

## **Soluble Proteins Delivered to Dendritic Cells Via pH-sensitive Liposomes Induce Primary Cytotoxic T Lymphocyte Responses In Vitro**

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### **Summary**

Effective immunity to many infectious agents, particularly viruses, requires a CD8<sup>+</sup> cytotoxic T lymphocyte (CTL) response. Understanding how to achieve CTL induction with soluble proteins is important for vaccine development since such antigens are usually not processed appropriately to induce CTL. In the present report, we have demonstrated that a potent primary CTL response against a soluble protein can be achieved by delivering antigen in pH-sensitive liposomes to dendritic cells (DC) either in vivo or in vitro. Since the pH-sensitive liposome delivery system is efficient and easy to use, the approach promises to be valuable both in the study of basic mechanisms in antigen processing, and as a practical means of immunization.

Class I-restricted CTL play a major role in immune defense particularly in recovery from infection (1). Consequently, inducing or priming for a CTL response is considered important in vaccine development. In this regard, subunit vaccines usually fall short because after administration, the usual means by which they are processed and presented leads at best to CD4<sup>+</sup> T lymphocyte and B cell induction but not to a CD8<sup>+</sup> T cell response. To obtain the latter almost invariably requires endogenous protein synthesis, as occurs with replicating agents (2–5). Studies in vitro reveal a similar scenario and it seems that attaining primary CTL induction with nonreplicating antigens has rarely been reported (6–7). In this report, we describe an efficient approach that results in primary CTL induction in vitro against the soluble protein, chicken OVA.

### **Materials and Methods**

**Antigens.** OVA (grade no. 5; Sigma Chemical Co., St. Louis, MO) was used. OVA was used in native form or encapsulated in liposomes. Treated OVA was material that was subjected to the same protocol as was used to make liposomal OVA (12). Labeling of OVA with <sup>125</sup>I was done using Iodogen (Pierce Chemical Co., Rockford, IL).

**Lipids.** DOPE (dioleoyl phosphatidylethanolamine), DOPC (dioleoyl phosphatidylcholine), DOSG (1,2-dioleoyl-sn-3-succinyl glycerol), PS (phosphatidyl serine), and cholesterol were used to prepare liposomes as described elsewhere (11).

**APC and Responder T Cells.** Splenocytes obtained from naive C57BL/6 (H-2<sup>b</sup>) female retired breeders were treated with ammonium chloride Tris buffer to deplete red blood cells. Splenocytes were layered over a metrizamide gradient and centrifuged at 600 *g* for 10 min. Cells from the interface were collected (7), and FACS<sup>®</sup> analysis showed 57% DC (mAb 33D1, kindly provided by Dr. Ralph Steinman, The Rockefeller University) (8), 1% macrophages (Mφ) (mAb F4/80) (9), 22% T cells, and 16% B cells. The pellet was resuspended and allowed to adhere for 1 h. More than 75% of the adherent population was identified as Mφ by morphology and nonspecific esterase staining with 5% lymphocytes and <5% DC (33D1 + C'). B cells were separated from the nonadherent population by panning on anti-IgG-coated plates. The separated cell population comprised of >80% lymphocytes was used as responder naive T cells. For in vitro pulsing, 3 × 10<sup>6</sup> DC or Mφ were incubated with 500 μl of the different liposomal preparations and incubated at 37°C for 3 h in a waterbath. The concentration of OVA in the liposomes was always between 200 and 600 μg/ml. The cells were washed twice and used as stimulators. Groups of three, young, female C57BL/6 mice were intravenously injected with liposomes and 3 h later their spleens were removed for isolation of DC and Mφ, and used as stimulators. In vivo pulsed DC and Mφ (10<sup>4</sup> each) were added to naive, responder T cells in 96-well U-bottomed plates at R/S ratios ranging from 100:1 to 3.13:1. Primary CTL induction was determined on day 5 with a standard 4-h <sup>51</sup>Cr release assay.

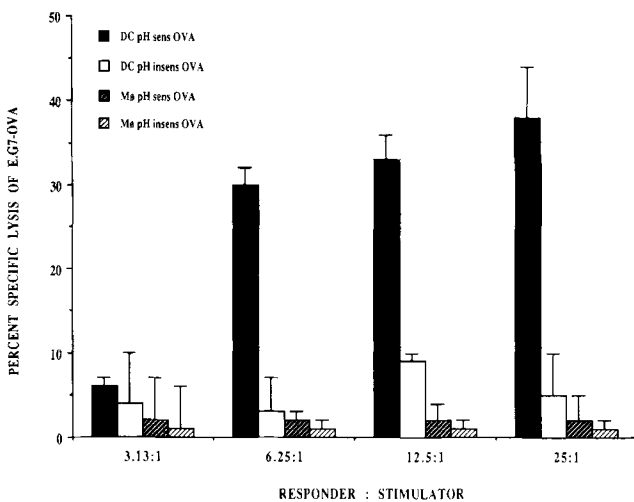
**Target Cells.** The target cells used were Ia<sup>-ve</sup> EL4 (C57BL/6, thymoma, H-2<sup>b</sup>), P815 (H-2<sup>d</sup>, mastocytoma), YAC-1 (H-2<sup>d</sup>), and E.G7-OVA (EL4 cells transfected with cDNA of chicken OVA) (10).

