

Induction of Cytotoxic T Lymphocytes in Mice against the Principal Neutralizing Domain of HIV-1 by Immunization with an Engineered T-Cytotoxic-T-Helper Synthetic Peptide Construct

JUAN-JOSÉ LASARTE,* PABLO SAROBE,* ARTURO GULLÓN,†
JESÚS PRIETO,* AND FRANCISCO BORRÁS-CUESTA*¹

*Departamento de Medicina Interna y †Departamento de Genética, Universidad de Navarra,
Facultad de Medicina, Apartado 273, 31080 Pamplona, Spain

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Peptide constructs were engineered by colinear synthesis of two short synthetic peptide determinants: a determinant recognized by T helper cells (TD_h) and a determinant recognized by T cytotoxic cells (TD_c). Three types of constructs were synthesized: TD_c-TD_h, TD_h-TD_c, and TD_h-KK-TD_c, where KK are two lysine residues. *In vivo* immunization with free construct induced cytolytic lymphocytes (CTL) only in the case of TD_c-TD_h. However, immunization with spleen cells to which these constructs had been internalized by hypertonic shock, induced CTL activity in all three cases. No CTL could be induced after immunization with free TD_c in either protocol. These results indicate that cell internalization of the construct might be essential for CTL induction, and also, that "help" from the TD_h seems to be required. © 1992 Academic Press, Inc.

INTRODUCTION

It is well established that two main types of immune responses are elicited after challenge with complex antigens; (i) the humoral, which leads to antibody production; and (ii) the cellular, which leads to the induction and proliferation of cytotoxic T lymphocytes (CTL).

Our understanding of how T-cell help is provided to antibody producing B-cells has greatly benefited from immunization studies using peptide haptens linked to a peptide determinant sequence (TD_h)² recognized by T-cells (1-6) as well as by immunization with mixtures of hapten and TD_h molecules injected in the same immunization site (7).

The cellular immune response has been studied using recombinant viral protein preparations (8-10) as well as peptides (11-17). Three different types of peptide preparations have been reported as being able to induce CTL *in vivo*. Carbone and Bevan (11) and Ishioka *et al.* (12) used trypsin-digested chicken ovalbumin fragments in C57BL/6 mice. Deres *et al.* (13) immunized BALB/c mice with a synthetic lipopeptide construct consisting of tripalmitoil-S-glycerylcysteinyl-seryl-serine bound to a viral

¹ To whom correspondence should be addressed.

² Abbreviations used: TD_h, determinant recognized by helper T cells; TD_c, determinant recognized by cytotoxic T cells.

cytolytic T-cell determinant peptide (TD_c). More recently, Schild *et al.* (14) found that the lipophilic moiety of lipopeptide construct of Deres *et al.* (13) was essential for an efficient *in vivo* priming of CTL. Aichele *et al.* (15) induced CTL in BALB/c mice after priming with a free 15-mer synthetic peptide encompassing a sequence of lymphocytic choriomeningitis virus nucleoprotein and Schulz *et al.* (16) found that this peptide was protective. Kast *et al.* (17) were able to induce protective CTL against Sendai virus after immunization of mice with a free 16-mer peptide from the nucleoprotein of the virus.

Based on the observations that the tripalmitoil-*S*-glycerylcysteinyl-seryl-serine moiety used in the construct of Deres *et al.* (13) behaves as a TD_h in another type of construct (18) and the ovalbumin tryptic fragment 229–276, which contains a predicted TD_h according to Sette's algorithm (19) and a proven TD_c (20), but not the 242–276 (which only contains the TD_c) is able to induce CTL *in vivo* (11), we speculated that synthetic colinear peptide constructs of the type TD_h–TD_c or TD_c–TD_h might be able to induce CTL *in vivo*. Ishioka *et al.* (12) have suggested a similar proposition; however, to our knowledge, no experimental evidence has yet been published to validate these hypothesis.

We show below that CTL induction *in vivo* depends on the type of peptide construct used.

MATERIALS AND METHODS

Peptide Synthesis

Peptides were synthesized by the solid phase method of Merrifield (21) using the Fmoc alternative (22). The synthesis was done manually and the ninhydrin test of Kaiser (23) was used to monitor every step. Couplings were repeated if necessary until a negative ninhydrin test was attained. All peptides were purified by HPLC on a reverse-phase C18 column.

