Induction of Antigen-Specific CTL Responses Using Antigens Conjugated to Short Peptide Vectors

Fiona H. Day, Yu Zhang, Philippe Clair, Kenneth H. Grabstein, Martine Mazel, Anthony R. Rees, Michel Kaczorek and Jamal Temsamani

*J Immunol* 2003; 170:1498-1503; doi: 10.4049/jimmunol.170.3.1498

http://www.jimmunol.org/content/170/3/1498

**References** This article cites 47 articles, 16 of which you can access for free at: http://www.jimmunol.org/content/170/3/1498.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
Induction of Antigen-Specific CTL Responses Using Antigens Conjugated to Short Peptide Vectors

Fiona H. Day,1* Yu Zhang,* Philippe Clair,† Kenneth H. Grabstein,* Martine Mazel,† Anthony R. Rees,† Michel Kaczorek,† and Jamal Temsaman†

Linear peptides (SynB vectors) with specific sequence motifs have been identified that are capable of enhancing the transport of a wide range of molecules into cells. These peptide vectors have been used to deliver exogenous peptides and protein Ags across the cell membrane and into the cytoplasm of cells. Specifically, in vitro analysis indicated that these SynB peptides enhanced the uptake of two 9-mer peptide Ags, NP147–155 and Mtb250–258 (T cell epitopes of influenza nucleoprotein and Mycobacterium tuberculosis, respectively) and the M. tuberculosis Ag Mtb8.4 protein, into K562 cells when covalently linked to the respective Ags. Furthermore, selected SynB vectors, when conjugated to these same Ags and used as immunogens, resulted in considerably enhanced Ag-specific CTL responses. Several SynB vectors were tested and resulted in varying levels of cellular uptake. The efficiency of uptake correlated with the ability of the SynB construct to deliver each epitope in vivo and induce specific CTL responses in mice. These data suggest that peptide vectors, such as SynB that transport target Ags across the cell membrane in a highly efficient manner, have significant potential for vaccine delivery. The Journal of Immunology, 2003, 170: 1498–1503.

The conventional pathway for Ag processing involves the release of short peptides, derived from protein degradation in the cytosol of endogenous protein Ags, which bind to MHC class I molecules (1–5). This occurs in the endoplasmic reticulum of professional APCs. In association with β2 microglobulin, this complex is then transported to the cell surface and presented to the CD8+ T cell. In vitro data have shown that both macrophages (6–9) and dendritic cells (10–13) are capable of presenting these short peptide derivatives of Ags within the context of MHC class I molecules.

There have been several reports on the delivery of exogenous proteins into cells using membrane permeable carrier peptides such as HIV-1 (6, 14–18), Tat (19), and antennapedia (20–22). Internalization of several of these peptides has been shown to be independent of endocytosis because internalization occurs at both 37°C and 4°C. Genetic or chemical coupling of these carrier peptides has led to efficient intracellular delivery of various oligopeptides and proteins. Futaki et al. (23) recently showed that the presence of arginine residues in these peptides significantly increased the membrane permeability, and therefore delivery, of associated proteins.

Recently, we have shown that the SynB peptide vectors are able to transport many different sorts of molecules efficiently across cell membranes (24, 25). This family of vectors derived from the linearization and subsequent optimization of protegrin and tachyplesin sequences which are themselves natural mammalian antimicrobial peptides. The internalization of SynB vectors is charge- and energy-dependent but does not require specific interactions with receptors on the cell membrane, although some cell types to cell type “tropism” has been observed (G. Drin, unpublished observations).

In this report, we first investigated the ability of these peptide vectors to deliver Ags into target cells (K562). The Ags used in this study consisted of a T cell epitope from a Mycobacterium tuberculosis Ag (Mtb39),6 Mtb250–258 (Mtbp) (26), and also a peptide from influenza nucleoprotein (H3N1/3–47 (H3N1/3–47) and a recombinant protein, also from M. tuberculosis, Mtb8.4. We then demonstrated that SynB vectors enhanced uptake of peptide and protein in vitro and that this correlated with the ability to deliver Ag in vivo, resulting in Ag-specific CTL responses.