**Preparation of Liposomes.** The pH-sensitive and pH-insensitive liposomes were made as described by Zhou et al. (11).

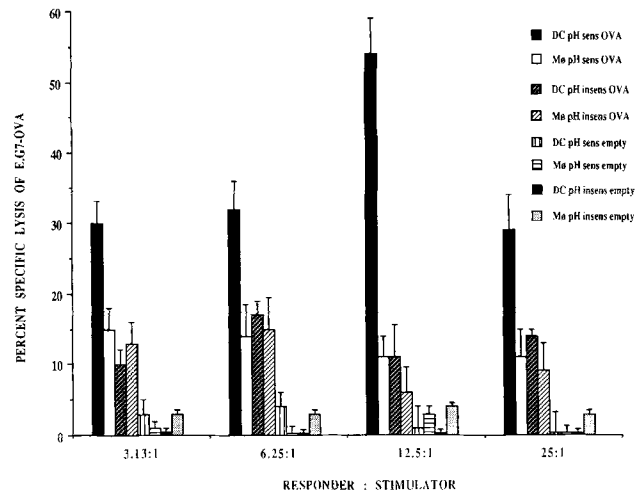
## Results and Discussion

Extensive studies by Bevan's group have demonstrated that CD8<sup>+</sup> MHC class I-restricted, OVA-specific CTL can be assayed in the C57BL/6 system using an EL4 thymoma transfected with the OVA gene (E.G7-OVA cells). This system was used to measure if successful OVA-specific CTL induction had occurred. As responder cells, splenic T lymphocytes from naive C57BL/6 were used. To achieve antigen delivery, OVA was incorporated into liposomes. Two types of liposomes were investigated: conventional liposomes (pH insensitive) and pH-sensitive liposomes. The latter were shown in previous studies to adopt a stable liposome structure under physiological conditions, but to destabilize upon protonation and release their entrapped contents (12). Previously, we demonstrated that OVA incorporated into pH-sensitive liposomes could deliver antigen to EL4 (H-2<sup>b</sup>) target cells and render such cells susceptible to lysis by OVA-specific CTL (13).

To present antigen we used syngeneic DC and M $\phi$  that were exposed to the antigen in vitro for 3 h. The results in Fig. 1 demonstrate vigorous primary in vitro OVA-specific CTL responses, but only in cultures stimulated with DC that had been exposed in vitro to antigen delivered by means of pH-sensitive liposomes. In contrast, DC exposed to antigen via pH-insensitive or empty liposomes, or treated or native OVA (not shown), failed to induce CTL. Even increasing the antigen dose given by means of pH-insensitive liposomes 10-fold beyond that effective with pH-sensitive liposomes failed to achieve significant CTL induction. Although antigen presented via pH-sensitive liposomes to DC was immunogenic, M $\phi$  similarly pulsed with antigen failed to induce CTL. Thus, these results demonstrate that primary CTL induction against a soluble protein can occur in vitro, but only



**Figure 1.** Primary CTL induction using DC pulsed in vitro with pH-sensitive liposomal OVA. DC, M $\phi$ , and T cells were isolated as described in Materials and Methods. DC and M $\phi$  treated with empty liposomes, treated, and native OVA induced no primary CTL. Control targets EL4, P815, and YAC-1 showed no significant lysis (data not shown). Each value represents the mean  $\pm$  SD of three replicates. The experiment was performed four times with similar results.



**Figure 2.** Induction of primary cytotoxic response by DC treated in vivo with pH-sensitive liposomal OVA. In vivo pulsed DC and M $\phi$  were added as stimulators to naive T cells and primary, OVA-specific cytotoxic response was determined on day 5. DC and M $\phi$  pulsed with treated and native OVA induced no response. No significant lysis of control targets EL4, YAC-1, and P815 was obtained (data not shown). Values represent the mean of triplicate cultures  $\pm$  SD. Data are representative of four independent experiments performed with similar results.

