In Vivo Cross-Presentation of a Soluble Protein Antigen: Kinetics, Distribution, and Generation of Effector CTL Recognizing Dominant and Subdominant Epitopes

Delia Nelson, Christine Bundell and Bruce Robinson

*J Immunol* 2000; 165:6123-6132;

doi: 10.4049/jimmunol.165.11.6123

http://www.jimmunol.org/content/165/11/6123

**References**

This article cites 40 articles, 14 of which you can access for free at:
http://www.jimmunol.org/content/165/11/6123.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
In Vivo Cross-Presentation of a Soluble Protein Antigen: Kinetics, Distribution, and Generation of Effector CTL Recognizing Dominant and Subdominant Epitopes

Delia Nelson, Christine Bundell, and Bruce Robinson

Cross-presentation of exogenous Ags via the MHC class I pathway is now recognized for its role in self-tolerance, tumor immunity, and vaccine development. However, little is known about the in vivo distribution and kinetics of cross-presented protein Ags, nor the subsequent development of CTL effector responses to dominant or subdominant epitopes. We examined the location and duration of cross-presented Ag by using 5,6-carboxy-succinimidyfluorescein ester-labeled T cells from class I-restricted Ag-specific TCR mice. Comparisons of results from an in vitro 51Cr release CTL assay with an in vivo CTL assay provided physiologically relevant insights into the functional capacities of CTL specific for epitopes with differing affinities. These data demonstrate that efficient cross-presentation of a dominant class I-restricted Ag is dose related and remains largely localized, but not limited to the draining lymph nodes for up to 3 wk following a single injection of soluble protein. Within this period, dominant peptide-specific CTL are fully functional in vivo throughout the secondary lymphoid system. However, no in vivo responses are seen to a subdominant or cryptic epitope. Prolonging Ag cross-presentation via use of IFA promoted persisting in vivo dominant epitope-specific CTL activity and revealed dose-responsive precursor CTL to the subdominant, but not to a cryptic epitope. Analysis of functional in vivo CTL responses demonstrated that, in the presence of strong ongoing responses to the dominant peptide, lytic activity of CTL directed at weaker epitopes is undetectable.

Cross-presentation defines the capacity of APCs to take up exogenous Ag and shunt them into the class I pathway for presentation to CD8+ T cells. Despite early data from transplantation models (1) suggesting that cross-presentation occurred, this process has been largely ignored in favor of the view that exogenous Ags generally enter the class II pathway and endogenous Ags enter the class I pathway (2). Recently, however, it has been realized that cross-presentation is important for host defense against infection by viruses and other organisms that do not infect APCs (3-5). Additionally, cross-presentation has been shown to play a significant role in the generation and maintenance of peripheral tolerance (6, 7), the induction of anti-tumor immune responses (8-10), and the generation of CD8 T cell responses using vaccination protocols (11, 12). Until now, the location and duration (within the secondary lymphoid system) after exposure to varying concentrations of cross-presented Ag was unclear. In particular, comparisons between persisting Ag vs a single exposure of a soluble (and therefore rapidly disseminated) Ag had not been undertaken.

Presentation of Ags via the classical class I pathway has demonstrated that CTL responses are often limited to a few, or even a single, dominant peptides, which, in some instances, may be associated with weaker responses to other epitopes, termed subdominant (reviewed in Ref. 13). Responses to subdominant epitopes have been shown to be biologically significant. In particular, studies examining viral infections have demonstrated T cell responses to epitopes of varying affinities (14, 15). Similarly, autoimmune models have demonstrated the sequential revealing of T cell responses to weaker epitopes during disease (16, 17). In some tumor systems, CTL to subdominant peptides have been detected during tumor growth (18) and, when these peptides are used as immunogens, they are able to confer protection (19). However, studies looking at hierarchical CTL responses to soluble proteins have generally been limited to in vitro systems (20, 21), and no studies to date have addressed Ag cross-presentation and its relationship to the in vivo generation and subsequent functional activity of CTL specific for subdominant or cryptic peptides.

