Influence of strong CD4 epitope on long-term virus-specific cytotoxic T cell responses induced in vivo with peptides

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Abstract
Free unmodified peptides are poor immunogens for cytotoxic T lymphocytes (CTL) in mice, unless they are injected with adjuvant. Although it is generally accepted that CD4+ T_h cells are essential for CTL priming with peptides, it is not clear how long sequences devoid of any CD4 epitope, or strict CD8 epitopic sequences too short to be presented in a MHC class II-restricted fashion, can generate such responses. We thus have examined the extent to which the immunization protocol affects the need for a CD4 epitope. Since peptides are potentially important for vaccination, we also examined the duration of the CTL responses using a set of peptides that contained a CD4 epitope, or a CD8 epitope, or both epitopes in the same sequence, and compared immunization protocols previously found to induce CTL responses with peptides. The in vivo injection protocol had a marked effect, since the same CD8 sequence could generate a CTL response in a T_h-dependent or T_h-independent fashion, depending on the protocol used. The T cell help provided by natural CD4–CD8 sequences was inefficient in T_h-dependent CTL priming and CTL generation required help from a stronger exogenous CD4 peptide. The peptides could be injected either as a single tandem CD8–CD4 peptide or as a mixture of two separate peptides. T_h-independent CTL responses primed in other conditions proved to be as strong as T_h-dependent responses, at least when the animals were killed shortly after the last injection. However, only CTL responses generated together with specific T_h cells persisted for several months. Moreover, the efficacy of CTL persistence seemed to be correlated with the strength of the CD4 epitope for priming. This has important implications for the design of peptide vaccines.

Introduction
The capacity of peptides to stimulate immune responses has been recognized for some time. Certain peptides with potentially protective epitopes are now considered to be potential synthetic vaccines in humans. Ongoing vaccine development has shown that immunization schedules must achieve optimal induction of both humoral and cytotoxic responses, particularly for protection against viral infections. However, although an astonishing amount of basic information has been accumulated in trying to understand the molecular basis of antibody production with peptides in vivo, the effective use of peptides to induce cytotoxic T lymphocytes (CTL) still remains more a deliberate art of empirical immunization. There is thus a need to incorporate more basic science into the development of techniques for triggering CTL, especially for producing long-term memory responses, which are an essential feature in the design of a vaccine.

Free short unmodified synthetic peptides are poor immunogens for CTL and several approaches have been used to overcome this difficulty. Published immunization schedules may be classified according to the presentation of the synthetic peptide [free unmodified, incorporated into (ISCOMs) or coupled to lipids (lipopeptides) (1)], the requirement for adjuvant, and the route and number of injections. It has also been shown that CD4+ T cell help is needed for optimal
in vivo triggering of specific CTL responses (2,3), although several studies have described T\textsubscript{n}-independent CTL induction in certain antigenic systems (4-9).

We and others have previously shown that only modified peptides (i.e. lipopeptides) are immunogenic for CTL in vivo without the use of adjuvant (10,11). By contrast, several groups have reported that free unmodified peptides are good immunogens in the presence of adjuvant (12-17). Peptide sequences that correspond to the minimum, naturally processed antigenic peptide are of particular interest (17-19). Their high affinity for MHC class I molecules makes these peptides highly immunogenic. However, although CD4\textsuperscript{+} T cells are also essential for a CTL response (12,13), it is unclear how short peptides devoid of any CD4 epitope can induce these responses. Both basic research and vaccine development thus depend on knowing the extent to which an immunization protocol can affect the need for a CD4 epitope. This can be divided into three questions. (i) Does this concept still hold for any approach for CTL induction with peptides? (ii) Can exogenous or intrinsic help be provided by any CD4 epitope? (iii) What are the consequences for the long-term persistence of CTL responses?

In order to answer these questions, we compared the capacities of several immunization schedules to induce virus-specific CTL responses by injecting mice with peptides plus adjuvant. The peptides all contained a CD4 epitope, a CD8 epitope or both epitopes in the same sequence. Our results show that the CD8 peptides required help from CD4\textsuperscript{+} T cells to prime mice for CTL responses under certain conditions. In contrast to previous data (15), both peptides did not need to be physically linked. Sequences which included a natural CD4 epitope and were likely to provide help for themselves did not seem to provide enough help, since induction of CTL responses occurred only in the presence of an exogenous strong CD4 peptide.

