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Induction of cytotoxic T-cell response by optimal-length peptides does not require $CD4^+$ T-cell help

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SUMMARY

In several experimental models, synthetic peptides were shown to activate efficiently cytotoxic T-lymphocyte (CTL) responses and therefore represent an attractive strategy to develop new vaccines. However, the mechanisms by which they induce CTL responses are not yet fully understood. Several studies using 15–16-mer peptides previously demonstrated that $CD4^+$ helper T cells are required to induce optimal CTL responses with synthetic peptides. However, recently, it was suggested that shorter 8–12-mer peptides could have an increased *in vivo* immunogenicity. In the present study, we therefore investigated if such optimal-length peptides still require $CD4^+$ T-cell help to activate CTL responses. To address this question, three synthetic peptides containing different viral CTL epitopes were injected into mice depleted of $CD4^+$ or $CD8^+$ T cells using specific monoclonal antibodies or into mice genetically deficient in those T-cell populations. Our results clearly established that activation of CTL responses by those short optimal peptides does not require $CD4^+$ T-cell help and therefore suggested that high-density binding of peptides to major histocompatibility complex class I molecules on the surface of antigen-presenting cells is required for direct activation of $CD8^+$ T cells, independently of $CD4^+$ T-cell help.

INTRODUCTION

Virus-specific cytotoxic T lymphocytes (CTL) play an important role in the resistance against many viral infections. The activation of strong CTL responses represents therefore a major objective for the development of efficient vaccines against many viral diseases. Recently, *in vivo* immunization with synthetic peptides was shown to be an efficient strategy to induce CTL responses against several viral antigens[1–4] or against a mouse malaria epitope.[5] Moreover, mice immunized with synthetic peptides containing CTL epitopes were shown to be protected against challenge with lymphocytic choriomeningitis virus (LCMV) or Sendai virus.[3,6]

Several of these studies also demonstrated that $CD4^+$ T-helper cells play a crucial role in the activation of CTL responses by these short synthetic peptides.[2,7] Moreover, hybrid peptides containing both helper and CTL epitopes were shown to have increased efficiency in inducing CTL responses as compared to peptides containing only CTL epitopes.[4,8] However, these studies were performed with 15–16 amino-acid long peptides whereas it was recently suggested that optimal-length 8–12 amino-acid long peptides, similar to endogenously produced peptides in virus-infected cells, could have higher efficiency to stimulate CTL responses.[9]

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In the present study, we therefore investigated the role of $CD4^+$ T cells in the activation of CTL responses by three different viral epitopes, previously defined as optimal for binding to major histocompatibility complex (MHC) class I molecules. Our results clearly demonstrated that these optimal-length peptides can activate CTL responses, independently of $CD4^+$ T-cell help.

MATERIALS AND METHODS

Mice

Inbred C57BL6/J (H-2^b) and BALB/c (H-2^d) female mice 6–8 weeks old were purchased from Iffa Credo (l'Arbresle, France). Mice lacking $CD4^+$ or $CD8^+$ T-cells[10,11] were kindly given by Dr Tak Mak.

Synthetic peptides

The synthetic LCMV (118–126) peptide RPQASGVYM corresponding to the optimal 9-mer H-2^d T-cell epitope of the nucleoprotein of LCMV[12] and the LCMV (118–132) peptide RPQASGVYMGNLTAQ[1] were synthetized by Neosystem (Strasbourg, France). The peptide SGPSNTPPEI corresponding to amino acid residues 234–243 of E1A region of adenovirus type 5[13] and the peptide RGYVYQGL corresponding to amino acid residues 52–59 of the nucleocapsid protein (NP) of vesicular stomatitis virus (VSV)[14] were synthesized using an applied Biosystems 430 peptide synthetizer (Applied Biosystems, Inc., Forster City, CA).[9,15]

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Cell lines and culture medium
EL4 (H-2^b, thymoma) and P815(H-2^d, mastocytoma) cells were purchased from ATCC (American Type Culture Collection, Rockville, MD). Cells were grown in complete RPMI-1640 medium (Seromed, Berlin, Germany) containing 10% heat-inactivated fetal calf serum (FCS), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine and antibiotics.

Immunization and in vivo *treatment of mice with anti-CD4 and anti-CD8 monoclonal antibodies*
Mice were subcutaneously injected in the base of the tail with 100 μg of synthetic peptides dissolved in phosphate-buffered saline (PBS) and emulsified with the same volume of incomplete Freund's adjuvant (IFA; Sigma, l'Isle d'Abeau Chesnes, France). For $CD4^+$ and $CD8^+$ depletion, mice were injected intraperitoneally (i.p.) on days −1, 0, +1 with 300 μg of CD4 (GK1–5) or CD8 (H35.17.2)-specific rat anti-mouse monoclonal antibodies (mAb), semi-purified from ascitic fluids as previously described.[7] Control mice received the same volume of PBS.