In this study, the in vivo relationship between local Ag cross-presentation and effector CTL responses after a single injection vs chronic exposure to a soluble Ag was assessed. Following a single exposure of OVA, Ag cross-presentation was maximal in, but not restricted to, the draining lymph nodes (DLN) and continued for 3 wk, a process much more prolonged than previously considered. Fully functional in vivo effector CTL recognizing the dominant peptide were readily detectable within, but not after, the period spanning Ag presentation, suggesting that loss of antigenic stimulation is directly associated with loss of effector function. The presence of IFA not only prolonged Ag presentation, promoted Ag presentation to low antigenic concentrations, and generated qualitative changes in functional effector CTL, but also revealed T cell precursor responses to a subdominant epitope. This work showed that induction of T cell responses to weaker epitopes requires continual cross-presentation of high antigenic concentrations within an adjuvant/inflammatory milieu. However, subdominant-specific

*Department of Medicine, University of Western Australia, Nedlands, Australia; and West Australian Institute for Medical Research, Queen Elizabeth II Medical Center, Perth, Australia
†Received for publication May 25, 2000. Accepted for publication August 29, 2000.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

This work was supported by the National Health and Medical Research Council of Australia, the State Government Insurance Commission, and the Raine Medical Research Foundation.

Address correspondence and reprint requests to Dr. Delia Nelson, Department of Medicine, University of Western Australia, Queen Elizabeth II Medical Center, 4th Floor, G Block, Nedlands, Western Australia, Australia, 6009. E-mail address: delian@cyllene.uwa.edu.au

Abbreviations used in this paper: DLN, draining lymph node; CFSE, 5,6-carboxyfluorescein succinimidyl ester; CM, complete medium; DC, dendritic cell.
CTL activity could not be seen in vivo, possibly due to the presence of a concomitant CTL response to the dominant epitope.

Materials and Methods

Mice

Female C57BL/6J mice (H-2b) were obtained from the Animal Resources Center (Canning Vale, Western Australia) and maintained under standard housing conditions in the University Department of Medicine (University of Western Australia) animal holding area. The TCR-transgenic mouse line OT-1, expressing a TCR recognizing an H-2b-restricted OVA epitope, SIINFEKL (22), was kindly supplied by F. Carbone (Monash University, Melbourne, Australia). For all experiments, mice between the ages of 6 and 12 wk were used.

OVA and peptides

OVA, grade V and grade VI (Sigma, St. Louis, MO), the model protein Ag used throughout these experiments, was regularly screened using a proliferation assay to ensure that the protein had not broken down to release the SIINFEKL peptide during routine handling procedures.

The dominant peptide OVA275–264 (SIINFEKL), a subdominant peptide OVA55–62, KVVRFDKL (21, 23), and a cryptic peptide OVA11–18, CFDVFKEL (20), were manufactured by the Center for Cell and Molecular Biology (University of Western Australia, Perth) at purities of 89, 90, and 95%, respectively.

Proliferation assay

Spleen cells from unmanipulated mice were irradiated (2100 rad), fixed with 4% formaldehyde (10 min at room temperature), washed twice, and pulsed with varying concentrations of OVA or SIINFEKL (90 min at 37°C under 5% CO2). Washed APC were plated in triplicate, with 2 × 105 OT-1 lymph node cells in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA), in 200 μl of complete medium (CM). CM consisted of RPMI 1640 supplemented with 10% FCS (both from Life Technologies, Gaithersburg, MD) containing 100 U/ml penicillin (CSL, Melbourne, Australia), 50 μg/ml gentamicin (David Bull Laboratories, Mulgrave North, Australia), and 5 × 10−5 M 2-ME (Sigma). The cells were incubated for 48 and 72 h and 1 μCi/well [3H]thymidine (Amersham, U.K.) was added 12–15 h before harvesting. In all tests, OVA grade V was found to remain free of peptide; however, on occasions, OVA grade VI did have peptide contamination and was therefore not used in our experimental procedures.