Surprisingly, modifying both the number, the route and the frequency of injections for the same peptides caused a T\textsubscript{n}-dependent CTL response to become a T\textsubscript{n}-independent status for priming. However, only CTL induced with a concomitant Th cell response were detected several months after the last injection, suggesting that CD4\textsuperscript{+} T cells play a direct role in the persistence of the responses.

**Methods**

**Peptide synthesis**

Peptides (Neosystem, Strasbourg, France) corresponding to three proteins in mice antigenic systems were selected to contain CD8 epitopes (Table 1). Peptide cl.12-26 contained only a CD4 epitope in the H-2\textsuperscript{d} haplotype (Table 1). The tandem peptide A2-cl was also synthesized by a conventional solid-phase ‘Boc-benzyl strategy’ (20) in an automated Applied Biosystem 470A peptide synthesizer (Applied Biosystems, Foster City, CA), by double coupling and systematic capping with acetic anhydride (Neosystem). The peptides were checked for homogeneity by analytical reverse-phase HPLC and for identity by amino acid analysis after acid hydrolysis.

**Viruses**

Three strains of influenza virus were used in CTL activity assays: strains A/New Texas (A/NT, H\textsubscript{3}N\textsubscript{2}) and A/Puerto Rico/8/34 (A/PR/8, H\textsubscript{1}N\textsubscript{1}) were indifferently used to assay splen cell from NP2 or NP2S immunized mice. Due to slight differences in the sequence NP365-379 between these two strains, only A/Puerto Rico/8/34 (A/PR/8, H\textsubscript{1}N\textsubscript{1}) was tested to assay the lytic activity of spleen cells from NP3 (corresponding to strain A/PR/8) immune mice. In all assays, a B strain (B/Yamagata (B/Yam)) was used as a negative control. In this strain, point mutations in every NP synthetic peptides used ensure both the inability to bind the MHC restricting molecules and the subsequent absence of recognition by A strain-specific CTL.

**Immunization of mice**

BALB/c (H-2\textsuperscript{b}), C57BL/6 (H-2\textsuperscript{b}) and C57BL/6xF1 (F\textsubscript{1}) (H-2\textsuperscript{bx}) mice were purchased from Iffa Credo (L’Arbresles, France). Two immunization protocols were used: The first, described in Aichelle et al. (27), consisted of three priming injections at the base of the tail [50 nm of peptide in incomplete Freund’s adjuvant (IFA)] at 1 week intervals. Spleens were removed 1 week after the last injection. In the second protocol, mice were primed by s.c. injection of 50 nm of free peptide in IFA on the abdomen. They were boosted 3 weeks later by the same technique. Their spleens were removed 2 weeks after the second injection.

Spleen cells from all the mice were used to study Th activity. However, in some experiments, Th cells were best induced using another protocol, in which mice were injected on the peptide in complete Freund’s adjuvant (CFA) at the base of the tail and into the footpads (28). The spleens of these mice were removed 10 days later and assayed for IL-2 specific release.

In all assays, mice were tested individually.

**IL-2 release assay**

Single cell suspensions were obtained from the spleens. The cells were washed twice in RPMI 1640, resuspended at 5x10\textsuperscript{6} cells/ml in culture medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, non-essential amino acids, 1 mM sodium pyruvate, 10 mM HEPES, 50 µM 2-mercaptoethanol) plus 1% Nutridoma SP (Boehringer-Mannheim, Germany). Triplicate aliquots of cell suspension (100 µl) were placed in round-bottomed microtiter wells (Costar), peptides were added at the indicated concentrations, and the cells cultured for 24 h at 37°C and 5% CO\textsubscript{2}. Aliquots of supernatant (100 µl/well) were removed and the IL-2 assays assayed using CTL L2 cells (10\textsuperscript{6} cells/well). [\textsuperscript{3}H]Thymidine was added (1 µCi/well) for the last 6 h of a 24 h incubation. The cells were harvested with an automatic cell harvester (Skatron, Sterling, VA) and [\textsuperscript{3}H]thymidine incorporation was quantified by scintillation counting.