In vitro *stimulation of* in vivo *primed effector cells and cytotoxicity assay*
Ten to fourteen days after immunization, mice were killed and spleens were removed aseptically. A single spleen cell suspension was prepared in RPMI-1640 medium (Seromed, Berlin, Germany) supplemented with 10% FCS, antibiotics, 2 mM L-glutamine, and 5×10^{-5} M 2-mercaptoethanol. Responder spleen cells (25×10^6) from *in vivo* primed mice were co-cultured with 25×10^6 irradiated (2500 rads) syngeneic spleen cells, in the presence of 0·05 μM peptides for 5 days at 37° in humidified air with 7% CO_2.

Cytotoxic effector populations were harvested after 5 days of culture. Target cells were obtained by incubating 2×10^6 cells with 50 μmol of peptide and 200 μCi of $Na_2{}^{51}CrO_4$ for 1 hr. After washing, 100 μl of target cells (10^5 cells /ml) were added to 100 μl of serial dilutions of effector cells in microtitre plates. Cultures were incubated for 4 hr at 37° with 7% CO_2. After incubation, the supernatants were collected and the percentage specific ^{51}Cr lysis was calculated by the formula: % specific lysis = 100 × (experimental − spontaneous)/(maximal − spontaneous) c.p.m. Spontaneous release was determined from target cells incubated without adding effector cells. Maximum lysis was determined from supernatants of cells that were lysed by adding 0·1 N HCl. Experimental lysis was expressed as the mean of duplicate or triplicate cultures. All the presented values have a standard error value of less than 10%. Spontaneous lysis was always less than 15–20% of the maximal lysis.

Assay for peptide-specific proliferative response of in vivo *primed T cells*
Mice were injected once as described and 2 weeks later, the animals were killed and the inguinal lymph nodes (LN) were removed aseptically. A single cell suspension of LN cells was prepared in complete RPMI-1640 medium containing 1·5% FCS and 0·5% normal mouse serum.

The cells were cultured in 0·2 ml in the presence of medium or peptide at a final concentration of $7{\cdot}5 \times 10^6$ cells/ml in 96-well flat bottom plates (Costar, Cambridge, MA) in triplicate. After 3 days at 37°, cells were pulsed for 18 hr with [^{3}H] thymidine ([^{3}H]TdR) (AS = 20 Ci/mmol; NEN Research Products, Boston, MA) and then harvested onto fibre glass filters (Wallac Oy, Turku, Finland) with an automatic cell harvester. Incorporated radioactivity was measured as background substracted geometric means. SD of triplicate cultures were less than 15% of the mean.

RESULTS

The nonapeptide LCMV(118–126) contains both $CD4^+$ and $CD8^+$ T-cell epitopes

The major $CD8^+$ T-cell response in BALB/c (H-2^d) mice infected with LCMV is directed against the amino acid 112–132 region of the (NP).[1] Moreover, the LCMV (118–132) peptide was shown by Aichele *et al.*[1] to induce antiviral cytotoxic T-cell response *in vivo*. Truncations of the N and C termini of the NP 112–132 sequence demonstrated that the nonapeptide 118–126 was efficiently recognized at 10^{-9} to 10^{-11} M concentration. This LCMV optimal peptide, LCMV(118–126) was recently shown to have a higher capacity to up-regulate L^d expression than LCMV(118–132) *in vitro* and also to elicit a stronger anti-peptide CTL response *in vivo*.[9,15]

In a previous study, we demonstrated that the LCMV (118–132) peptide contains both $CD4^+$ and $CD8^+$ T-cell epitopes. Moreover, the *in vivo* elimination of $CD4^+$ T cells by treatment with a mAb was shown to strongly reduce the anti-peptide CTL response induced by the LCMV (118–132) peptide.[7] In the present study, we therefore examined whether $CD4^+$ T-cell help is still required for CTL induction by the optimal nonapeptide LCMV (118–126). To address this question, we first tested if this minimal $CD8^+$ T-cell peptide is recognized by $CD4^+$ T lymphocytes.