Vaccination protocol

Mice were given varying doses of OVA either dissolved in PBS or as an emulsion in IFA. Mice were s.c. injected with 100 μl at two sites on either side of the base of the tail.

Cytoplasmic loading of OVA into APC using osmotic shock

Osmotic loading was conducted as previously described (24). Briefly, after erythrocytes were removed by lysis, C57BL/6 spleen cell suspensions were washed and pulsed with 1 mg/ml OVA in 1 ml of hypertonic media (0.5 M sucrose, 10% (w/v) polyethylene glycol, 10 mM HEPES, in RPMI 1640 at pH 7.2) for 10 min at 37°C. Then, 14 ml of hypotonic medium (60% RPMI 1640 and 40% H2O) was added for 2 min at 37°C, followed by centrifugation at 1200 rpm for 5 min, washed twice, and placed into culture.

Murine tumor cell lines

The EL4 thymoma cell line and the OVA-transfected EL4 cell line EG7 (24) were both purchased from the American Type Culture Collection (Manassas, VA) and maintained in CM.
Table 1. Location, duration, and levels of Ag presentation after a single inoculation of varying concentrations of OVA

<table>
<thead>
<tr>
<th>Days after Immunization</th>
<th>OVA Dose (µg)</th>
<th>DLN (%)</th>
<th>LN (%)</th>
<th>Spleen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>200</td>
<td>65</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>14</td>
<td>200</td>
<td>35</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>21</td>
<td>200</td>
<td>24</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>28</td>
<td>200</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>PBS controls</td>
<td>20</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

5,6-Carboxy-fluorescein succinimidyl ester (CFSE) labeling of OT-1 TCR-transgenic lymph node cells

CFSE (Molecular Probes, Eugene, OR) labeling was performed as previously described (25). SIINFEKL-specific lymph node cells from TCR-transgenic OT-1 mice were resuspended in 20 ml of RPMI 1640 at 1 x 10⁷ cells/ml and incubated with 1 µl of CFSE stock solution (5 mM in DMSO) for 10 min at room temperature. Cells were washed through FCS four times and PBS twice, then resuspended in PBS before 1 x 10⁷ cells were i.v. injected into the tail vein of each recipient mouse. In all experiments, CFSE-labeled cells were recovered 3 days after adoptive transfer and analyzed by FACS analysis. CFSE-labeled cells were adoptively transferred at time points such that the degree of proliferation was analyzed on days 7, 14, 21, and 28 after OVA immunization. In a few instances, other time points were also examined (as described in Results and the figure legend).

FACS analysis

Lymph node or spleen cells were stained for two-color FACS analysis using the PE-conjugated mAb anti CD8 (clone 53-6.7; Pharmingen, San Diego, CA). Analysis was performed on a FACSscan (Becton Dickinson, Mountain View, CA) using CellQuest software. For analysis of CFSE-labeled cells, 100,000 events were collected and analyzed using the ModFit LT cell cycle analysis software (Verity Software House, Topsham, ME).

In vitro cytotoxic assay

Spleens were removed and prepared as single-cell suspensions. To ensure that there were enough effector CTL for the assay, cells from each experimental group (of three mice) were pooled. These effector cells were then expanded in vitro with a 1:1 ratio of splenic APC taken from naive mice that were either osmotically loaded with whole native OVA or pulsed with 90 min with 10⁻⁶ M of the dominant, subdominant, or cryptic peptides. All populations of APC were thoroughly washed before addition to the effector cells, then resuspended in PBS before use. Effector cells were added to corresponding targets at varying E:T cell ratios and incubated at 37°C for 4 h. After incubation, the supernatants were harvested and ⁵¹Cr release was determined. The mean of duplicate samples was calculated and the percent specific ⁵¹Cr release was determined as follows: percentage of specific cytolyis = ([experimental ⁵¹Cr release − control ⁵¹Cr release]/(maximum ⁵¹Cr release − control Cr⁵¹ release)) x 100%. Experimental ⁵¹Cr release represents counts from target cells mixed with effector cells, control ⁵¹Cr release represents counts from targets incubated with medium alone (spontaneous release), and maximum ⁵¹Cr release represents counts from targets exposed to 5% Triton X-100.