Internalization of Peptides in Cells

Peptides were internalized in the cytoplasm of homogenized spleen cells from BALB/c mice by osmotic lysis of pinosomes induced by hypertonic shock as described (20, 24) using 300 µg/ml of peptide.

Induction of CTL

Groups of three 6-week-old female BALB/c mice were primed by ip injection of 100 µg of relevant peptide dissolved in 0.5 ml of RPMI 1640 solution at Days 1, 8, and 15. Alternatively, mice were immunized ip with a single dose of syngenic spleen cells (5×10^6 cells in 0.5 ml of RPMI 1640) having been submitted to hypertonic treatment in the presence of peptide. A week after the third injection of peptide or 3 weeks after immunization with cells, the animals were killed and their spleens were removed. Spleen cells were restimulated with 5 µg/ml of the relevant peptide according to Ishioka *et al.* (12). Cytolytic activity was measured 5 days after initial culture and after establishing CTL lines by repeated restimulations.

Cytotoxicity Assay

CTL activity was measured using the conventional cytotoxicity assay (12). Target cells P815 (H-2^d-restricted) or EL-4 (H-2^b-restricted) previously incubated with 5 µg/

ml of relevant peptide were used. The assays were done in triplicate; the spontaneous release was in all cases below 20% of total release.

Immunization Experiments for Antibody Induction

BALB/c mice were immunized by ip injection as previously described (6).

Assessment of the Topography of Peptides with Antibodies

The degree of "surface exposure" of regions from the peptide constructs was assessed by ELISA (6) using specific anti-peptide antibodies for each region. For the competition experiments, the antibodies were incubated with free competitor peptide in solution for 1 hr at 37°C before adding this mixture to the ELISA well containing the peptide adsorbed on the plastic.

RESULTS

To test if constructs of the type TD_c-TD_h or TD_h-TD_c could induce CTL *in vivo* we synthesized KQIINMWQEVGKAMYA (from now on KQI) and RIQRGPGRAFVTIGK (RIQ), encompassed respectively by amino acids 428-443 and 315-329 from HIV-1-IIIB gp120 (positions according to Ratner *et al.* (25)). We also synthesized the constructs RIQRGPGRAFVTIGKKQIINMWQEVGKAMYA (RIQ-KQI) and KQIINMWQEVGKAMYARIQRGPGRAFVTIGK (KQI-RIQ) representing the two possible N-terminal to C-terminal permutations (TD_c-TD_h or RIQ-KQI and TD_h-TD_c or KQI-RIQ) of the above peptides. KQI and RIQ have been reported as TD_h and TD_c in BALB/c mice, respectively (9, 26).

Induction of CTL by Immunization with Free Peptides

To attempt CTL induction *in vivo*, five groups of BALB/c mice were immunized with peptides RIQ, KQI, RIQ-KQI, KQI-RIQ, and a mixture of KQI and RIQ, respectively. The spleen cells of each group of mice were isolated and cultured *in vitro* for 5 days under stimulation with different combinations of the above peptides. These cell cultures were next assayed for cytolytic activity against radioactively labeled target cells previously incubated with different peptides.

Figure 1 shows that specific cytolytic activity could be induced by *in vivo* priming with peptide RIQ-KQI (panels a and b) but not with free RIQ or with KQI-RIQ (panels d and e), respectively. Immunization with a mixture of RIQ and KQI gave lower, but significant levels of CTL. No CTL could be induced with free KQI (data not shown).

A peptide KQI-**KK**-RIQ, having two potential cleavage sites at the frontier between KQI and RIQ (the two lysines in bold), was synthesized and tested. This peptide was unable to induce CTL *in vivo* (data not shown), a result that was confirmed in another group of experiments (Table 2).