BALB/c mice were immunized with the LCMV (118–126) peptide and draining lymph node cells were restimulated *in vitro* either with the priming 9-mer peptide or the 15-mer LCMV (118–132) peptide previously shown to elicit both cytotoxic and proliferative responses.[7] As shown in Fig. 1(a), lymphocytes from mice primed with the LCMV (118–126) peptide proliferated in response to both the 9-mer and the 15-mer LCMV peptides. Moreover, lymph node cells from

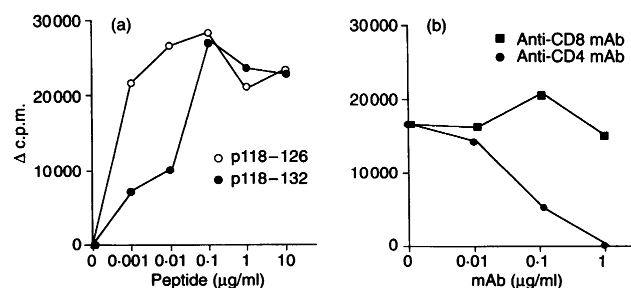

Figure 1. The optimal-length LCMV (118–126) peptide contains a $CD4^+$ T-cell epitope. BALB/c mice (three per group) were immunized subcutaneously with 100 μg of LCMV (118–126) peptide emulsified in IFA, and 2 weeks later, LN cell proliferative responses to various concentrations of LCMV (118–126) or LCMV (118–132) peptides were assayed (a). The proliferative response of primed LN cells to LCMV (118–126) peptide (10 μg/ml) was tested in the presence of various concentrations of anti-CD4 or anti-CD8 mAb (b).

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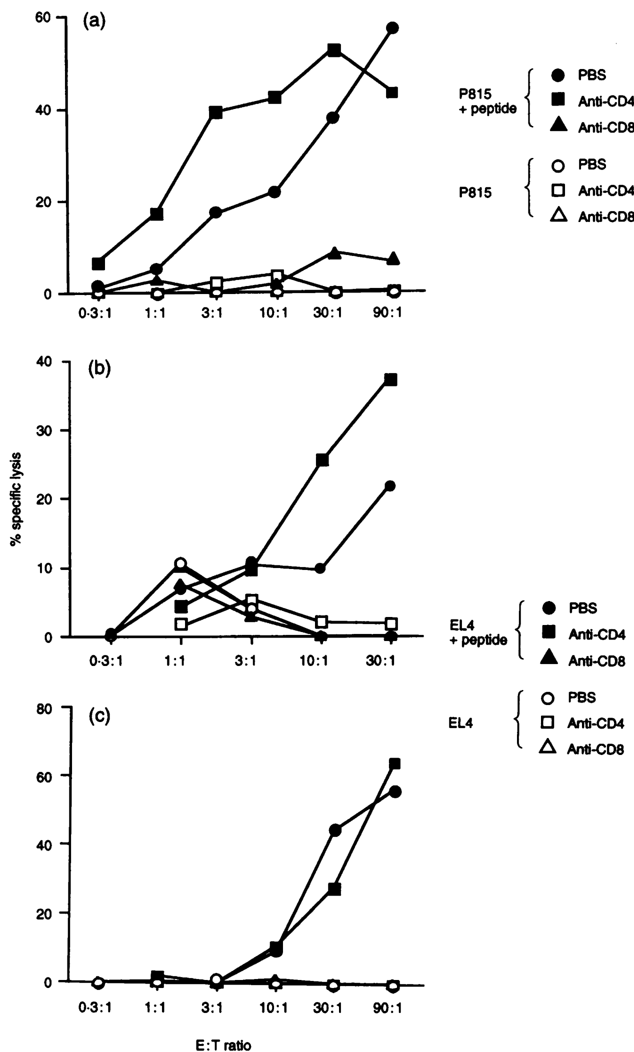

Figure 2. Optimal-length peptides induce strong CTL responses in the absence of $CD4^+$ T cells. On day 0, BALB/c (a) or C57BL/6 mice (b and c) were immunized subcutaneously with 100 μg of LCMV (118–126) (a) or VSV (NP52–59) (b) or E1A (234–243) (c) peptides emulsified in IFA. On days −1, 0, +1, mice received intraperitoneal injections of PBS or of 300 μg of either anti-CD4 or anti-CD8 mAb. On day 10, mice were killed and CTL responses were determined after *in vitro* stimulation of primed spleen cells with irradiated syngeneic spleen cells and 0·05 μM of LCMV (118–126) (a), or VSV (NP52–59) (b) or E1A(234–243) (c) peptide. After 5 days in culture, cytotoxic activity of spleen cells was tested either on P815 target cells coated with LCMV (118–126) peptide (a) or on EL4 target cells coated either with VSV (NP52–59) (b) or E1A (234–243) (c) peptide or left untreated. Results are expressed as the mean percentage of specific lysis of duplicate cultures.