Materials and Methods

Mice and cell lines

C57BL/6 and BALB/c mice (female, 6–8 wk of age) were purchased from Charles River Breeding Laboratories (Wilmington, MA) and maintained in a pathogen-free environment in the Fred Hutchinson animal facility (Seattle, WA).

The EL4 cell line expressing the entire Mtb39 gene (E1) was generated by stable transfection (using electroporation) of the EL4 cell line (C57BL/6, H-2b-derived thymoma cell line) with a Mtb39 pcDNA3 plasmid. Positive transfectants were selected using G418 (Geneticin, Life Technologies, Gaithersburg, MD)–supplemented medium. Transfectants were subsequently cloned using limiting dilution analysis. Preparing lysates from these cells and using this as an Ag source to stimulate a Mtb39-specific human CD4 T cell line assessed expression of Mtb39 on the transfectants. Long-term cultures of E1 cells were maintained in G418-supplemented medium (1 mg/ml).

The EL4/Mtb8.4 and EL4/GFP cell lines were generated using retroviral transduction systems as previously reported (29).

K562 and P815 cells (DBA/2, H-2b-derived mastocytoma cell line) were obtained from American Type Culture Collection (Rockville, MD).

Peptide synthesis

T cell epitope peptides were synthesized on a Rainin peptide synthesizer (Rainin Instrument, Woburn, MA) using standard F-moc chemistry. Cleavage and purification (via reverse-phase HPLC) were performed using conventional methods.

---

1 Address correspondence and reprint requests to Dr. Fiona H. Day at the current address: Amgen, 51 University Street, Seattle, WA 98101. E-mail address: dayf@amgen.com

2 Abbreviations used in this paper: Mtb, Mycobacterium tuberculosis; NP, nucleoprotein; NB, 2,1,3-benzodioxole; DMF, dimethylformamide; DIEA, diisopropylethylamine; TFA, trifluoroacetic acid.

Received for publication July 30, 2002. Accepted for publication November 21, 2002.

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.

*Corixa Corporation, Seattle, WA 98104; and †Syntem, Parc Scientifique Georges Besse, Nimes, France.

Copyright © 2003 by The American Association of Immunologists, Inc.
All SynB vectors used for delivery were synthesized according to Fmoc-tBu strategy using an AMS 422 (Abimed, Langenfeld, Germany) synthesizer. Peptide sequences are shown below (Table 1). Labeling of the N terminus of the peptides with the 7-nitro-2,1,3-benzoxadiazole (NBD) probe was conducted as described elsewhere (30). Briefly, resin-bound peptide was treated with piperidin (20% (v/v) in dimethylformamide (DMF)) to remove the N-terminal F-moc protecting group, then the resin was washed with DMF to remove piperidin. NBD-Cl was added in dry DMF (5-fold molar excess) in the presence of disopropylethylamine (DIEA) (2-fold molar excess) for 6 h under agitation in the dark to selectively label the N-terminal amino group. The resin was washed with dichloromethane and treated with a deprotecting mixture to cleave peptides from the resin and deprotect the side chains.

Peptide purification was accomplished by reverse-phase HPLC (Water-prep LC 40; Waters, Guyancourt, France) under a trifluoroacetic acid (TFA) 0.01%/acetoniitride gradient condition. Purification was over 95% for all peptides by the criterion of UV absorbance at 220 and 460 nm.

Synthesis of Mtbb8.4 conjugates

Recombinant Mtbb8.4 was generated as previously reported (29). The SynB peptides were derived with N-succinimidyl pyridyl dithiopropionate in dry DMF containing two equivalents of DIEA, precipitated, and then purified by reverse-phase HPLC purification on a C18 preparative column (Waters).