Mapping the Surface of Peptides with Antibodies

The degree of "surface exposure" of KQI and RIQ in the above peptide constructs was tested by ELISA with anti-RIQ- and anti-KQI-specific antibodies (induced by immunization with free RIQ and KQI, respectively). Table 1 shows that the KQI

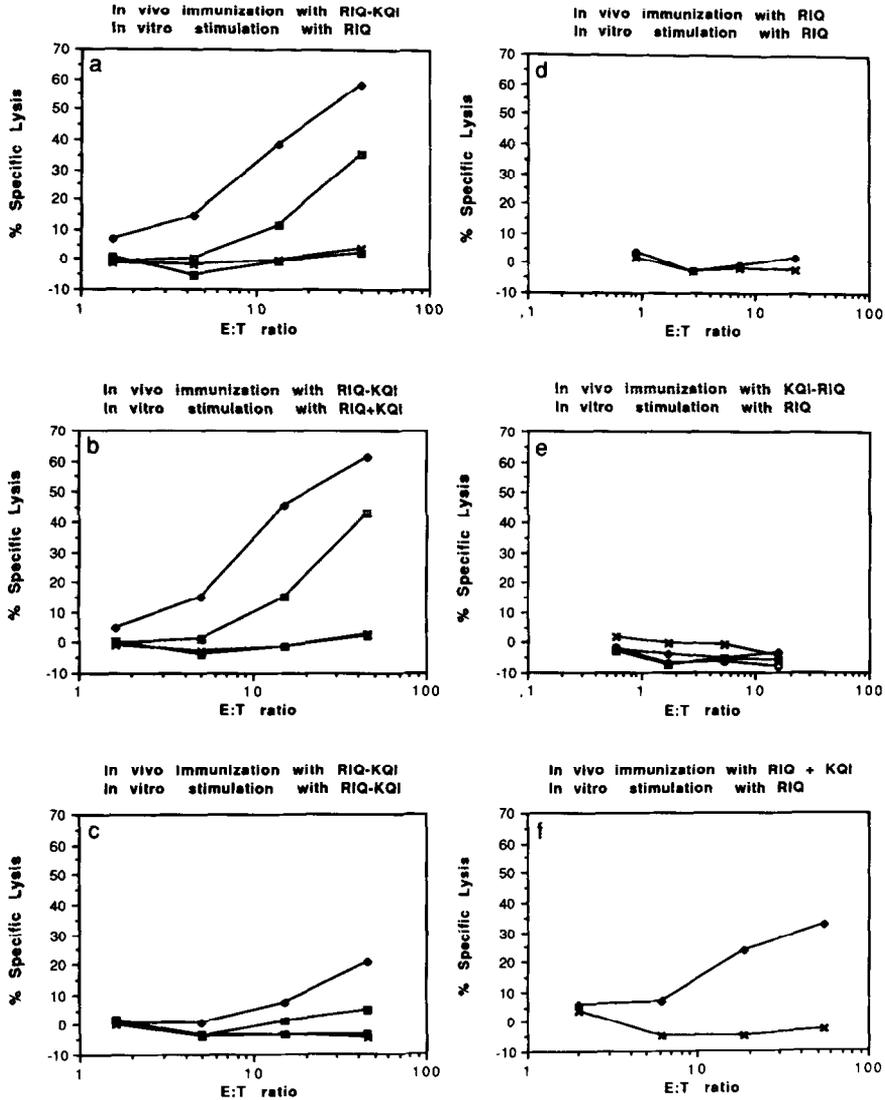


FIG. 1. CTL activity of spleen cells of BALB/c mice after *in vivo* immunization and *in vitro* restimulation with the peptides shown on top of each panel. Activity is expressed as the percentage of specific lysis of P815 target cells preincubated with peptides (□) RIQ-KQI, (○) KQI-RIQ, (◆) RIQ, (■) KQI, (×) irrelevant control peptide. This lysis was induced by effector cells at the effector-to-target ratios (E:T) shown. The irrelevant control peptide corresponds to FERFEIFP, which encompasses amino acids 111-118 of influenza virus hemagglutinin.

moiety is well recognized by anti-KQI antibodies in construct RIQ-KQI but not in KQI-RIQ or KQI-KK-RIQ. By contrast, the RIQ moiety is well recognized by anti-RIQ antibodies in KQI-RIQ, KQI-KK-RIQ but not in RIQ-KQI. Qualitatively equivalent results were obtained in inhibition experiments using these peptide constructs to neutralize the recognition of RIQ and KQI (adsorbed on the ELISA plate) by anti-RIQ and anti-KQI antibodies (data not shown).

