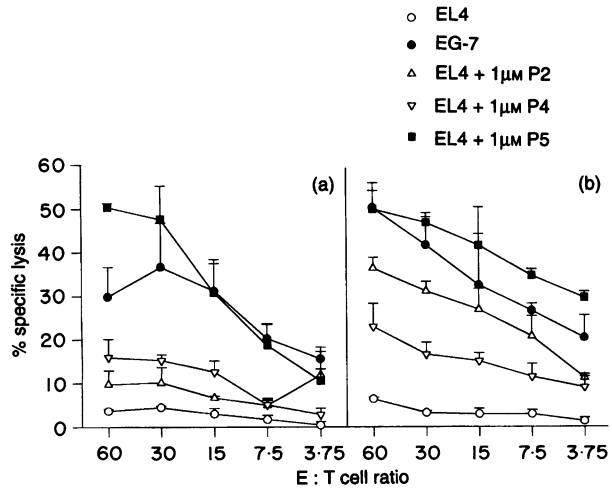

Figure 2. Peptide specificity of CTL induced by OVA_{hd}. Mice were injected with 30 μg of OVA_{hd} (a) or liposomes entrapping OVA (b). The peptide specificity of the induced CTL was tested against EL4 target cells pulsed with the indicated peptides.

P5 (257–264).[19,20] As can be seen from Fig. 2b, CTL induced by the liposome technique recognized P5 and, to a lesser extent, the minor epitopes P2 and P4, corroborating previous reports.[19,20] CTL induced by OVA_{hd}, however, recognized the immunodominant P5 yet showed only a limited reactivity against P2 and P4 (Fig. 2a). Hence, immunization with denatured OVA_{hd} induced MHC class I-restricted peptide-specific $CD8^+$ cytolytic T cells.

Denaturation by SDS conveys immunogenicity for CTL to OVA

As denaturation by heat of OVA conveyed immunogenicity for MHC class I-restricted $CD8^+$ CTL to OVA, we tested whether denaturation of OVA by SDS would allow the sensitization of CTL *in vivo* by similar means. OVA was treated with varying concentrations of SDS and 300 μg of the treated protein (OVA_{SDS}) was injected into mice. As can be seen from Fig. 3, OVA_{SDS} induced specific CTL with similar efficacy as OVA_{hd}. CTL populations immunized by OVA_{SDS} recognized the immunodominant H-$2K^b$-restricted OVA P5 (Fig. 3) and were $CD8^+$ (data not shown). Thus we concluded that denaturation of proteins conveyed immunogenicity for MHC class I-restricted peptide-specific $CD8^+$ CTL. Denatured exogenous proteins obviously enter the MHC class I-restricted pathway of antigen presentation *in vivo* and sensitize $CD8^+$ T lymphocytes.

Denatured OVA enters MHC class I-restricted pathway of antigen presentation *in vitro*

To reveal how OVA_{hd} enters the class I-restricted pathway of antigen presentation, we tested whether OVA_{hd} would sensitize EL4 cells for lysis by OVA-specific $CD8^+$ CTL. OVA-specific $CD8^+$ CTL populations were induced by the liposome technique[14] and then used as a probe to determine the efficacy of MHC class I-restricted presentation of OVA-derived peptides by EL4 cells. EL4 cells were incubated with OVA or OVA_{hd} and tested for susceptibility of lysis by OVA-specific CTL. While native OVA failed to sensitize EL4 cells even at high concentrations (10 mg/ml; data not shown), EL4 cells could be readily sensitized by OVA_{hd} in a dose-dependent manner (Fig. 4a). Kinetic analyses revealed that 150–180 min were required for reaching plateau values for sensitization of EL4 cells (Fig. 4b). Note that incubation of OVA_{hd} with EL4 cells for 30 min was too short to allow efficient presentation.

It has been suggested that proteolytic activity of cell culture components might cleave proteins and thus permit binding of peptides to empty class I molecules.[5] We therefore tested whether sensitization with OVA_{hd} was dependent on serum components within the cell culture medium. EL4 cells were