In vivo cytotoxicity assay

Target cells, which included the OVA transfectant cell line EG7, and EL4 cells pulsed with either SIINFEKL, KVVRFDKL, or the cryptic peptide were labeled with 150 µCi of ⁵¹Cr for 90 min and washed four times before use. Effector cells were added to corresponding targets at varying E:T cell ratios and incubated at 37°C for 4 h. After incubation, the supernatants were harvested and ⁵¹Cr release was determined. The mean of duplicate samples was calculated and the percent specific ⁵¹Cr release was determined as follows: percentage of specific cytolyis = ([experimental ⁵¹Cr release − control ⁵¹Cr release]/(maximum ⁵¹Cr release − control Cr⁵¹ release)) x 100%. Experimental ⁵¹Cr release represents counts from target cells mixed with effector cells, control ⁵¹Cr release represents counts from targets incubated with medium alone (spontaneous release), and maximum ⁵¹Cr release represents counts from targets exposed to 5% Triton X-100.

Results

Location and duration of Ag presentation is dose related

The first series of experiments was designed to dissect out the relationship between Ag concentration and duration of Ag presentation (in this case, the dominant class I peptide, SIINFEKL) in the absence of an adjuvant. A kinetic study was undertaken in which CFSE-labeled OT-1 lymph node cells were adoptively transferred into recipient mice treated with a single s.c. injection of varying dose.
concentrations of OVA diluted in PBS. Throughout these experiments, the proliferative responses of CFSE-labeled OT-1 cells in secondary lymphoid compartments of recipient animals were analyzed 3 days after adoptive transfer. Responses were determined weekly over 1 mo.

A strong in vivo OT-1 proliferative response was seen in the DLN of all mice given 200 mg of OVA (Fig. 1). This response continued for 2 wk, diminished at day 21, and disappeared by day 28. In mice given an intermediate dose (20 mg OVA), only one of three mice demonstrated a weak response 7 days after OVA immunization, and no response was observed at any other time point. No OT-1 proliferation was seen in the DLN at any time point in mice given the lowest concentration (2 mg of OVA).

Table I compares the magnitude of the proliferative response determined by quantifying the percentage of daughter cells relative to those remaining in the parental peak in different lymphoid tissues. The highest concentration of OVA induced a strong proliferative response in the DLN (65% of cells had undergone at least one division), in association with significant but lower levels of proliferation in the nondraining LN (29% daughter cells), and an even weaker, but positive response in the spleen (11% proliferating cells). Log-fold dilutions of OVA induced correspondingly lower levels of SIINFEKL-specific OT-1 proliferation at day 7 in all of the secondary lymphoid organs examined. Unlike the highest OVA dose, variable OT-1 proliferative responses were seen in the two lower doses at days 14 and 21 after inoculation. Interestingly, all doses at days 7 and 21 after immunization showed weak, but above background levels of proliferation within the spleen, implying low levels of Ag presentation. However, by day 28, no proliferation was seen in any tissue site. These data imply that high doses of soluble protein are required before consistent Ag presentation can be observed and that, at this concentration, cross-presentation is observed not only within the DLN, but also throughout the secondary lymphoid system.

The generation of OVA-specific CTL is dose related

Based on the above observations, we assessed whether peptide-specific effector CTL cells had been generated in mice given the same doses of OVA using an in vitro 51Cr release CTL assay. In this assay, spleens were pooled from each experimental group (of three mice) and expanded in vitro with splenic APC taken from naive mice. These APC were either osmotically loaded with whole native OVA or pulsed with the dominant peptide.