**Cellular cytotoxicity assay**

CTL were generated by in vitro stimulation performed by mixing 3x10\textsuperscript{6} responding cells with 6x10\textsuperscript{5} irradiated (4000 rad) stimulating cells in 2 ml culture medium supplemented with 10% heat-inactivated FCS. These stimulating cells were...
dependence of long-lasting CTL induced with peptides

I-A2 in BALB/c mice NP2S in BALB/c mice NP3 in C57BL/6 mice

Th-independent protocol for CTL priming

Effector to target ratio

Fig. 1. Role of \( T_h \) activation in CTL induction with peptide following different immunizing conditions. BALB/c or C57BL/6 mice were injected either thrice at the base of the tail (A) or twice s c (abdomen) (B) with 100 \( \mu \)g of peptides A2, NP2S or NP3. Spleen cells of these mice were re-stimulated twice in vitro with A2- or NP2S-pulsed BALB/c or NP3S-pulsed C57BL/6 spleen cells, and CTL activity was tested 5 days after the second re-stimulation on syngeneic P815 and EL4 target cells pretreated as indicated (ENV irrelevant HIV-1 V3.312-327, A2-TC HLA-A2 P815 transfected cells)

obtained by incubating \( 1 \times 10^7 \) syngeneic spleen cells from naïve mice in 1 ml medium containing 30 \( \mu \)g synthetic peptide for 3 h at 37°C. They were washed before use. A weak, but significant CTL activity could be generally observed after 5 days of in vitro re-stimulation. However, in order to assay this activity on several target cells, a second re-stimulation was necessary to allow amplification of the in vivo primed precursors. In most experiments, an additional in vitro stimulation was performed 1 week later using stimulating cells prepared as described above, in culture medium supplemented with 5% human IL-2-containing supernatant prepared elsewhere (29). Cytotoxicity was evaluated 5–6 days after each in vitro stimulation, using appropriate tumor cell lines as targets: P815 (H-2b, DBA/2) and EL4 (H-2b, C57BL/6). Influenza virus-infected cells were obtained by incubating cells for 90 min with 100 HU influenza virus [A/New Texas (A/NT, H3N2), A/Puerto Rico/8/34 (A/PR/8, H1N1) or B/Yamagata (B/Yam)]. These cells were used as targets after a further 2–3 h incubation to allow virus replication. Peptide-pulsed target cells were prepared by a similar incubation with peptide (final concentration 20 \( \mu \)g/ml) and used immediately. P815-A2 cells expressing the whole HLA-A2 molecule after gene transfection (A2-Tc) (25) were also used. The target cells were labeled with 100 \( \mu \)Ci \( \text{Na}_2^{51}\text{CrO}_4 \) (CEA, Gif sur Yvette, France) for 90 min at 37°C and washed twice.

Target cells (5000 in each well) and serial dilutions of effector cells were then incubated together in 200 \( \mu \)l culture medium in round-bottomed microtiter plates for 4 h at 37°C. The assay was terminated by counting the \( ^{51}\text{Cr} \) released into the supernatants. The specific lysis was calculated as: \( 100 \times (\text{experimental} - \text{spontaneous release})/(\text{total}^{51}\text{Cr incorporated} - \text{spontaneous release}) \). Spontaneous release in the absence of effector cells was <20% of total \( ^{51}\text{Cr} \) incorporated in all experiments.

Results

\( T_h \)-independent CTL responses induced with CD8 peptides under certain immunization conditions.