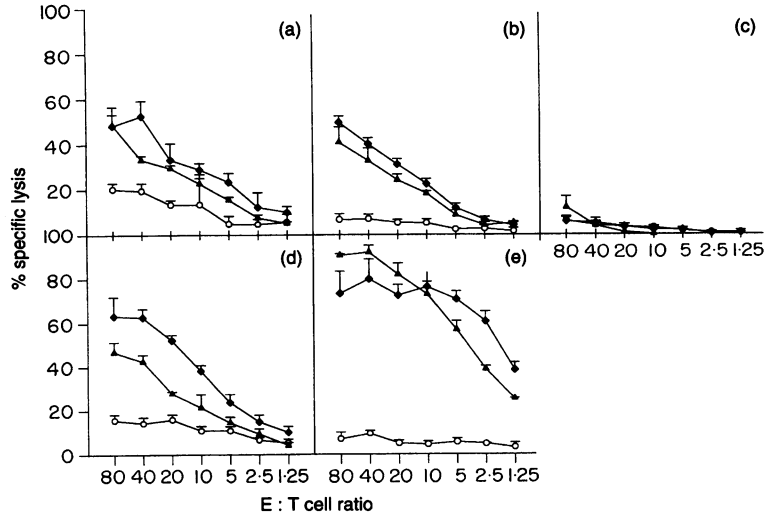

Figure 3. Detergent- or heat-denatured OVA primes $CD8^+$ cytotoxic T cells *in vivo*. CTL were induced *in vivo* by injecting either 300 μg OVA suspended in PBS containing 1% (a), 0·5% (b) or 0·1% (c) SDS, 30 μg OVA_{hd} (d) or liposomes containing OVA (e). The CTL populations primed *in vivo* were tested for cytolytic activity against EG-7 (▲), EL4 (○) and peptide (P5)-pulsed EL4 target cells (◆).

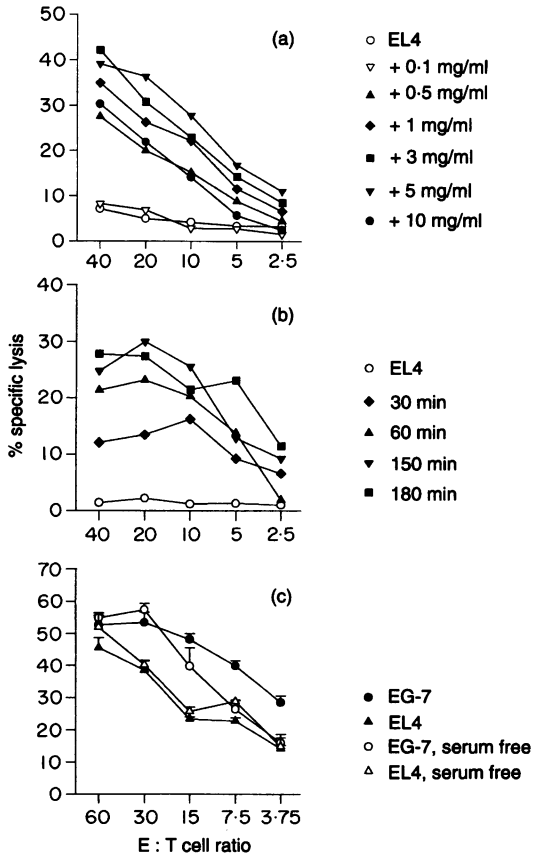

Figure 4. OVA_{hd} sensitizes *in vitro* EL4 target cells for lysis by OVA-specific CTL independent of serum components. (a, b) EL4 target cells were incubated at 37° with increasing concentrations of OVA_{hd} for 150 min (a) or with 1 mg/ml OVA_{hd} for different time periods (b). Thereafter cells were washed and then tested for susceptibility for lysis by OVA-specific CTL. (c) EL4 cells (▲, △) or EG-7 cells (●, ○) were grown in FCS-containing cell culture medium (closed symbols) or serum-free (AEM V) culture medium (open symbols). EL4 cells were then preincubated with 1 mg/ml of OVA_{hd} for 150 min at 37° in serum-free or serum (5% FCS)-containing medium. Thereafter cells were washed and tested for lysis by OVA-specific CTL in FCS-containing (closed symbols) or serum-free (open symbols) culture medium.

grown in serum-free medium and then incubated with OVA_{hd} for 150 min. A cytolytic assay was then carried out under serum-free conditions (as control EG-7 cells). As can be seen from Fig. 4c, OVA_{hd} sensitized EL4 cells independent of the type of medium used. Thus sensitization of EL4 cells was independent of external proteolytic activity provided by serum components.