At all time points examined (Fig. 2), the highest concentration of OVA yielded the strongest CTL response to the dominant peptide. However, in contrast to Ag presentation within the DLN, this response was maintained well beyond 28 days and remained readily detectable 8 wk later, declining to a weak, but detectable CTL response at 3 mo after immunization. There appears to be some interexperimental variation with responses to the lower OVA concentrations. For example, at day 7, a higher CTL response was seen to the 2-mg dose than to the 20-mg dose. Days 28 and 56 were more consistent, with the two lower OVA doses demonstrating equivalent CTL responses.
Fig. 2 shows the percent lysis by effector cells cultured in vitro with SIINFEKL-pulsed APC and tested on SIINFEKL-pulsed EL4 targets. However, similar data were generated when the effector cells were pulsed with osmotically loaded APC (data not shown), as well as when the OVA-transfected cell line EG7 was used as a target (data not shown). These data demonstrated that SIINFEKL-specific CTL were detectable in spleens taken from experimental mice that had received all three OVA doses.

In vivo effector CTL are present throughout the lymphoid system

The use of in vitro antigenic re-exposure to expand peptide-specific CTL precursors (seen in Fig. 2) may not be an accurate reflection of in vivo physiological events. Therefore, an in vivo CTL assay was used to assess the location and duration of effector CTL in mice immunized with 200 μg of OVA in PBS. In this assay, differentially CFSE-labeled target cells from naive mice were pulsed with (CFSEhigh) or without SIINFEKL (CFSEiso), i.v. injected into experimental mice and CTL responses within secondary lymphoid organs examined 18 h later.

A strong in vivo SIINFEKL-specific effector CTL response (illustrated in an individual mouse in Fig. 3 and as group means in

FIGURE 4. Duration of in vivo CTL activity in different lymphoid sites. From the in vivo CTL described and visualized in Fig. 3, the ratio between the percentages of uncoated vs SIINFEKL-coated (CFSEiso/CFSEhigh) was calculated to obtain a numerical value of cytotoxicity with data from each experimental group of three mice averaged and normalized to allow for interexperimental comparisons. These experiments were reliably repeated three times.

FIGURE 5. IFA prolongs Ag presentation. FACS analysis of CFSE-labeled class I-restricted OVA-specific TCR-transgenic T cells adoptively transferred into mice s.c. inoculated with varying concentrations of OVA in IFA. CFSE cells were reisolated from the DLN of mice 3 days after transfer, such that analysis by FACS was on days 7, 14, 21, and 28 after OVA inoculation. All profiles obtained were gated on CD8 T cells. These results shown are from individual mice that are representative of three experiments (three mice per group).
Fig. 4) was seen in the DLN in association with a weaker response in the spleen at day 7. Variable splenic responses were seen, with one mouse showing equivalent effector responses throughout the secondary lymphoid system (Fig. 3) and others demonstrating weaker responses (Fig. 4). We were surprised to see strong CTL activity throughout the secondary lymphoid system at day 7. However, unlike the in vitro detection system, in vivo peptide-specific CTL responses rapidly declined to barely detectable levels by day 20.

Taken together, these data imply that a single injection of OVA results in the Ag being transported and presented in local DLN and other lymphoid organs, resulting in SIINFEKL-specific CTL that, after a second in vitro antigenic signal, could be detected for up to 3 mo. However, only transient in vivo effector CTL activity was observed throughout the lymphoid system that peaked at day 7 and was barely detectable 3 wk after immunization. Therefore, the next series of experiments was designed to address how these responses are altered when cross-presentation is prolonged.

