Recombinant adenylate cyclase toxin of
*Bordetella pertussis* induces cytotoxic T
lymphocyte responses against HLA*0201-
restricted melanoma epitopes

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Abstract

The adenylate cyclase (CyaA) of *Bordetella pertussis* is able to deliver CD8+ T cell epitopes into the
cytosol of CD11b+ dendritic cells (DC) following its specific interaction with the α2β1 integrin
(CD11b/CD18). This delivery results in intracellular processing and presentation by MHC class I
molecules of the CD8+ T cell epitopes inserted into CyaA. Indeed, we previously showed that CyaA
toxins carrying a single cytotoxic T lymphocyte (CTL) epitope can induce efficient protective and
therapeutic antitumor immunity in mice. With a view to elaborating cancer immunotherapy in
humans using CyaA, we constructed two recombinant CyaA carrying HLA*0201-restricted
melanoma epitopes. Here we show that these recombinant CyaA induce strong anti-melanoma CTL
responses in HLA*0201 transgenic mice, even after a single i.v. immunization without adjuvant.
These responses are long lasting, since they were also detected 5 months after the last injection.
Finally, human DC treated with the recombinant CyaA were shown to process and present
efficiently the melanoma epitopes to human CTL clones. Altogether, our results demonstrate that
tumoral epitopes inserted into CyaA are efficiently processed and presented in association with
human MHC molecules. These observations suggest that CyaA is capable of activating antitumoral
CTL in humans and highlight the potential of CyaA for use in cancer immunotherapy.

Introduction

T cells play an important role in tumor rejection in many animal
tumor models, and a variety of tumor antigens recognized by
CD4+ or CD8+ tumor-reactive T cells have been identified both
on murine and human tumors (1). CD8+ cytotoxic T lympho-
cytes (CTL) are of particular interest since these cells
cspecifically recognize tumor cells and kill them. An important
goal in cancer immunotherapy is therefore to activate tumor-
specific CTL. The study of antigens recognized by CD8+ T
cells on human melanoma has resulted in the molecular
identification of several MHC-restricted tumor epitopes (2)
which correspond to non-mutated or mutated peptides
derived from various self-proteins. Among these peptides,
several are derived from non-mutated differentiation proteins
such as tyrosinase (Tyr), Melan-A/Mart-1 and gp100. These
proteins are specifically expressed in most melanocytes/
melanomas and thus the HLA-restricted epitopes are pre-
sented by most melanoma cells from patients expressing the
relevant HLA molecules. These antigens could therefore
constitute the targets of immunotherapeutic strategies based
on immunization against tumor epitopes. Others antigens
expressed on tumoral cells were also described, such as a
peptide derived from an intron sequence of the gene that
codes for N-acetylgalactosaminyl-transferase V (GnT-V) (3).
This intron is specifically expressed in melanoma cells and is

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present in ~50% of melanoma cells, and thus induction of CTL against this epitope could be very useful in immunotherapy. Various vaccination protocols designed to induce specific antitumor CTL responses against these epitopes have been developed, including the use of free peptide in incomplete Freund’s adjuvant (4), recombinant viral vectors (5–7) or dendritic cells (DC) (8–11). Nevertheless, the application of these approaches to human vaccination remains limited due to the potential toxicity of adjuvants, bias towards the potential toxicity of adjuvants, bias towards the potential toxicity of adjuvants, bias towards the potential toxicity of adjuvants. Additionally, the availability of different immunotherapeutic strategies will be of interest to amplify specifically CTL responses against tumoral antigens while controlling those directed at vector-derived epitopes using several vectors carrying the same tumor epitopes.