BALB/c mice immunized with the LCMV (118–132) peptide proliferated equally well to the LCMV (118–126) and LCMV (118–132) peptides (data not shown). Altogether, these data suggest that the minimal LCMV (118–126) peptide contains a $CD4^+$ T-cell epitope. This was confirmed by the total inhibition of proliferative response observed in LN cell cultures stimulated with the LCMV (118–126) peptide and incubated with an anti-CD4 mAb (Fig. 1b). These results therefore suggest that the nonapeptide LCMV (118–126) binds to MHC class I and II molecules and stimulates both $CD4^+$ and $CD8^+$ T-cell responses. It remains at this stage, however, to determine if the $CD4^+$ T-cell response induced by the LCMV (118–126) peptide plays a role in the activation of $CD8^+$ CTL activity, as previously shown for LCMV (118–132) peptide.[7]

In vivo *depletion of $CD4^+$ T cells does not abrogate the induction of CTL responses by the optimal length LCMV (118–126) peptide*

We therefore next determined if $CD4^+$ T cells are required for the induction of CTL responses. Mice were primed with one injection of LCMV (118–126) peptide and *in vivo* elimination of $CD4^+$ or $CD8^+$ T cells was performed by chronic treatment of mice with mAb as previously described, using the same batch of mAb than in our previous study with the LCMV (118–132) peptide.[7] Data shown in Fig. 2(a) demonstrate that the peptide-specific CTL response was totally abolished in mice treated with the anti-CD8 mAb. In contrast, this response was not inhibited in BALB/c mice treated with the anti-CD4 mAb and was even slightly increased, presumably due to the higher percentage of $CD8^+$ T cells found in mice treated with this mAb.[7]

These results strongly suggest that the CTL response induced by this optimal nonapeptide does not require additional help from $CD4^+$ T lymphocytes. This discrepancy with our previous results obtained with the LCMV (118–132) peptide could be related to the increased binding to MHC class I molecules of the nonapeptide as compared to the 15-mer peptide. It could therefore be suggested that optimal length peptides, binding with a high affinity to MHC class I molecules, may accumulate on the surface of antigen-presenting cells. The increase in density of MHC molecules complexed with the peptide could lead to direct stimulation of $CD8^+$ cytotoxic T cells, without requirement for any additional $CD4^+$ T-cell help.

To test this hypothesis, we investigated the role of $CD4^+$ T cells in the induction of CTL responses by two other short peptides, previously defined as being optimal for binding to K^b and D^b molecules. The first one corresponds to the amino acid residues 52–59 of the NP of VSV[14] whereas the second is located between amino acid residues 234–243 of the E1A region of adenovirus type 5.[13] None of the two peptides stimulated proliferative responses when injected to C57BL/6 mice suggesting that they did not stimulate $CD4^+$ T-cell responses (data not shown). However, after a single subcutaneous injection of these peptides emulsified in IFA, C57BL/6 mice developed strong peptide-specific CTL responses (Fig. 2b and c). *In vivo* depletion of $CD8^+$ T cells totally abrogated these responses (Fig. 2b and c). In contrast, strong CTL responses were observed in $CD4^+$-depleted mice immunized either with VSV (NP52–59) or E1A (234–243) peptides, confirming the results obtained with the LCMV (118–126) peptide. In order to verify these results, we immunized H-2^b mice genetically defective in CD4[10] or CD8.[11] Results shown in Fig. 3 confirm our previous conclusion that optimal-length peptides induce $CD8^+$ CTL responses, independently of $CD4^+$ T-cell help. It should, however, be noted that only low levels of CTL responses were observed in control B6/J mice immunized with E1A (234–243) and that these responses were further decreased in $CD4^{-/-}$ mice.