Sensitization of EL4 cells by denatured OVA is independent of the classical MHC class I-restricted pathway of antigen presentation

Presentation of peptides via the MHC class I-restricted pathway of antigen presentation requires proteolytic degradation of the antigen within the cytosol, followed by passage of the peptide fragments into the ER by specialized peptide transporters. There antigeneic peptides form heterotrimeric complexes with class I molecules and β_2-microglobulin, which are transported through the Golgi apparatus to the cell surface. To determine whether these steps were involved in class I-dependent presentation of OVA_{hd}, we first tested whether transporter-deficient RMA-S cells could be sensitized by OVA_{hd}. RMA-S cells were grown at 24° overnight to allow expression of class I molecules. The cells were then incubated at 37° for 150 min with OVA_{hd} and tested for lysis by OVA-specific CTL. The results depicted in Fig. 5a demonstrate that transporter-deficient RMA-S cells efficiently processed and presented OVA_{hd} to OVA-specific CTL.

Next we tested whether *de novo* protein synthesis and protein transport via the Golgi apparatus were required for sensitization of EL4 cells by OVA_{hd}. EL4 cells were preincubated with CHX or BFA, respectively, and then incubated with OVA P5 (Fig. 5b) or OVA_{hd} (Fig. 5c) to sensitize for lysis. As can be seen from Fig. 5c, neither CHX nor BFA blocked sensitization of EL4 cells by OVA_{hd}. External pulsing of class I molecules by OVA P5, however, was partially sensitive to CHX (Fig. 5b). Hence we concluded that sensitization of EL4 cells by OVA_{hd} was independent of protein synthesis, protein transport and peptide translocation into the ER. Thus OVA_{hd} was not processed by the classical MHC class I-restricted pathway of antigen presentation.

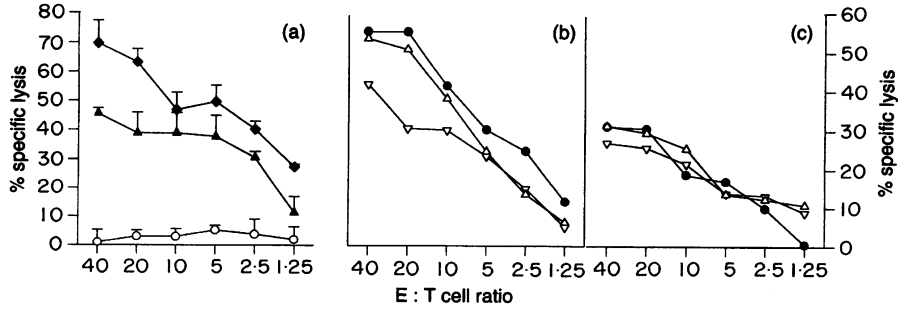

Figure 5. Protein synthesis and protein transport is not required for class I-restricted presentation of OVA_{hd}. (a) RMA-S cells were grown overnight at 24° and then incubated with 1 μM P5 (◆) or 1 mg/ml OVA_{hd} (▲) for 150 min .Then the cells were washed and tested for lysis by OVA-specific CTL. (○) Control, RMA-S. (b, c) EL4 cells were preincubated with 10 μg/ml CHX (▽) or 10 μg/ml BFA (△) for 30 min 37°. Then 1 μM P5 (b) or 1 mg/ml OVA_{hd} (c) was added and cells were incubated for additional 150 min at 37°. Thereafter EL4 cells were washed and tested for lysis by OVA-specific CTL. (●) Control, EL4 without CHX or BFA.