The Mtbb8.4 protein was derived by adding 10 volumes of DMF, followed by addition of 10 molar equivalents of 2-iminothiolane hydrochloride in water/DMF (1/1). About 20 equivalents of DIEA were then added. The reaction was monitored by matrix-assisted laser desorption ionization-time of flight spectrometry. The derived protein was precipitated by addition of 20 volumes of diethyl ether, and resuspended in water containing 0.1% TFA, and submitted to gel filtration on a Pharmacia Sephadex G10 (Piscataway, NJ) using Water/0.1% TFA as elution solvent. The protein fractions were lyophilized. The 2T-Mtbb8.4 lyophilizate was resuspended in water containing 0.1% TFA, and 10 volumes of DMF were added. About two to five equivalents of N-succinimidyl pyridyl dithiopropionate peptide in DMF were added, followed by 10 equivalents of DIEA. The coupling reaction was monitored by matrix-assisted laser desorption ionization-time of flight spectrometry. The conjugated protein was precipitated with 20 volumes of diethyl ether, then submitted to gel filtration as indicated above.

Protein concentration in the eluate was assayed by the BCA reagent (Pierce; Per Bio Science, Bezons, France).

Cellular uptake studies

K562 cells were cultured in RPMI supplemented with 10% FBS. Cells were diluted at 0.3 × 10^6 cells per ml 1 day before the experiment. Cell uptake was measured by flow cytometry using a FACScan (BD Biosciences, Mountain View, CA). NBD was used as a fluorescent marker to assess the cell uptake of peptide vectors (31). It is introduced on the N-terminal part of the peptides by nucleophilic attack of the 4-chloro derivative. NBD-label free or vectorized peptides (3 μM) were incubated with K562 cells (5 × 10^6 cells per ml) in OptiMEM medium (Invitrogen, Cergy Pontoise, France) at 37°C for various periods of time (0, 5, 20, 30, 60, or 120 min) in a final volume of 0.5 ml. Thereafter, the cells were washed twice and then resuspended in 0.5 ml of ice-cold PBS for FACS analysis. Cell-associated fluorophores were excited at 488 nm and fluorescence measured at 525 nm. A histogram of fluorescence intensity per cell (1 × 10^4) was obtained and the calculated mean of this distribution was considered as representative of the amount of cell-associated peptide.

In vivo model systems

Mice (BALB/c or C57BL/6) were immunized with SynB peptide, either free or conjugated to the specific immunogen (peptide or whole protein) (75 μg/100 μl/animal), interdermally at the base of the tail. The SynB-conjugated immunogen was formulated in the presence of heparin (15 μg/animal; Sigma-Aldrich, St. Louis, MO).

BALB/c Flu NP model

Three weeks postimmunization, BALB/c mice were sacrificed, spleens were removed, and single cell suspensions were prepared. APCs for in vitro stimulations were prepared by activating naïve BALB/c spleen cells (3 × 10^7/ml) with dextran sulfate (7 μg/ml) and LPS (25 μg/ml) for 3 days at 37°C/5% CO2, in complete medium (RPMI 1640; Life Technologies) containing 10% FCS, 2 mM glutamine (Life Technologies), sodium pyruvate (Life Technologies), nonessential amino acids (Life Technologies), 2 × 10^-3 M m2-ME, 50 U/ml penicillin and streptomycin. On day 3, the LPS blasts were harvested and irradiated (3000 rad), washed in serum-free RPMI, then pulsed with 5 μg/ml flu NP and 10 μg/ml β2 m or 90 min at 37°C/5% CO2. Cells were then washed three times and resuspended in complete medium. Immune spleen cells (6 × 10^6/well) and stimulator cells (3 × 10^5/well) were incubated in wells of 24-well plate (2-ml final volume, 4 wells per immune spleen). Cells were assayed for lytic activity on day 6 (see CTL assay protocol) and restimulated on day 7 (see restimulation protocol). Target cells for the CTL assay were either peptide-pulsed or unpulsed P815 cells.

C57BL/6 Mtbb39 model

Three weeks after immunization, C57BL/6 mice were sacrificed. Spleens were removed, and single cell suspensions were prepared. Immune spleens were stimulated in vitro with irradiated (20,000 rad) E1 cells (EL4 tumor cells expressing Mtbb39) or EL4 cells pulsed with Mtbp. Immune spleens (6 × 10^6/well) were stimulated with 3 × 10^5/well stimulator cells in a final volume of 2 ml in each well of a 24-well plate. Cells were assayed for lytic activity on day 6 (see CTL assay protocol) and restimulated on day 7 (see restimulation protocol). Target cells for the CTL assay were either E1 cells, or peptide-pulsed or unpulsed EL4/GFP cells.