CTL responses were generated with free peptides in vivo by a classical three-injection protocol into the base of the tail (27). In contrast with the dogma of CD4+ T cells (\( T_h \)) requirement for successful priming of mouse anti-viral CTL, several peptides that contained only a CD8 epitope for a given strain of mice were immunogenic in vivo for CTL (13,14,17–19). We
T<sub>H</sub> dependence of long-lasting CTL induced with peptides

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**Fig. 2.** Restoration of CTL induction by injection with CD8 peptide plus a strong CD4 peptide. (A) BALB/c mice were injected s.c. (abdomen) with either the tandem peptide A2-cl or a mixture of peptides A2 or NP2S and cl. Spleen cells from these mice were re-stimulated in vitro as in Fig. 1 and tested for cytolysis activity on P815 target cells pretreated as indicated. (B) The same responding cells were also assayed for their helper activity upon in vitro re-stimulation with the indicated peptides. IL-2 in supernatants of a 24 h culture of these cells was titrated by [<sup>3</sup>H]thymidine incorporation into CTL L2 cells.

attempted to reproduce these unexpected findings with three different peptides (Table 1): NP3 (as previously described) injected into C57BL/6 and NP2S from the influenza virus NP injected into BALB/c mice; A2 from the HLA-A2 molecule was also injected in BALB/c mice. Spleen cells from mice immunized with these three peptides all produced high levels of specific CTL lysis (Fig. 1A). Effector cells efficiently recognized target cells sensitized by both the exogenous peptide and the naturally processed peptide (HLA-A2 transfected or influenza virus-infected syngeneic cells). These results were reproduced with several individual tested mice (not shown). Thus CTL responses could be generated by this immunization protocol without simultaneous specific activation of CD4<sup>+</sup> T<sub>H</sub> cells. This was confirmed by the absence of specific IL-2 release in these experimental conditions (not shown).

**T<sub>H</sub>-dependent CTL responses induced under other immunization conditions**

The ability of the same three peptides to induce CTL responses in mice was then evaluated using a route more relevant to human vaccination (two s.c. abdominal injections 3 weeks apart). This protocol efficiently induces CTL with lipopeptides (11). Very few mice produced CTL specific for the tested peptides (Fig. 1B and Table 2), suggesting that a concomitant T<sub>H</sub> cell response is required under these immunizing conditions.

The required help was provided by the construct A2-cl, which consisted of peptide A2 followed by peptide cl, which is known to induce strong CD4<sup>+</sup> T helper cell responses in the H-<sup>2</sup>d haplotype (Table 1). BALB/c mice injected with this tandem peptide in IFA generated strong, reproducible A2-specific CTL responses (Fig. 2A and Table 2), plus cl-specific T<sub>H</sub> cells, as measured by the release of IL-2 from the spleen cells of these mice (Fig. 2B and Table 2). Thus the immunogenicity of the two peptides can be maintained, even when they are combined in a single peptide construct. Also, the peptides do not need to be physically linked, since a mixture of the peptides is also immunogenic for both types of effectors with the same efficiency (Fig. 2A and B, and Table 2). Similar results were obtained with peptide NP2S: only mice injected
Table 2). Similarly, a mixture of peptides NP3 and cl generated a non-responding mouse, Table 2), and all attempts to reproducibly prime NP3-specific CTL responses (Fig. 3 and Table 2). This indeed occurred with peptide NP3 injected into C57BL/6 mice, whose H-2^K^ hybrid haplotype allowed the presentation of the peptide by both class I and class II molecules (Table 1). However, it was inefficient, since only two out of four F, mice responded (as illustrated in Fig. 2A and B, and Table 2).

**Insufficient help from naturally occurring CD4–CD8 sequences**

Exogenous help enables free CD8 peptides to generate in vivo CTL responses. Hence, natural CD4–CD8 peptides may be able to provide internal help for themselves and induce CTL responses using the same immunization protocol. This indeed occurred with peptide NP3 injected into C57BL/6×CBA F1 mice, whose H-2^D^x^K^ hybrid haplotype allowed the presentation of the peptide by both class I and class II molecules (Table 1). However, it was inefficient, since only two out of four F1 mice responded (as illustrated in Fig. 3 for one non-responding mouse, Table 2), and all attempts to generate CTL in BALB/c mice with peptide NP2, which includes the sequence of NP2S and an additional CD4 epitope (Table 1), failed (Fig. 3 and Table 2). However, injection of NP2 plus strong CD4 peptide like cl elicited CTL (Fig. 3 and Table 2). Similarly, a mixture of peptides NP3 and cl reproducibly primed NP3-specific CTL responses (Fig. 3 and Table 2).