Prolonged exposure to Ag extends the period of Ag presentation and enhances responses to lower doses

Peptides, proteins, heat-killed intact cells, or cell lysates in the presence of adjuvants such as IFA are used for a number of vaccination purposes, including tumor immunotherapy. Based on the above results, we evaluated how responses to OVA in the presence of IFA altered the immune response. As IFA acts as an oil/emulsion Ag depot, we were not surprised to find that, at the highest OVA concentration used (200 μg), Ag presentation within the DLN persisted well past 28 days (Fig. 5), diminishing to below detectable levels 8 wk after inoculation (data not shown). However, the presence of IFA also promoted increased sensitivity to lower antigenic concentrations. Immunization with 20 μg of OVA in IFA also resulted in persistent Ag presentation throughout the time period examined (28 days). Weak levels of Ag presentation were detected 7 days after exposure to 2 μg and 0.2 μg (data not shown), but not at any other time. No Ag presentation was seen when mice were inoculated with 0.02 μg of OVA in IFA (data not shown).

IFA enhances the quality of effector CTL

Effector/memory CTL responses to SIINFEKL, determined by a 51Cr release in vitro CTL assay, demonstrated equivalent responses to the two higher OVA concentrations in IFA for up to 2 wk after inoculation (Fig. 6). These responses became dose responsive 3 wk after exposure and persisted past 28 days. Peptide-specific CTL responses generated by exposure to the lowest dose demonstrated variable results between experiments; nevertheless, they were significantly reduced at the earlier time points and diminished to background levels 3 wk later.

IFA promotes persisting in vivo effector CTL cells that are present throughout the lymphoid system

Comparison of the in vivo CTL response following administration of 200 μg of OVA in IFA to those seen after OVA alone treatment (Fig. 7) showed that IFA only marginally increased the level of peptide-specific CTL in the DLN and LN at day 7. However, the splenic response was almost doubled (to 65% lysis) at this time point. These responses diminished to relatively constant levels for the duration of the experiment. In contrast to Ag exposure in the absence of IFA, effector activity within the DLN consistently demonstrated higher effector CTL activity throughout the experiment.

IFA promotes CTL responses to a subdominant peptide but not to a cryptic epitope

We evaluated the generation of CTLs with specificity for a defined subdominant peptide using the standard 51Cr release CTL assay in which effector cells were expanded in vitro with the subdominant peptide, and target cells were pulsed with the same peptide. Fig. 8 shows that a sustained subdominant response was detected in spleens taken from mice given 200 μg or 20 μg, but not to mice given 2 μg of OVA in IFA. A weak and transient subdominant-specific CTL response was also seen in mice given 200 μg of OVA only. This CTL response to the subdominant peptide was quantitatively weaker than those seen to the dominant peptide within the same groups of mice (Fig. 8 vs Fig. 6), suggesting the generation of lower, but not insignificant numbers of subdominant vs dominant precursor CTL. Additionally, effector cells expanded in vitro with the cryptic peptide did not demonstrate lytic activity toward targets pulsed with the cryptic peptide (data not shown), indicating that CTL specific to this epitope were not generated. Hence, the generation of responses to a subdominant peptide requires cross-presentation from a persistent and highly concentrated source of Ag.

Examination of in vivo responses to the same peptide after exposure to 200 μg of OVA/IFA, using differential CFSE labeling of cotransferred dominant and subdominant peptide-pulsed target populations, revealed a strong lytic response to the dominant epitope; however, little or no subdominant specific effector function within the DLN (Fig. 9) or other secondary lymphoid sites (data not shown) examined at any time point. Taken together, these data suggest that, although CTL to the subdominant peptide are
generated in mice given high OVA doses plus IFA, in vivo these CTL are poor effectors.

Discussion

Cross-presentation has recently been described as a general mechanism whereby protein Ags from nonhemopoietic cells gain access to the class I pathway and are presented to host CD8\(^+\) T cells for activation (6, 7). However, both the biology of this process and the physiological role of cross-priming are not yet clearly understood. Inducing CD8 effector cells against exogenous Ags is obviously important for defense against infectious agents. Many self-Ags may also be cross-presented under healthy conditions, as well as in diseases such as cancer and autoimmunity. Additionally, cross-presentation may be involved in the generation of an immune response after prophylactic or immunotherapeutic immunization. We used a soluble Ag that is not cell associated and rapidly removed after injection because it represented an ideal tool to determine the threshold requirements for the generation of CTL after cross-presentation.