A new approach for CTL activation has recently been developed based on bacterial toxins capable of delivering antigenic epitopes across the plasma cell membrane into the cytosol, where appropriate processing and interaction with MHC class I molecules can occur. The adenylate cyclase (CyaA) toxin of Bordetella pertussis has the capacity to deliver its catalytic domain into the cytosol of eukaryotic cells (12). Delivery of a CD8+ T cell epitope inserted into the catalytic site of CyaA results in intracellular processing and presentation of the epitope by MHC class I molecules at the surface of antigen-presenting cells (APC) (13). Furthermore, we recently demonstrated that the CyaA specifically binds to the α5β2 integrin (CD11b/CD18) (14) and thus targets the CD11b+ DC subpopulation, which very efficiently induces primary immune responses (15). Thus, immunization of mice with a recombinant CyaA toxin bearing a viral epitope led to the induction of strong CTL responses and to full protection against a lethal viral challenge (16). Moreover, CyaA toxins carrying a single CTL epitope could also stimulate protective and therapeutic antitumor immunity in mice (17). Importantly, genetically detoxified CyaA toxoids retain the property to induce protective antiviral or antitumoral immunity in mice (17,18). Thus, CyaA seems to be a safe and efficient non-replicating vector to induce specific immune responses in mice. However, with a view to elaborating cancer immunotherapy using CyaA, it is of particular importance to demonstrate that human tumoral epitopes inserted into CyaA are efficiently processed and presented in association with human MHC molecules. To that end, we constructed two recombinant CyaA carrying HLA*0201-restricted melanoma epitopes derived either from Tyr or GnT-V. In this study, we evaluated the potency of these recombinant CyaA to induce in vivo HLA*0201-restricted CTL responses against the inserted epitopes and the ability to deliver these epitopes to human APC.

**Methods**

**Mice**

HHD mice are H-2Db/b2m−/− double-knockout mice expressing the HHD transgene comprising the α1 (H) and α2 (H) domains of HLA*0201 linked to the α3 transmembrane and cytoplasmic domains of H-2Dα (D), with the α1 domain linked to human β2-microglobulin. Thus, the only MHC class I molecule expressed by the HHD mice is the modified HLA*0201 molecule (19). HHD mice were bred and housed in the animal facilities of the Pasteur Institute.

**Peptides**

The synthetic peptides pTyr (YMDGTMSQV) corresponding to the melanoma HLA*0201-restricted epitope from the 369–377 region of Tyr (20,21) and pGnT-V (VLPDVFIRCGT) corresponding to the HLA*0201-restricted epitope NA17-A derived from an intron of the GnT-V gene (3) were purchased from Neosystem (Strasbourg, France).

**Construction of recombinant B. pertussis CyaA toxins and toxoids carrying melanoma epitopes**

Recombinant CyaA toxin CyaA-Tyr harbors a 14-amino-acid long polypeptide sequence (PASYMDGTMSQVGT, one-letter code for amino acid) genetically inserted between residues 224 and 235 of CyaA. This sequence contains a single copy of the HLA*0201-restricted melanoma epitope derived from Tyr (amino acid 369–377, underlined sequence above). Recombinant CyaA toxin CyaA-GnT-V harbors a 14-amino-acid long (PASVLPDVFIRCGT) insert at the same position and contains a single copy of the HLA*0201-restricted melanoma epitope NA17-A derived from the GnT-V gene (underlined sequence above).

These recombinant toxins were produced in the Escherichia coli strain BLR (Novagen, Madison, WI) by using expression vectors that are derivatives of the pTRACG plasmid (22), modified by insertion between the Nhel and Kpnl restriction sites of appropriate synthetic double-stranded oligonucleotides encoding the indicated polypeptide sequences (nucleotide sequences are available upon request). The E. coli strain XL1-Blue (Stratagene, La Jolla, CA) was used for all DNA manipulations that were performed according to standard protocols. The recombinant proteins were purified to homogeneity from inclusion bodies by a two-step procedure that includes DEAE–Sepharose and phenyl–Sepharose chromatography, as described previously (23).

The recombinant toxins CyaA-Tyr and CyaA-GnT-V are enzymatically active and therefore cytotoxic. The recombinant toxoids CyaA-E5-Tyr and CyaA-E5-GnT-V are enzymatically inactive, detoxified variants of CyaA-Tyr and CyaA-GnT-V respectively. They are unable to synthesize cAMP as a result of a dipeptide insertion within a critical region of the catalytic site (23). CyaA-E5-Tyr and CyaA-E5-GnT-V toxoids were produced in E. coli by using expression vectors that are derivatives of the pTRACES plasmid: this plasmid was obtained by insertion of the hexanucleotide CTGCAG in an intron of the GnT-V gene (3) were purchased from Neosystem (Strasbourg, France). This results in an in-frame insertion of the dipeptide Leu–Gln between Asp188 and Ile189 of CyaA (23). The same synthetic double-stranded oligonucleotides described above were inserted between the Nhel and Kpnl sites of pTRACES to create plasmids pTRAC-E5-Tyr and pTRAC-E5-GnT-V. The recombinant CyaA-E5-Tyr and CyaA-E5-GnT-V toxoids were purified to homogeneity as described (23).