These results suggest that the intrinsic CD4 epitope in peptide NP2 was not strong enough to provide help for itself, in agreement with the observation that only cl-specific Th cells were detected in mice immunized with both peptides (not shown). The helper activities provided by the two peptides were compared by injecting BALB/c mice under experimental conditions designed for optimal detection of the Th cell activity. Much more IL-2 was released by the spleen cells of mice immunized with 100 μg peptide cl than by spleen cells from mice immunized with 100 μg NP2 (Fig. 4). This helper activity was also detected in mice immunized with as little as 1 μg peptide cl, whereas mice given 10 μg peptide NP2 produced no IL-2. Peptide NP3 (100 μg) injected into F1 mice gave an intermediate response which could reflect the moderate CTL-inducing capacity of this peptide.

**Short-lived CTL in the absence of specific Th cells**

The second immunization protocol showed the strict dependency of the CTL priming with free peptides on CD4+ T cell help. This was also observed with lipopeptides using the same conditions for CTL priming (30) and these responses persisted for over 6 months. Therefore, for vaccinal purposes, it was of interest to study the duration of the CTL responses induced without T cell help with the first immunization protocol. The strong CTL responses specific for the three CD8 peptides were short-lived, since they were undetectable 3 months after the last injection. Indeed, no cytolytic activity was found in four out of four BALB/c mice immunized with A2 (as illustrated in Fig. 5A), in four out of four C57BL/6 mice immunized with NP3 (as illustrated in Fig. 5C) and in five out of five BALB/c mice immunized with NP2S (not shown). However, the response of mice primed with the CD8 peptide plus the strong CD4 peptide cl was still present at 3 months in five out of six BALB/c mice tested in the A2 antigenic system (as illustrated with a positive mouse in Fig. 5B) and in two out of two F1 mice injected with NP3 alone had less persistent CTL activity, since only one out of four mice remained positive (not shown). This may be due to the moderate helper activity of this peptide. In conclusion, when the results from the different antigenic systems were pooled together, no detectable CTL response could be observed in mice (0/13) injected 3 months previously with the CD8 peptide alone, but CTL still persisted in seven out of eight mice co-immunized with the CD8 and the strong CD4 cl peptide and in only one out of four mice injected with a natural CD8–CD4 peptide.

**Discussion**

Free peptides are weak immunogens for CTL in vivo unless they are given as lipopeptides. However, defined immunizing conditions, especially injections with adjuvant efficiently elicit CTL responses to peptides, and protective responses have been obtained with several mouse viral systems (14,16–18). The present study examines the cellular and molecular requirements for in vivo priming of CTL responses with unmodified peptides, by analyzing the influence of several parameters in the immunization protocol on CTL induction, especially with regard to the need for CD4 T cell help. Although it is generally accepted that induction of CTL...
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Fig. 4. \( T_h \)-inducing capacities of different CD4 epitopes. BALB/c or F1 mice were injected once at the base of the tail and in footpads with 100, 10 or 1 \( \mu \)g peptides cl, NP2 or 100 \( \mu \)g peptide NP3. Splenic cells were removed from immune mice 10 days later and re-stimulated \textit{in vitro} with the immune peptides. The IL-2 content of a 24 h culture was titrated by measuring the \(^{3}H\)thymidine incorporation into CTL L2 cells.

Table 1. Synthetic sequences used

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Protein</th>
<th>Peptide</th>
<th>Sequence*</th>
<th>MHC restriction</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP2</td>
<td>influenza A NP</td>
<td>NP 147-158</td>
<td>TYQTRALVTG</td>
<td>( K^d )</td>
<td>Class I</td>
</tr>
<tr>
<td>NP2S</td>
<td>influenza A NP</td>
<td>NP 147-155</td>
<td>TYQTRALY</td>
<td>( K^d )</td>
<td>Class II</td>
</tr>
<tr>
<td>NP3</td>
<td>influenza A NP</td>
<td>NP 365-379</td>
<td>IASNENMAMESSTL</td>
<td>( D^b )</td>
<td>(23)</td>
</tr>
<tr>
<td>A2</td>
<td>HLA-A2</td>
<td>A2 170-185</td>
<td>RYLENGKETQLT0DAP</td>
<td>( K^d )</td>
<td></td>
</tr>
<tr>
<td>cl</td>
<td>lambda phage cl repressor protein</td>
<td>cl 12-26</td>
<td>-</td>
<td>-</td>
<td>(22, 24)</td>
</tr>
<tr>
<td>A2-cl</td>
<td>HLA-A2 tandem</td>
<td>cl 12-26</td>
<td>RYLENGKETQLT0DAP-LEDARRLKNAIYKMM</td>
<td>( I-A^d, I-A^k )</td>
<td>(26)</td>
</tr>
</tbody>
</table>