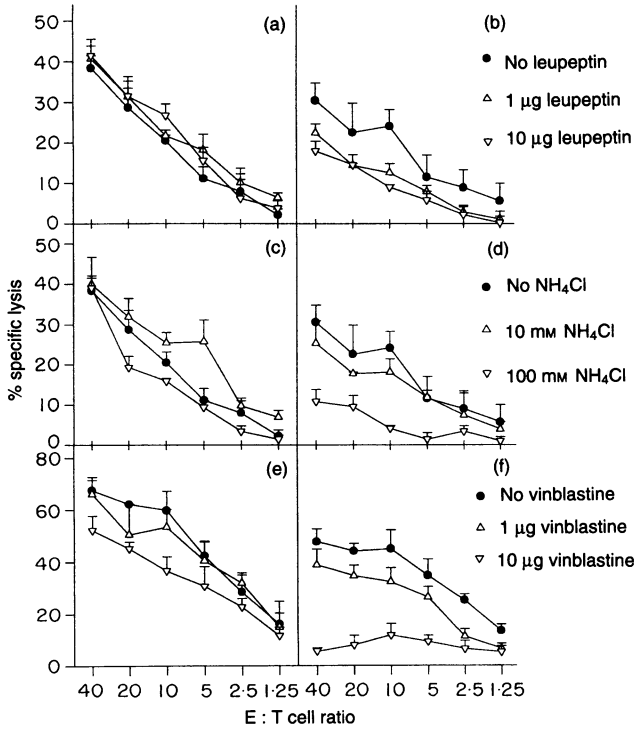

Figure 6. Lysosomotropic agents block sensitization of EL4 cells by OVA_{hd}. EL4 cells were incubated with 1 μM P5 (left column) or 1 mg/ml OVA_{hd} (right column) for 150 min in the presence of inhibitors (a, b: leupeptin; c, d: NH_4Cl; e, f: vinblastine) at the indicated concentrations. Thereafter cells were washed and tested for lysis by OVA-specific CTL. The inhibitors were present during the cytotoxicity assay as follows: leupeptin 1 μg/ml and 5 μg/ml, respectively, NH_4Cl 10 mM, vinblastine 1 μg/ml and 5 μg/ml.

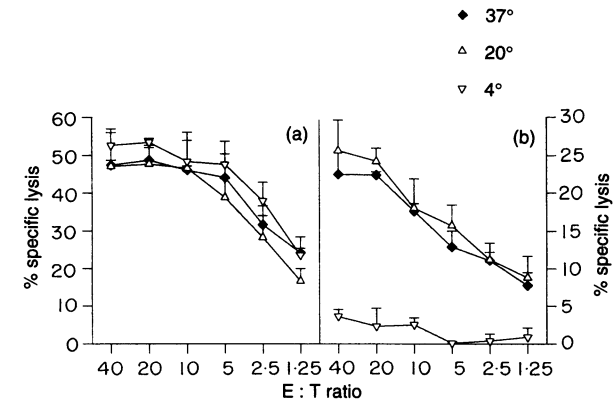

Figure 7. Sensitization of EL4 cells by OVA_{hd} is dependent on the temperature. ^{51}Cr-labelled EL4 cells were incubated for 150 min with 1 μM P5 (a) or 1 mg/ml OVA_{hd} (b) at the indicated temperatures. Then the cells were washed four times and tested for lysis by OVA-specific CTL.

Presentation of heat-denatured OVA is dependent on lysosomal function

In order to identify the alternative presentation pathway that is required for processing and class I-restricted presentation of OVA_{hd}, we analysed whether lysosomal function was necessary for effective presentation. EL4 cells were incubated with agents that block lysosomal fusion (vinblastine) or lysosomal proteases (leupeptin and NH_4Cl) together with OVA_{hd}. At high doses all three inhibitory reagents were cytotoxic to cells; however, as a control we incubated EL4 cells with the agents and OVA P5, which binds externally to class I molecules on the cell surface without being processed. The results in Fig. 6a, c and e indicate that the doses of the inhibitors used did not interfere with cytolytic activity of OVA-specific CTL against P5-pulsed EL4 cells, i.e. they were not toxic. In contrast, incubation with leupeptin (Fig. 6b), NH_4Cl (Fig. 6d) and vinblastine (Fig. 6f) effectively prevented, in a dose-dependent manner, the processing and class I-restricted presentation of OVA_{hd} to OVA-specific CTL. Hence the data indicated that the endosomal compartment was involved in the processing and presentation of OVA_{hd} to OVA-specific CTL.

Heat-denatured OVA enters cells via fluid-phase endocytosis

Transition of exogenous proteins into endosomal compartments cells might be achieved through fluid-phase endocytosis or through clathrin-coated pits.[21] While both processes are temperature dependent, endocytosis via clathrin-coated pits is sensitive to K^+-depletion[22] and inhibited by sucrose.[21] We therefore analysed the temperature dependency of the sensitization of target cells by OVA_{hd}. As can be seen from Fig. 7, incubation with OVA_{hd} at 4° completely failed to sensitize EL4 target cells, while pulsing with the OVA peptide P5 was still effective. In addition, we measured the uptake of FITC-conjugated OVA_{hd} by cytometry. The uptake of LY, known to enter cells via fluid-phase endocytosis,[17] was used as a control. The results depicted in Fig. 8 show that uptake of FITC-conjugated OVA_{hd} was inhibited at low temperatures (Fig. 8a) yet still occurred in the presence of hyperosmotic concentrations of sucrose. In both respects the uptake of OVA_{hd} was similar to that of LY (Fig. 8b).