C57BL/6 Mtbb8.4 model

Mice were immunized as indicated in the figure legends. Three weeks after the last immunization, mice were bled and sacrificed. Spleen cells were prepared as for the Mtbb39 model system but were stimulated with 3 × 10^5/well irradiated EL4 cells expressing Mtbb8.4.

Restimulation protocol

On day 7 of culture, two wells from each immune spleen group were harvested and each was divided into two wells. Irradiated (20,000 rad) stimulator cells (flu NP peptide-pulsed P815 cells or E1 cells) were added to each well (3 × 10^5/well). Also irradiated (3,000 rad) feeder cells (naive BALB/c or C57BL/6 spleens, for the flu NP and Mtbp models, respectively) were prepared and added to each well (1–3 × 10^5/well). The final volume in each well was 2 ml. This restimulation protocol was conducted on day 7 for no more than two restimulations.

CTL assays

CTL analysis was performed on day 6 of each stimulation cycle for no more than three stimulations using the standard 4-h 51Cr-release assay. Briefly, cells were harvested from the stimulation cultures and serial dilutions (1/2) were prepared starting with 2 × 10^5 T cells/well in 96-well round bottom plates. Targets were labeled with Na23CrO4 and pulsed with 5 μg/ml specific peptide where appropriate. Target cells were added to the effector cells at 2 × 10^5 cell/well (resulting in various E:T ratios). After a 4-h incubation at 37°C, the supernatants were harvested using macrowell tube strips (Skatron, Sterling, VA) and counted in a Cobra II gamma counter (Packard Instrument, Downers Grove, IL). Spontaneous release was determined by adding 0.1% Triton X-100 to target cells. Specific lysis = ((experimental release – spontaneous release)/(maximum release – spontaneous release)) × 100%.

Results

Comparison of cell uptake of SynB conjugated to NPP or Mtbb39 and free peptide

Fig. 1 shows the uptake by K562 cells of fluorescently labeled SynB1, 3, and 4 conjugated peptides (A, NPP or B, Mtbp), free SynB peptide, and free antigenic peptides. For NPP or Mtbp alone, the level of internalization was low. Coupling the two peptides with SynB1, 3, and 4 vectors increased their cell penetration significantly. The kinetics of uptake of all peptides was rapid with maximum uptake reached between 10 and 30 min. The enhancement of maximal internalization was ~2- to 8-fold, depending on the peptide. Assuming comparable levels of labeling efficiency, the

---

Table 1. Sequences of SynB vectors and T cell epitopes, Mtbb250–258 (Mtbb39p) and NPP247–255 (NPP)

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Nomencature</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGGRL5YSRRRFSTSTG</td>
<td>SynB1</td>
</tr>
<tr>
<td>RRLSY5RRRF</td>
<td>SynB3</td>
</tr>
<tr>
<td>AMSFVRS5YGISYRRSR</td>
<td>SynB4</td>
</tr>
<tr>
<td>SMNTSIMGVM</td>
<td>Mtbp</td>
</tr>
<tr>
<td>TYQRTALVV</td>
<td>NPP</td>
</tr>
</tbody>
</table>
The apparent maximal level of uptake of the flu NPp conjugates was higher than for the Mtbp conjugates.

The SynB4 vector enhanced cell penetration slightly better than SynB1 or SynB3 for both Mtbp and NPp. Similar data were observed in a different cell line (data not shown).

Comparisons of the different SynB conjugates

Mice were immunized with the various formulations of SynB conjugates (SynB1, 3, and 4; 75 μg/mouse) or peptide emulsified in IFA (75 μg/mouse), all delivered in the presence of heparin. Heparin was added in our experiments to enhance the protection and diffusion of the SynB vector. Because heparin is negatively charged, it may interact with the positively charged peptide vector, and this electrostatic interaction may protect the complex from degradation and favors its diffusion. Protection against degradation due to electrostatic charge has been proposed for other molecules (21, 32).