TABLE 1

Recognition Antibody Titers^a ($\times 10^{-3}$) of RIQ and KQI in Three Different Peptide Constructs by Antisera Specific for Each of These Moieties

Peptide construct	Recognition by anti-KQI antibodies	Recognition by anti-RIQ antibodies
RIQ-KQI	10	0.9
KQI-RIQ	0.8	13
KQI-KK-RIQ	2	13

^a The titers shown are the mean from three different mice.

Induction of CTL with Peptide-Containing Cells as Compared to Immunization with Free Peptides

To study the effect of peptide internalization in the cell on CTL induction *in vivo*, mice were immunized with one of the following peptides: RIQ, RIQ-KQI, KQI-RIQ, KQI-KK-RIQ, and a mixture of KQI and RIQ or with syngenic spleen cells subjected to peptide internalization.

In Table 2 we summarize the results obtained with both types of immunizations. In the group immunized with free peptides, specific cytolytic activity could be induced by *in vivo* priming with a peptide construct consisting of a colinear combination of a TD_c-TD_h (RIQ-KQI) amino acid sequences, and to a lower extent after *in vivo* priming with a mixture of RIQ and KQI, thus confirming the results of immunization experiments shown in Fig. 1. No induction was achieved by priming with KQI-RIQ (as in Fig. 1) or with KQI-KK-RIQ. By contrast, immunization with spleen cells containing RIQ-KQI, KQI-RIQ, or KQI-KK-RIQ could all induce CTL *in vivo* whereas immunization with spleen cells containing a mixture of RIQ and KQI gave a marginal response. No response could be induced after immunization with free RIQ or with RIQ-containing cells. No significant response was obtained after *in vivo* immunization with RPMI alone, or with spleen cells subjected to hypertonic shock in the absence of peptide, and *in vitro* restimulation with peptides.

TABLE 2

Percentage of Specific Lysis Induced after Immunization with Free Peptide or with Peptide-Containing Syngenic Cells

Peptide tested	Induced with free peptide	Induced with peptide-containing cells
RIQ-KQI	31	28
KQI-RIQ	13.5	22
KQI + RIQ	18	14
RIQ	13.2	11
KQI-KK-RIQ	6	30
None	7	10

Note. Results were obtained after a single *in vitro* restimulation with free RIQ and testing CTL activity using P815 cells incubated with RIQ. The effector-to-target ratio was 30:1 in both experiments.

Surface Phenotype and Class I MHC Restriction of Induced CTL

To characterize the lymphocytes induced after *in vivo* priming with RIQ-KQI, these lymphocytes were treated with fluorescently labeled anti-CD4 and anti-CD8 antibodies and analyzed by flow cytometry. This showed that around 95% of them were conventional CD8⁺ CD4⁻. Moreover, only P815 target cells (H-2^d-restricted) but not EL-4 cells (H-2^b-restricted), incubated with RIQ, were lysed by these lymphocytes (data not shown). Since these target cells are Ia⁻, it can be concluded that the induced CTL are H-2^d class I-restricted.

DISCUSSION

CTL Induction with Peptides, Effect of Epitopes within Constructs

Figures 1a and 1b show that specific cytotoxic activity can be induced by *in vivo* priming with a peptide construct consisting of a colinear combination of a TD_c-TD_h (RIQ-KQI) amino acid sequence. This induction cannot be obtained in the absence of KQI, that is, induction of CTL requires help from KQI (compare Fig. 1a with Fig. 1d). The *in vitro* restimulation does not require KQI since similar percentages of specific lysis can be obtained with and without KQI in the medium (compare Fig. 1a with Fig. 1b). However, as expected, the presence of RIQ during restimulation is essential for cytotoxic activity because if only KQI is used no lysis is observed (data not shown).