Next we tested whether depletion of K^+ would interfere with the uptake and processing of OVA_{hd}. EL4 target cells were first depleted for K^+ and then incubated with OVA P5 (Fig. 9b) or OVA_{hd} (Fig. 9c). Thereafter the cells were washed intensively and tested for recognition by OVA-specific CTL. As can be seen from Fig. 9, K^+ depletion did not prevent uptake and processing of OVA_{hd}. We then tested whether endocytosis of OVA_{hd} could be inhibited by sucrose; however, sucrose treatment resulted in high spontaneous lysis of EL4 cells and could not be used (data not shown). Collectively these data suggested that OVA_{hd} enters target cells via fluid-phase endocytosis and not via clathrin-coated pits.

DISCUSSION

It has been recognized that effective presentation of endogenous antigens to $CD8^+$ T cells requires several rate-limiting steps, including cytoplasmic degradation, transport into the ER, complex formation of the peptides with newly synthesized MHC class I molecules, and finally transition of the complex through the Golgi apparatus to the cell surface.[1] The results presented here show that this canonical pathway of class I-restricted antigen presentation can be bypassed by exogenous antigens *in vivo* and *in vitro*. We found that denaturation of the

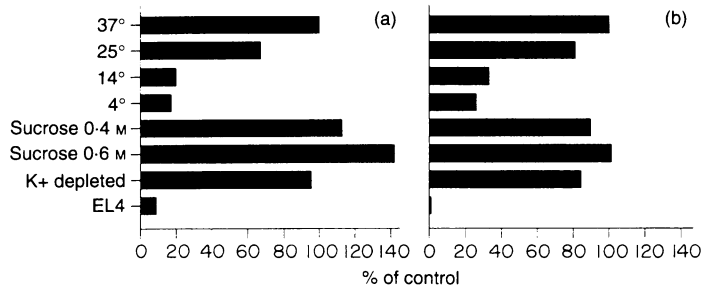

Figure 8. Fluid-phase endocytosis of OVA_{hd} and LY. EL4 cells were incubated with 6·6 mg/ml LY (b) or 1 mg/ml FITC-conjugated OVA_{hd} (a) at the indicated temperatures or in the presence of a hyperosmotic sucrose solution. Thereafter the cells were washed four times and subsequently fixed in paraformaldehyde and analysed cytometrically. The data are expressed as percentage mean fluorescence intensity relative to staining of EL4 cells at 37°.

exogenous antigen (OVA) either by heat or SDS was a prerequisite for entering an alternative class I-restricted pathway of antigen presentation. Partial denaturation might explain why immunization with exogenous proteins[6] or peptides[7] has occasionally resulted in effective priming of class I-restricted CTL. Denatured OVA sensitized target cells for recognition and lysis by class I-restricted CTL *in vitro* via an endosomal-dependent pathway. Furthermore, injection of denatured OVA induced class I-restricted $CD8^+$ T cells, indicating that the alternative class I-restricted pathway of antigen presentation is operative *in vivo* and represents a new and effective way of vaccination of $CD8^+$ T cells.

The evidence for a discrete exogenous class I-restricted pathway of antigen presentation is manifold.[6,23–29] We show here that inhibition of rate-limiting steps of the endogenous presentation pathway (protein synthesis, Golgi transport; Fig. 5) does not affect class I-restricted presentation of denatured OVA. Moreover, TAP-deficient RMA-S cells presented OVA_{hd} to class I-restricted CTL (Fig. 5), indicating that transport into the ER is not rate limiting for class I-restricted presentation of exogenous antigens. In contrast, lysosomotropic agents (vinblastine, NH_4Cl, leupeptin) inhibited presentation of OVA_{hd} but not externally added OVA P5 (Fig. 6). Hence, we assume that the endosomal compartment is involved in the class I-restricted exogenous pathway of antigen presentation. Similar conclusions can be drawn from recent reports.[23–25] It has been shown that endosomal processed SV40-derived peptides are presented in a class I-restricted manner by transporter-deficient RMA-S cells.[24] In addition, carrier-mediated uptake of influenza matrix protein-derived peptides sensitized transporter-deficient T2 cells for recognition and lysis by class I-restricted CTL.[25]