These experiments were conducted in both the flu NP and the Mtb39 model systems. Results indicate (Fig. 2) that for the flu NP system, intradermal immunization with SynB3 and SynB4 conjugates of this peptide was able to induce consistent CTL, in three experiments performed. These immunogens induced higher levels of specific CTL activity compared with the flu NP in IFA. SynB1 generated responses similar to those obtained for peptide in IFA. Data is shown after two in vitro stimulations with Flu NPp-pulsed cells as stimulators. However, CTL activity was detectable after a single in vitro stimulation (data not shown). Neither the free flu NPp, nor IFA alone (data not shown), nor naive spleens (Fig. 2E) were able to generate flu NP-specific CTL.

Similar data was obtained in the Mtb39 model system (Fig. 3). Using this epitope, no specific CTL activity was detectable in any of the groups after a single in vitro stimulation (in all experiments performed). Percent-specific lysis is shown using peptide (Mtbp) pulsed EL4 cells as targets after two in vitro stimulations. CTL activity was induced only in the SynB4/Mtbp-immunized animals (in two experiments performed comparing the vectors). The free Mtbp peptide immunization was unable to induce any measurable CTL responses in mice (data not shown).

Therefore, these experiments indicate the ability of the SynB conjugates to enable the generation of in vivo CTL responses in two independent model systems. Taken as a whole, SynB4 was the consistent conjugate that worked with both model systems, while SynB3 worked well in the flu NP model system.
Induction of CTL that recognize naturally processed Ags

An important consideration when delivering peptides, formulated in any way, is whether the induced T cell is capable of recognizing cells expressing and presenting the naturally processed epitope. This is a necessary requirement for induction of antitumor or antipathogen responses in vivo. For the Mtbp system, we addressed this issue because we were able to immunize mice with the SynB4/Mtbp then restimulate in vitro with EL4 tumor cells expressing the entire Mtbp Ag (E1 cell line). After two rounds of in vitro stimulation, CTL assays were performed using E1 cells as targets (and EL4 cells as negative control). Fig. 4 indicates that CTL were identified that could indeed lyse these Mtbp39-expressing targets.

Delivery of recombinant protein (Mtbp8.4) using SynB constructs

The data reported in this study so far indicate that SynB constructs can be used to efficiently deliver CD8 T cell epitopes. However, the delivery of peptides may be a strategy of limited utility for vaccine development. Additional experiments were performed to determine whether this approach could be extended to the delivery of whole proteins. The protein used in these studies was an 8.4-kDa protein identified from M. tuberculosis, termed Mtbp8.4 (29, 33). SynB3 and SynB4 were conjugated to Mtbp8.4 as described in Materials and Methods. Fig. 5A shows the ability of these SynB peptides to enhance uptake of Mtbp8.4 into K562 cells. SynB3 and SynB4 enhanced the uptake of the protein 4- to 8-fold, respectively. Interestingly, as observed for antigenic peptides, SynB4 was more effective than SynB3. Animals were also immunized and the protein conjugate and Mtbp8.4-specific responses were measured. Fig. 4B indicates that SynB4 was able to induce a more efficient Ag-specific CTL response compared with protein alone. Ag-specific Ab responses were also studied. However, SynB4 Mtbp8.4 conjugates were unable to alter the level of Mtbp8.4-specific IgG1 or IgG2a Abs induced (data not shown).