All purified recombinant toxins and toxoids were ≥90% pure as judged by SDS–PAGE analysis (not shown). The toxin concentrations were determined spectrophotometrically from...
the absorption at 280 nm using a molecular extinction coefficient of 142,000 M\(^{-1}\) cm\(^{-1}\).

Oligonucleotide synthesis and DNA sequencing were performed by Genaxis (Nimes, France). Cultures in fermentors were performed by the ‘Service des Fermentations’ facility from the Pasteur Institute.

Mouse immunization
Female HHD mice (6–10 weeks old) were immunized with two or three i.p. injections at 21-day intervals of either 50 μg CyaA or recombinant CyaA carrying melanoma epitopes in the presence or in the absence of 1 mg alum. In some experiments, mice were immunized with one i.v. injection of 50 μg of the recombinant CyaA in PBS. Detoxified recombinant CyaA-E5 was used in the same conditions. Spleens were surgically removed 7 days after the last injection except for the analysis of long-lasting responses, where spleens were removed 3 or 5 months after the last injection.

In vitro cytotoxic assay
Spleen cells from immunized mice were stimulated in vitro with 10 μg/ml of pTyr or pGnT-V peptides corresponding to the priming epitope in the presence of syngeneic naive spleen cells in complete medium (RPMI 1640 medium containing L-Ala–L-Glu dipeptide supplemented with 10% FCS, 5 × 10\(^{-5}\) M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 20 mM HEPES) during 5 days. The cytotoxic activity of these effector cells was tested in a 4-h \(^{51}\text{Cr}\)-release assay on HHD transfected TAP\(^{−}\)/RMA-S cells (RMA-S-HHD) loaded with the relevant peptides as target cells. \(^{51}\text{Cr}\)-labeling was performed as follows: 1 day before the cytotoxic test, RMA-S-HHD cells were incubated overnight at room temperature in 7% CO\(_2\) equilibrated RPMI 1640 medium supplemented with 20 mM HEPES. Then, cells were incubated 3 h at room temperature with or without 20 μg/ml of the relevant peptide, washed once and radiolabeled with 100 μCi of \(^{51}\text{Cr}\) for 1 h at 37°C.

Various E:T ratios were used and all assays were performed in duplicate. The radioactivity released in the supernatant of each well was measured. The percentage of specific lysis was calculated as 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). Maximum release was generated by adding 10% Triton X-100 to target cells and spontaneous release was obtained with target cells incubated in medium alone. Mice are considered as responders when at least 20% specific lysis was observed at the highest E:T ratio. Results are expressed as means ± SD of responder mice per group. No specific CTL activity was obtained with splenocytes from immunized mice stimulated in vitro with an irrelevant peptide.

Human CTL clones
CTL clone IVS-B directed against the HLA*0201-restricted Tyr epitope (positions 369–377) of Tyr was previously described (24). It was weekly stimulated with 50 μl human IL-2, irradiated HLA*0201 transfected MZ2-MEL melanoma cells pulsed with 2 μg/ml pTyr peptide and irradiated LG2-EBV cells as feeder cells. The CTL clone CMU 579 6/3 specific for the HLA*0201-restricted epitope derived from GnT-V was obtained from the blood of a healthy donor following a recently described method (25). Briefly, the PBMC were stimulated for 2 weeks with the antigenic peptide pGnT-V, human IL-2, IL-4 and IL-7. On day 13, PBMC were stained with an HLA*0201 tetramer folded with the pGnT-V peptide. Tetramer\(^{+}\) lymphocytes were cloned using flow cytometry. They were stimulated for 2 weeks with irradiated allogeneic HLA*0201*–Epstein–Barr virus-transformed B cells pulsed with the peptide, irradiated allogeneic peripheral blood lymphocytes, IL-2, IL-4 and IL-7, and then maintained by weekly stimulation with irradiated HLA*0201* peptide-pulsed allogeneic tumor cells and irradiated allogeneic Epstein–Barr virus-transformed B cells. Both CTL clones were maintained in Iscove’s medium supplemented with 10% of human serum, amino acids and antibiotics.