Dose, distribution, and duration of cross-presented protein Ag

Until now, the relationship between dose and location of Ag presentation of noninfectious protein Ag in lymphoid tissues following a single inoculation was unknown. When the threshold antigenic concentration required for in vivo cross-presentation was examined, we were surprised to find that a single inoculation of a high dose of OVA, without adjuvant, results in Ag presentation that is detectable in the DLN for up to 3 wk. Similarly, Kurts et al. (7) have shown that cross-presentation of protein from an intact organ continued in the DLN for \(\sim\)10 days following removal of the organ. We believe that the cell responsible for cross-presenting OVA (the most probable candidate being the dendritic cell (DC);
Refs. 8 and 27) remained in the DLN and, after 3 wk, declined to below detectable levels for Ag presentation. This is supported by Garcia et al. (28) who reported that DC presenting a specific Ag to CD4 T cells remained within the spleen for 3 wk before disappearing, possibly via postmaturation, programmed DC apoptosis (29) or T cell-mediated DC lysis (30).

Interestingly, very high doses also generated weaker, but significant TCR T cell proliferation in other lymph nodes and in the spleen. We have recently shown that high viral loads, as well as varying levels of tumor Ags, generate significant levels of TCR T cell proliferation that are restricted to the DLN (9, 10); therefore, we believe that proliferation in other lymphoid sites represents local Ag presentation and not migrating T cells. How the Ag was transported to these distal sites remains unknown. Soluble protein may have “leaked” into the blood supply or lymphatics, alternatively the protein may have been transported in association with a cell. It is also unclear whether the Ag was transported directly from the inoculation site or via the DLN. Promoting Ag persistence at the injection site (via IFA) not only prolonged cross-presentation (to 8 wk) but also enhanced sensitivity to lower OVA concentrations. This may be due to an increase in local DC numbers in response to inflammatory signals, as described in other models (31).

The relationship between cross-presentation and generation of CTL recognizing dominant vs subdominant epitopes

To date, the bulk of work addressing hierarchical responses to class I epitopes has been undertaken using infectious models, in particular viral systems (3, 4). Research on responses to weaker epitopes of soluble proteins has been limited to in vitro systems or artificial in vivo systems (e.g., using proteins forced into the cytosol; Ref. 32). We found that in vivo effector CTL activity specific for the dominant epitope reflected Ag concentration and directly paralleled the kinetics of Ag presentation. In vitro assays showed that dominant-specific CTL precursors could be detected beyond the point at which Ag presentation had ceased. However, we were unable to generate long-term memory CTL after exposure to OVA minus IFA, a process believed to be under the control of IL-2 and Ag concentration (Refs. 33 and 34 and reviewed in Ref. 35).

Even in the absence of detectable Ag presentation, low antigenic concentrations resulted in the generation of dominant epitope-specific CTL, which may reflect 1) the ability of T cells to recognize vanishingly small numbers of peptide-class I complexes (36); 2) that we did not reach threshold detection levels; or 3) the Ag was rapidly processed, presented, and cleared within 7 days, implying a highly efficient system. In physiological terms, these data suggest that cross-priming Ag from infected cells requires small antigenic

FIGURE 8. Induction of CTL responses to the subdominant peptide. Splenocytes prepared from mice given log-fold dilutions of OVA with or without IFA (200 μg represented by ■, 20 μg by ●, and 2 μg by ○, respectively) were in vitro restimulated with APC pulsed with 10^{-6} M KVVRFDKL (the subdominant peptide). Five days later, a standard 51Cr in vitro CTL assay was performed using EL4 target cells pulsed with the same peptide. Controls included mice given PBS or IFA only (▲). Data are from one representative experiment of three, all of which showed the same pattern.