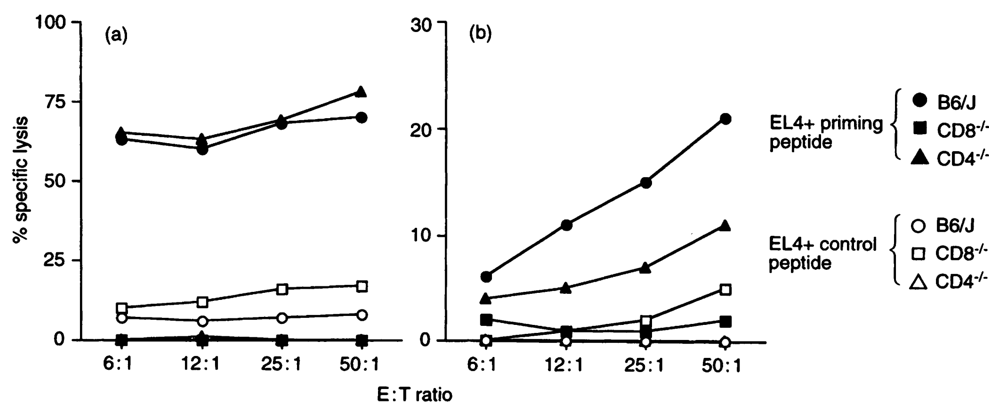

Figure 3. Induction of CTL responses by optimal-length peptides in mice lacking $CD4^+$ T cells. On day 0, B6/J, $CD8^{-/-}$ and $CD4^{-/-}$ mice were immunized subcutaneously with 100 μg of VSV (NP52–59) or control peptides (a) or E1A (234–243) or control peptides (b), emulsified in IFA. On day 10, mice were killed and CTL responses were determined after *in vitro* stimulation of primed spleen cells with irradiated syngeneic spleen cells and 0·05 μmol of VSV (NP52–59) (a) or E1A (234–243) (a) peptide. After 5 days in culture, cytotoxic activity of spleen cells was tested on EL4 target cells coated with VSV (NP52–59) (a) or E1A (234–243) (a) peptide or left untreated. Results are expressed as the mean percentage of specific lysis of triplicate cultures.

DISCUSSION

In the present study, we investigated the capacity of three different short-optimal-length peptides to stimulate CTL response in the absence of $CD4^+$ T-cell help. Using two different mouse haplotypes and two experimental models (depletion of $CD4^+$ T cells by specific mAb and mice genetically defective in CD4), our results clearly demonstrate that, in contrast to previous results obtained with longer peptides,[2,7] short optimal-length peptides do not require $CD4^+$ T-cell help to activate CTL responses.

The three peptides used in this study differ by their immunological properties since one of them, the LCMV(118–126) peptide contains both $CD4^+$ and $CD8^+$ T-cell epitopes whereas the two others do not stimulate $CD4^+$ T-cell response. In a previous study, we demonstrated that the longer peptide, the LCMV(118–132) peptide, also contains both $CD4^+$ and $CD8^+$ T-cell epitopes.[7] Our results therefore support the view that a nonapeptide can bind both to MHC class I and class II molecules and activates simultaneously both subsets of T cells. However, in contrast to our previous results using the LCMV(118–132) peptide,[7] the shorter peptide was shown in the present study to stimulate CTL response in the absence of $CD4^+$ T cells. The only difference between LCMV(118–126) and (118–132) peptides is the higher capacity of the nonapeptide to bind the L^d molecules.[9,15] Therefore, our results suggest that the presentation to T-cell receptors of high-density of MHC–peptide complexes could optimally trigger $CD8^+$ T-cell activation without requirement for additional help. These results are in good agreement with the recent observation that RMA-S cells expressing a high density of MHC class I molecules complexed with relevant peptide are highly efficient in activating CTL responses *in vitro*.[17]

It should be noted that similar results were recently obtained by Dyall *et al.*[16] using several 8–10 amino-acid long class I-restricted peptides emulsified in adjuvant. The discrepancy with previous studies showing a $CD4^+$ T-cell dependence of CTL responses induced by longer peptides[2,7] could therefore be linked to the optimal fit of these short peptides into the antigen-binding groove of empty class I molecules, increasing the density of peptides presented at the surface of cells.[15] Conflicting results were obtained in the past concerning the role of $CD4^+$ T cells in CTL activation.[18–21] The present study suggests that the density of MHC class I molecules complexed to a given peptide may control the capacity of antigen-presenting cells to activate directly $CD8^+$ T cells independently of $CD4^+$ T-cell help. If the required density threshold is not reached, $CD4^+$ T cells may be required to give appropriate signals to activate $CD8^+$ T cells. Depending upon the virus and the infected target cells, the density of peptides associated with MHC class I molecules may vary, thus explaining differences in helper activity requirement observed in different experimental systems.

The results of the present study therefore strongly suggest that reaching a high density of peptide–MHC class I molecule complexes at the antigen-presenting cell surface is an important requirement for CTL activation strategies. It remains however to determine if optimal-length peptides can stimulate long-lived memory CTL since it was recently demonstrated that $CD4^+$ T cells play an important role in the maintenance of antiviral memory CTL.[22]

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