Discussion

The data presented in this study illustrate a novel method for delivery of potentially whole proteins for the induction of T cell immune responses. Specifically these data indicate the potential utility of using the SynB peptides for delivery of peptides and proteins into the class 1 pathway for induction of Ag-specific CTL activity. Uptake of the conjugates was studied in vitro (Figs. 1 and 5A). When the available constructs were compared it was found that SynB4 was not only the better choice for enhancing uptake (in both the flu NPp, Mtbp, and Mtbp8.4 systems) but was also more efficient at inducing both NPp- and Mtbp-specific CTL responses. Classically, adjuvants have been used for the generation of such responses in vivo, therefore experiments were performed comparing the delivery of SynB/peptide constructs to T cell epitopes formulated with IFA adjuvant. IFA was identified as a relatively poor adjuvant for induction of peptide-specific immune responses. For the NPp system, SynB1 was unable to generate CTL responses and was also the least effective at delivering the Ag in vitro. SynB3 generated responses in vivo but was less efficient than SynB4 at delivering the peptide to cells in vitro. For the Mtbp system only SynB4 was able to induce peptide-specific responses and as indicated, the SynB4 conjugate enhanced uptake in vitro to levels superior to the other constructs. Therefore, there appears to be a direct correlation between cell uptake of particularSynB constructs and also the induction of CTL in this system. Therefore, this shows that basic cell uptake assays could be used as a major part of the screening process before in vivo analysis. When designing a vaccine, an important point to consider is whether the T cell generated is able to recognize peptide-pulsed targets (i.e., high density of peptide on cell surface) or whether the T cells recognize naturally processed peptides on the surface of the target cell (e.g., tumor cells expressing the target Ag or pathogen-infected APC). One way to determine whether SynB vectors could generate such T cell responses was to see whether the resulting T cells from the vaccination in vivo with SynB/peptide constructs could recognize cells expressing the whole protein. The NPp system is unable to address this question because throughout the experiment, T cells are continuously stimulated with peptide-pulsed tumor cells. However, the data presented in Fig. 4A show responses generated from animals immunized with an Mtbp/SynB construct and then stimulated in vitro and detected in the assay using cells expressing the whole Ag (E1 cells). Therefore, these data indicate that the SynB peptide can deliver a T cell epitope to generate T cells that recognize a naturally processed T cell epitope. It is not clear why...
generation of CTL required two rounds of in vitro stimulation with E1 cells. This may be a function of level of Ag expression on the E1 cells or immunogenicity of the Mtb39 Ag.

The delivery of peptides into the class I pathway, although an important discovery, is limited in its utility. Many studies have investigated the need to deliver whole Ags either in the form of protein (reviewed in Refs. 34–36), recombinant viruses (37, 38), or DNA (reviewed in Refs. 37, 39–45) into APCs for the generation of potent Ag-specific responses. In these strategies the dominant T cell epitope does not need to be known, rather the APC can select and present the immunogenic peptides. Therefore, we chose to conjugate the SynB4 construct to a recombinant protein and test its ability to induce Ag-specific CTL responses. The Ag that was chosen was another TB Ag, Mtb8.4 (29, 33). This protein was conjugated to SynB3 and SynB4 because they were the best peptides for delivery of the T cell epitope peptides. SynB4 was able to enhance uptake of the Mtb8.4 protein into cells compared with Mtb8.4 protein alone. SynB3 was also able to deliver Ag but less efficiently than SynB4. Fig. 5B clearly illustrates that immunization of mice with SynB4/Mtb8.4 results in the generation of Mtb8.4-specific CTL responses. CTL were also obtained for SynB3/Mtb8.4, however, a higher dose had to be used (data not shown). Therefore, SynB4 appeared to be the most efficient peptide for uptake and generation of CTL. In these assays, the target for the CTL assay were tumor cells expressing the Mtb8.4 transcripts and therefore once again the recognition of naturally processed epitopes occurred. This response occurred in the absence of any adjuvant indicating the potency of such a delivery system. There did not appear to be any augmentation in the humoral immune responses to the Mtb8.4 Ag (data not shown). Mtb8.4 is a relatively small Ag (8.4 kDa), and therefore it remains to be seen if other, larger proteins can be delivered in a similar way.

In conclusion, the data presented in this study identify, for the first time, the ability of SynB vectors, when conjugated to an Ag of interest (either T cell epitope, or whole protein), to be able to induce Ag-specific CTL responses. The potential use of this technology is significant to the field of vaccine development. Generation of CTL responses is an important part of induction of the protective immune responses to various cancers (46–48). Therefore, it is conceivable that this technology could be used for the
induction of a CTL response to a tumor Ag that is known to be present on a cancer cell.

Acknowledgments

We thank Liqing Zhu for generation of the E1 tumor cell line, Dr. Sally Mossman for critical review of the manuscript, and Dr. Mark Alderson (Corixa) for valuable discussion regarding this work.

References