*CTL epitopes are shown in bold type.

requires CD4\(^+\) T cell help (2,3), there have been conflicting data from some experimental virus infections. For example, in vivo depletion of CD4\(^+\) T cells did not affect CD8\(^+\) CTL priming following infection with ectromelia virus (5), influenza virus (31), vaccinia virus (32), LCMV (33-35) or HSV (6). Likewise, infection of CD4 knockout mice with LCMV did not prevent the generation of virus-specific CTL responses (36). To investigate the in vivo requirement of such T cell help with peptides, we have used several 15- to 16-mer long CD8 peptides which are unable to trigger T\(_h\) cells (A2 and NP3), and a 9-mer peptide corresponding to the naturally presented CTL epitope (NP2S). The requirement for T cell help in CTL generation with peptides was found to depend greatly on the immunization schedule.

Subcutaneous injections of CD8 peptides did not prime CTL without specific help. However, natural CD8-CD4 sequences (NP2 in BALB/c mice, NP3 in F1 mice) did not provide such help for themselves. Similar observations were made in another antigenic system, peptide V3.312-327 of HIV-1 (not shown), which also contains a CD8 epitope and a weak CD4 epitope (11). Only mice immunized with the CD8 peptide plus a potent activator of CD4\(^+\) T\(_h\) cells (as measured by specific IL-2 release), like peptide cl, generates strong CTL responses. Remarkably, lipopeptides with a weak CD4 epitope are the only peptides that work s.c. in the absence of adjuvant (30).

Conversely, injection into the base of the tail, a route usually used in mouse immunization protocols, produced efficient CTL priming with all the peptides tested, and seems to bypass the need for specific help from CD4\(^+\) T cells. The fact that the same peptides were used indicates that this difference is due to the immunizing protocol and not to different antigenic systems. However, in vivo depletion of CD4\(^+\) T cells has been
shown to have dramatic effects on CTL priming under the same immunizing conditions (12,13), suggesting that Th cells are still essential in this particular experimental approach. However, our results agree with several studies that used long antigenic peptides and showed that CTL responses can be primed using this protocol in vivo without specific CD4+ T cell help (13,14). Similar results were obtained with short peptides that correspond to endogenously processed CD8 peptides and are too short to be presented by MHC class II molecules (17–19). The strong in vivo immunogenic potency of these short peptides is correlated with their high-affinity binding to the restricting MHC class I molecule in vitro (19,37). This is not an absolute criterion and other factors are involved (38). The high-affinity binding of the peptide could influence the avidity of the TCR for antigen generated by different densities of class I–peptide complexes on the surface of antigen-presenting cells (APC), as recently described in a transgenic mouse model (39).

None of the experiments performed with short peptides addressed the duration of the CTL protective effects. Indeed, experimental infections with four viruses revealed that CD4+ Th cells are more important after the initiation of the CTL responses (6,7,32,40–42). Likewise, MHC class-II-deficient mice can generate CD8+ CTL that transiently control a primary infection with Leishmania major, but are unable to resolve such infections (43). The increase in Th dependency over time could reflect a progressive loss of the ability of CD8+ CTL to secrete their own growth factors, especially IL-2, as shown in vaccinia-infected mice (32). This occurred in Sendai virus infection, where Th clones cooperated with Tc clones inoculated at the same time to provide help to virus-specific Tc by producing IL-2 (40). The same conclusions were drawn for the induction of male antigen-specific CTL responses in transgenic mice (44).