FIGURE 9. Analysis of in vivo effector CTL specific for the dominant and subdominant epitopes. Target cells taken from the spleens and lymph nodes of naive syngeneic mice were pooled and divided into three populations. One population was pulsed with 10^{-6} M SIINFEKL, a second population was pulsed with 10^{-6} M KVVRFDKL for 90 min at 37°C, and labeled with high (CFSE high) or low CFSE concentrations, respectively. Control uncoated target cells were labeled with an intermediate concentration of CFSE (CFSE int). Then 1 × 10^7 cells of each population were mixed before i.v. injection into recipient mice that had been previously treated with 200 μg of OVA/IFA. Histograms are from representative individual animals at each time point. These experiments were repeated five times with three animals per group.
concentrations to induce an immune response and that the higher the dose the stronger the CTL response will be. It also implies that when local Ag presentation can be demonstrated in the absence of CTL, as reported in tumor models (37), there is a defect in CTL generation/maintenance rather than a failure of Ag cross-presentation to reach a threshold required for CTL generation. However, we have also shown that the context of Ag delivery may be crucial when a strong and persisting CTL is the desired outcome. Prolonging Ag presentation not only generated CTL responses, but also promoted increased CTL responses to lower antigenic doses. Hence, when low doses of OVA are used in the absence of IFA, a few DC may transport and present Ag to a limited number of T cells; however, IFA may recruit larger numbers of DC that, once in the DLN, reach threshold detection levels. Furthermore, as more local T cells are activated, the resulting T-DC interaction may promote DC survival (29), leading to sustained generation of effector CTL. This observation may be valuable when designing immunotherapeutic vaccines for use in diseases such as cancer, where large amounts of tumor Ag may be difficult to obtain. Thus, use of low concentrations of tumor Ag with an adjuvant that prolongs Ag presentation may be more likely to promote a promising clinical outcome.

A weak CTL response to the subdominant peptide was seen, after in vitro restimulation, in spleens taken from mice given the highest OVA concentration. However, changing the context of Ag presentation (and possibly Ag processing) to a persistent source revealed a dose responsive subdominant response. IFA may generate a local site of inflammation that is accompanied by a cellular infiltrate or enhance the APC capacity of cells already identified as either presenting a wider range of Ags or preferentially presenting weaker Ags, such as B cells (38) or fibroblasts (39), respectively. Analysis via a 51Cr release in vitro assay demonstrated that the subdominant epitope had been processed and presented, resulting in the generation of a significant number of specific precursor CTL. These observations may help explain the high numbers of dominant epitope-specific CTL generated after acute viral infection (35), as well the emergence of T cell responses to other (often weaker) epitopes during chronic infection (40) in which higher viral antigenic concentrations are reached within an inflammatory microenvironment in association with prolonged Ag cross-presentation. However, these CTL responses were only detectable following in vitro restimulation with subdominant peptide-pulsed APC in the absence of the dominant peptide. Examination of CTL responses in vivo showed that subdominant-specific effector CTL could not be detected in association with strong dominant-specific functional CTL, implying either that these precursors did not become fully functional in vivo or that CTL recognizing the dominant epitope are potent suppressors, as suggested by others (13). It has been suggested that cryptic epitopes may be revealed when there is a sufficiently strong and prolonged inflammatory or humoral response. However, in these experiments, despite the induction of significant responses to other epitopes and detectable levels of circulating anti-OVA Abs (data not shown), no response to a cryptic epitope was unmasked.

In conclusion, we have shown that the threshold levels of Ag concentration required for the generation of dominant epitope-specific CTL, after cross-presentation, are low, and that the in vivo duration of their lytic activity parallels the kinetics of Ag presentation. In contrast, generating CTL to a weaker epitope requires a high Ag dose and persisting Ag cross-presentation. These findings have important implications for vaccination and immunotherapeutic strategies, and we are currently studying some of these issues using our tumor immunology models (9, 10).

Acknowledgments
We thank Drs. F. Carbone and W. Heath for providing breeding stock of the TCR-transgenic mice and Dr. A. Marzo for technical advice.

References