**Table 1.** Efficiency of Primary CTL Induction by DC Pulsed with Liposomal Antigens In Vivo and In Vitro

Groups	Responder/stimulator			
	25:1	12.5:1	6.25:1	3.13:1
DC + pH-sensitive liposomes (in vivo)	46 $\pm$ 3*	53 $\pm$ 3	39 $\pm$ 3	36 $\pm$ 4
DC + pH-sensitive liposomes (in vitro)	33 $\pm$ 3	32 $\pm$ 1	31 $\pm$ 4	15 $\pm$ 4
DC + pH-sensitive liposomes (in vivo) + 33D1 Ab <sup>†</sup>	3 $\pm$ 1	2 $\pm$ 1	2 $\pm$ 1	0 $\pm$ 1
DC + pH-sensitive liposomes (in vitro) + 33D1 Ab <sup>†</sup>	1 $\pm$ 2	1 $\pm$ 1	1 $\pm$ 1	1 $\pm$ 1

3  $\times$  10<sup>6</sup> DC were pulsed in vitro with 500  $\mu$ l of pH-sensitive liposomal OVA (250  $\mu$ g OVA) at 37°C for 3 h. In vivo pulsed DC were obtained 3 h later from groups of three mice injected intravenously with 500  $\mu$ l pH-sensitive liposomes containing OVA (250  $\mu$ g OVA per mouse). 10<sup>4</sup> DC pulsed in vivo or in vitro were added to naive T cells at varying R/S ratios, and primary cytotoxic response was determined 5 d later. Control target EL4 showed no significant lysis.

\* Percent specific lysis of E.G7-OVA  $\pm$  SD.

† DC population pulsed in vivo and in vitro were treated with mAb 33D1 (anti-DC) and complement in vitro to deplete all DC and then added to naive T cells as described above.

when antigen is presented via DC that have been exposed to the antigen appropriately, such as occurs with pH-sensitive liposomes.

Others have shown that DC taken from animals exposed to antigen *in vivo* have potent antigen-presenting activity as judged by their ability to stimulate T cell clones (14). Accordingly, we intravenously injected young C57BL/6 mice with different liposomal preparations and 3 h later their spleens were removed for the isolation of DC and M $\phi$ . It is evident from Fig. 2 that excellent primary CTL responses resulted in cultures stimulated with DC taken from mice given OVA-containing pH-sensitive liposomes. In contrast, DC from mice that received OVA in pH-insensitive liposomes provided a much weaker stimulus for CTL induction. DC from animals given native OVA, treated OVA, or empty liposomes failed to respond. Not shown is the fact that when cytotoxicity occurred it was mediated by CD8<sup>+</sup> T cells. DC from mice that received antigen via pH-sensitive liposomes were efficient antigen presenters for primary CTL induction, but M $\phi$  from the same mice were far less effective. Thus, as noted *in vitro*, the most effective means of attaining a primary CTL response against a soluble protein was to deliver antigen to DC via pH-sensitive liposomes.

It is known that after injection of antigens via liposomes the bulk of the material locates in the liver and that liposomes that arrive in the spleen are primarily taken up by macrophages (15, 16). Consequently, it is likely that DC exposed to antigen *in vivo* interact with far smaller amounts (estimated to be 100–1,000-fold less) of antigen than those exposed *in vitro*, although we have yet to perfect the technology to measure the comparative uptake of OVA by the two cell

populations. However, a comparison was made between the antigen-presenting activity of DC exposed to the same population of antigen containing pH-sensitive liposomes *in vitro* and *in vivo*. Results, shown in Table 1, indicate that even though *in vivo* pulsed DC are likely exposed to substantially less antigen, they nonetheless were more effective than were *in vitro* pulsed DC at inducing a primary CTL response. Also shown in Table 1 is further evidence that the APC population used to stimulate primary CTL induction are DC. Thus treatment of the population with mAb 33D1 and C' *in vitro* to deplete DC removed the CTL-inducing activity of the cell population.

In conclusion, we have demonstrated that pH-sensitive liposomes provide a valuable means of delivering a soluble antigen to DC and that this is an effective method of inducing primary CTL responses. Why the approach is so successful needs to be established. However, we know from work with other systems that antigen delivered to a cell via pH-sensitive liposomes results in a fraction of the material gaining access to the nonendosomal processing pathway necessary for CTL induction and recognition (13). It is far from certain how antigen delivered *in vivo* gains access to DC in the form that is effective at inducing CTL. We suspect that the liposome-bound antigen may be delivered to macrophages and that such cells may preprocess the material and pass on appropriate antigen fragments to the DC. Another possibility could be that DC pulsed *in vivo* may retain the antigen more efficiently, or they may require much less antigen for efficient presentation because of the higher density of class I molecules, or DC may provide a stronger accessory signal (14). Experiments to test these ideas are currently underway.

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