Our results show for the first time that CTL primed with peptides without specific help are also short lived. This could explain why Sendai virus-specific CTL can be raised in C57BL/6 mice with peptide NP.321–336 independently of specific Th cells and why the unprimed status of Th cells at the time of challenge was suspected to leave some time for the virus to initiate signs of disease in peptide-immunized mice (14). The persistence of CTL responses requires the presence of Th cells. These can be induced by using a CD4–CD8 tandem peptide or more interestingly by injection of the CD8 peptide together with a CD4 peptide. The strength of the specific help, as measured by IL-2 release, also influences the duration of the CTL response. This is of prime importance in the design of a vaccine.

This raises the question of what feature of the immunization protocol determines a CD8+ CTL Th-dependent or Th-independent phenotype and, consequently, a short or long lasting CTL response. The influence of either the route or the number and frequency of injections was examined. It appeared that the route was the most important parameter since injections at the base of the tail were always successful in CTL priming even with two spaced injections, whereas no peptide was immunogenic when three closely injections were made s.c. (not shown). The nature of the APC was shown to be critical in the recruitment of specific T lymphocyte subpopulations. When injections are made at the base of the tail, the antigen

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**Table 2. Role of strong Th epitope for CTL priming.**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Epitope</th>
<th>No of responding mice</th>
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<tbody>
<tr>
<td>A2</td>
<td>CTL</td>
<td>1/5</td>
</tr>
<tr>
<td>NP2S</td>
<td>CTL</td>
<td>0/4</td>
</tr>
<tr>
<td>NP3 in C57BL/6</td>
<td>CTL</td>
<td>0/4</td>
</tr>
<tr>
<td>A2-cl</td>
<td>CTL + Th\textsuperscript{strong}</td>
<td>5/5</td>
</tr>
<tr>
<td>A2 + cl</td>
<td>CTL + Th\textsuperscript{strong}</td>
<td>4/5</td>
</tr>
<tr>
<td>NP2S + cl</td>
<td>CTL + Th\textsuperscript{strong}</td>
<td>3/3</td>
</tr>
<tr>
<td>NP3 in F1</td>
<td>CTL + Th\textsuperscript{endogenous}</td>
<td>2/4</td>
</tr>
<tr>
<td>NP2</td>
<td>CTL + Th\textsuperscript{endogenous}</td>
<td>0/4</td>
</tr>
<tr>
<td>NP2 + cl in F1</td>
<td>CTL + Th\textsuperscript{endogenous}</td>
<td>2/2</td>
</tr>
<tr>
<td>NP2 + cl</td>
<td>CTL + Th\textsuperscript{strong}</td>
<td>2/2</td>
</tr>
</tbody>
</table>

Mice were injected twice s.c. and spleen cells were assayed for CTL activity 2 weeks after the last injection. Results are expressed as the number of mice with a significant specific cytotoxic activity (at least 20 and 10% more lysis) on both the infected and peptide-pulsed target cells than in negative control targets, at E:T ratios of 100:1 and 20:1 respectively) over the number of mice injected.
is rapidly transported to local lymph nodes where dendritic cells (DC) are present. DC are known to be able to recruit T<sub>H</sub>-independent CTL (4,45). These properties seem to correlate with an important surface representation of specific adhesion molecules such as B7/B81 interacting with CD28, a critical step in T<sub>H</sub>-independent activation of CD8<sup>+</sup> CTL (6,9). The expression of such molecules on Langerhans cells is up-regulated by pro-inflammatory cytokines (47), some of which are probably released locally by injection of IFA. Such an in vivo effect of IFA may explain why Mutsunori et al. concluded that a CD4 epitope is essential for CTL priming with CD8 peptides when using another adjuvant such as QS21 (15). The same authors also concluded that both CD4 and CD8 peptides needed to be linked to prime CTL responses, whereas our results show the possibility to trigger CTL by separate peptides. This could also be explained by an intrinsic property of IFA to co-sequestrate the two peptides in a same emulsion.

The present results show that the maintenance of a CTL response induced with peptides depends on whether the specific help provided at the time of priming is strong enough. This has important implications in vaccinal strategies, as synthetic peptides are very good candidate as immunogens.

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Abbreviations
APC antigen-presenting cell
CFA complete Freund's adjuvant
DC dendritic cell
IFA incomplete Freund's adjuvant
NP nucleoprotein

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