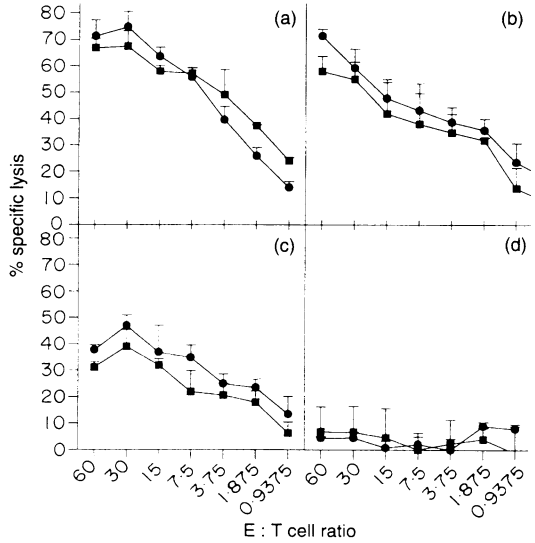

Figure 9. K^+-depletion does not prevent sensitization by OVA_{hd}. EG-7 (a) or EL4 (b, c, d) cells were depleted for K^+ by repeated washing with culture medium lacking K^+. The cells were then incubated with P5 (b), OVA_{hd} (c) or medium only (d) for 90 min. Thereafter the cells were washed four times in complete medium and tested for lysis by OVA-specific CTL. (●) Incubation with conventional medium; (■) incubation without K^+.

Assuming that the endocytic compartment is critically involved in the alternative class I pathway of antigen presentation, it is so far elusive how exogenous antigens enter this compartment. We show here that denaturation of OVA either by heat or SDS channels OVA into the endosomal route of class I presentation. Moreover, sensitization of target cells by OVA_{hd} correlated with the endosomal uptake via fluid-phase endocytosis of an indicator molecule, LY (Fig. 8). Neither sucrose nor K^+ depletion, known to inhibit uptake via clathrin-coated pits, interfered with uptake (Fig. 8) or effective presentation (Fig. 9) of OVA_{hd}. Although the evidence is so far not conclusive, we speculate that OVA_{hd} enters the target cell *in vitro* via fluid-phase endocytosis.

However, other mechanisms of antigen uptake may also be operative. These include phagocytosis (e.g. of antigens complexed on beads),[26] endocytosis of carrier–antigen complexes[25] or hydrophobic proteins,[28] or stress protein-mediated uptake of antigens.[24] Subsequent to these events, proteins would be processed within the endosomes, leading to generation of antigenic peptides. Processing by endosomal proteases, however, might generate peptide fragments that differ from cytosolic degradation products, thus endosomal processing would generate a different set of immunodominant epitopes. We addressed this question when we compared the peptide specificity of CTL induced *in vivo* via the classical (OVA–liposomes) or via the endosomal route (OVA_{hd}) of antigen presentation. We found that a similar set of MHC class I-binding peptides was recognized by these CTL. Thus, at least

for OVA, the processing compartment imposes similar constraints on the selection immunodominant epitopes. Internalization and recycling of MHC class I molecules is mandatory for endosomal presentation as considered here. It has been demonstrated convincingly for cells of the lymphocytic lineage that class I molecules are internalized and recycled.[30–33] Whether other cell types are also able to recycle class I molecules is not known.

Class I-restricted presentation of exogenous proteins, however, might not be confined to lymphocytes only. Recent reports show that specialized macrophages present phagocytosed antigens in a class I-restricted manner,[26] although optimal presentation was reached only after 24hr.[26] Moreover, this pathway was resistant to chloroquine but was blocked by BFA and required TAP1–TAP2 transporters.[29] In these respects antigen presentation to $CD8^+$ T cells by macrophages differs significantly from the presentation pathway described here and might thus represent a third independent pathway of class I MHC antigen presentation.

It has been suggested that empty class I heavy chains are preferentially internalized and recycled.[18,34] Consequently, recycling should be prevented by loading empty class I molecules on the cell surface with binding peptides and thus inhibit presentation of class I-restricted peptides via the endosomal pathway. Indeed, our preliminary data show that stabilization of class I molecules on the surface prevents effective presentation of OVA_{hd} (data not shown).

Vaccination with OVA_{hd} induces effective class I-restricted $CD8^+$ CTL *in vivo* without addition of further adjuvant (Fig. 1). Other examples of exogenous $CD8^+$ T-cell vaccines have been reported recently. SDS-treated recombinant surface antigen hepatitis B virus (HB)[28] and recombinant SV40 large T antigen[23,27] have induced class I-restricted CTL *in vivo*. Furthermore SDS-treated recombinant antigens from lymphocytic choriomeningitis (LCM) virus vaccinated $CD8^+$ T cells and induced protective immunity *in vivo*.[35] Therefore immunization with denatured exogenous proteins may offer a simple and reliable method for vaccination and induction of cytolytic class I-restricted $CD8^+$ effector cells *in vivo*.

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