An interesting aspect of this work is the finding that although CTL priming *in vivo* is efficiently attained with RIQ-KQI, restimulation *in vitro* prefers free RIQ over RIQ-KQI (compare Figs. 1a and 1b with 1c). Moreover, the relative position of RIQ and KQI has a dramatic effect on CTL induction *in vivo*. Thus, RIQ-KQI is active as judged by the percentages of specific lysis induced after *in vitro* restimulation with either RIQ alone or with a mixture of RIQ and KQI (see Figs. 1a and 1b) whereas KQI-RIQ does not show any sign of activity after *in vitro* restimulation with RIQ (compare Fig. 1a with Fig. 1e). These results suggested to us that enzymatic processing to free active RIQ might be more favorable in RIQ-KQI than in KQI-RIQ, a similar result to the one found for TD_h-hapten peptide constructs concerning the induction of anti-hapten antibodies (6). It is interesting to note that in the sequence RIQRGPGRAFVTIG**KK**QIINMWQEVGKAMYA (construct RIQ-KQI) two residues of K (in bold) are side by side in positions 15 and 16. These residues might define a more accessible trypsin-like cleavage site to generate free RIQ, a situation which would not be as favorable in peptide KQIINMWQEVGKAMY-ARIQRGPGRAFVTIGK. Thus, active TD_c-TD_h or TD_h-TD_c peptide constructs might need to have a "cleavable" site between the TD_c and TD_h sequences. The understanding of how these constructs are processed would greatly facilitate the engineering of tailor-made constructs for a given individual, that is, of how to use TD_c and TD_h sequences recognized by class I and class II MHC molecules of this individual. Irrespective of interpretation, the experimental results discussed above, stress the importance of choosing the most adequate position of both TD_c and TD_h to generate active peptide constructs.

Another aspect concerning the induction of CTL *in vivo* is whether the TD_c and TD_h need to be covalently linked to induce CTL, or perhaps it might suffice to prime with a mixture of them, a result that might resemble the induction of antibodies using

another type of peptide constructs (7). Comparison of Figs. 1a and 1b with Fig. 1f show that CTL induction is clearly more efficient after priming with RIQ-KQI than after priming with a mixture of RIQ and KQI. In this last case, lower but significant CTL induction seems to have taken place.

Since efficient processing of the constructs inside the cell, to liberate the TD_c (RIQ in the above constructs), might be important for the induction of CTL, we synthesized the peptide KQI-**KK**-RIQ, having two added potentially cleavable sites at the frontier between KQI and RIQ (the two lysines in bold) to attempt to improve processing. However, this peptide was unable to induce CTL *in vivo*. This result made us think that the lack of activity of KQI-**KK**-RIQ and of KQI-RIQ might perhaps be related to poor internalization of these constructs rather than to inefficient processing.

Surface Exposure of Epitopes

Since internalization of peptides inside the cells might be related to the topography of peptides, we thought of mapping the surface of our constructs with anti-RIQ and anti-KQI antibodies. Table 1 shows that the KQI moiety is well "surface exposed" only in RIQ-KQI, which is the sole active peptide construct. Since the KQI moiety but not RIQ is surface exposed in RIQ-KQI, it would seem logical that if internalization of RIQ-KQI inside the cell takes place, this should happen via KQI.

Effect of Peptide Internalization on CTL Induction

To study the effect of peptide internalization in the cell on CTL induction *in vivo*, we "forced" internalization of our constructs by hypertonic shock (20, 24). The cells thus obtained were used for immunization experiments. Table 2 shows that by contrast with immunization with free peptides, where only RIQ-KQI is active, immunization with peptide-containing cells (RIQ-KQI, KQI-RIQ, and KQI-**KK**-RIQ) are all active. This proves that peptide internalization render KQI-RIQ, and KQI-**KK**-RIQ active. It is interesting to note that KQI-**KK**-RIQ is as active as RIQ-KQI but more active than KQI-RIQ, this might be related to improved processing due to the added lysines. Moreover, because CTL activity was not induced by RIQ alone in either of the two groups of immunized mice, it would seem likely that in addition to a potential role of KQI in the internalization of RIQ-KQI, help from KQI might be required for CTL induction. However, KQI seems to be more effective when covalently linked to RIQ. These observations are in agreement with the results reported by Schild for a lipopeptide, where the lipophilic moiety seems to play a role in the internalization (14) and has TD_h character in another type of construct (18). For similar reasons, it would seem that shorter peptides (15-17) able to induce CTL *in vivo* might contain both properties (internalization ability and TD_h character) as the lipophilic moiety or the KQI of the above constructs.

We believe that our results have important implications in vaccination and in the treatment of chronic infections, i.e., AIDS and hepatitis B and C. Thus, if the major TD_c and TD_h determinants (of the corresponding viral antigens) recognized by class I and class II MHC molecules of most individuals were known (a research field which is quickly developing) constructs like the ones described above could be engineered to protect against natural infection and to treat chronically infected individuals.

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