In vitro stimulation assay of human CTL clones
For the stimulation assay, 10\(^{9}\) immature DC were seeded in U-bottom microplates in 25 μl of X-Vivo 10 medium (Whittaker Bioproducts, Walkersville, MD). CyaA preparations (25 μl) diluted in X-Vivo 10 medium at different concentrations were added to the wells. After 30 min of incubation, the corresponding CTL clones were incubated with these cells (75 μl of X-vivo medium containing 10\(^4\) anti-Tyr CTL clone IVS-B or 104 anti-GnT-V CTL clone CMU 579 6/3) and IL-2 (at a final concentration of 25 U/ml). The supernatants were collected after 20 h and IFN-γ content determined by ELISA (Biosource, Camarillo, CA). To control the ability of DC incubated with the various detoxified recombinant toxoids to stimulate the CTL clones, they were exogenously loaded with the relevant antigenic peptides, incubated with the relevant CTL clones
and the production of IFN-γ was similarly assessed (data not shown).

Results

Induction of melanoma-specific CTL responses by immunization of HHD transgenic mice with recombinant CyaA carrying HLA*0201-restricted melanoma epitopes

To determine whether the CyaA toxin is capable of inducing specific CTL responses against human tumoral antigens, two recombinant CyaA carrying HLA*0201-restricted human melanoma epitopes were constructed. The first recombinant CyaA expresses the epitope 369–377 from the Tyr antigen (CyaA-Tyr) and the second one expresses the epitope NA17-A derived from an intron of GnT-V (CyaA-GnT-V). The ability of recombinant CyaA to induce CTL responses against these two epitopes in vivo was assessed in HHD mice, which are transgenic for the human MHC class I molecule HLA*0201 and have been shown to develop HLA*0201-restricted CTL responses against tumoral peptides (26). HHD mice were immunized by three i.p. injections of 50 μg control CyaA toxin (circles) or recombinant CyaA toxins carrying melanoma epitopes (squares) (A, CyaA-Tyr; B, CyaA-GnT-V) in the presence of 1 mg alum. Seven days after the last injection, spleen cells from immune mice were stimulated in vitro with the priming peptide pTyr (A) or pGnT-V (B) in the presence of irradiated syngeneic spleen cells. The cytotoxic activity of these effector cells was measured on 51Cr-labeled RMA-S-HHD target cells pulsed with the respective peptide (solid symbols) or incubated with medium alone (open symbols). Data represent mean values of duplicates (SD < 10%). Quadrants represent the number of positive mice versus number of tested mice and curves represent means ± SD of responder mice per group from three experiments.

![Fig. 1](image1)

Fig. 1. In vivo induction of CTL responses by recombinant CyaA carrying HLA*0201-restricted melanoma epitopes. HHD mice received i.p. injections on days 0, 21 and 42 of either 50 μg control CyaA toxin (circles) or recombinant CyaA toxins carrying melanoma epitopes (squares) (A, CyaA-Tyr; B, CyaA-GnT-V) in the presence of 1 mg alum. Seven days after the last injection, spleen cells from immune mice were stimulated in vitro with the priming peptide pTyr (A) or pGnT-V (B) in the presence of irradiated syngeneic spleen cells. The cytotoxic activity of these effector cells was measured on 51Cr-labeled RMA-S-HHD target cells pulsed with the respective peptide (solid symbols) or incubated with medium alone (open symbols). Data represent mean values of duplicates (SD < 10%). Quadrants represent the number of positive mice versus number of tested mice and curves represent means ± SD of responder mouse per group from three experiments.

![Fig. 2](image2)

Fig. 2. Induction of melanoma-specific CTL responses by recombinant CyaA carrying melanoma epitopes using different routes of immunization. (A and B) HHD mice were immunized i.p. twice on days 0 and 21 with 50 μg wild-type CyaA (circles) or recombinant CyaA-Tyr (squares) in the presence (A) or in the absence of 1 mg alum (B). (C and E) HHD mice were immunized by one i.v. injection with 50 μg control wild-type CyaA (circles) or recombinant CyaA-Tyr (squares) (C) or recombinant CyaA-GnT-V (squares) (E) in the absence of adjuvant. (D and F) HHD mice were immunized by one i.v. injection with 50 μg control detoxified CyaA-E5 (circles) or detoxified recombinant CyaA-E5-Tyr (squares) (D) or recombinant CyaA-E5-GnT-V (squares) (F) in the absence of adjuvant. Seven days after the last injection, spleen cells from immune mice were stimulated in vitro with priming peptides in the presence of irradiated syngeneic spleen cells. The cytotoxic activity was measured on 51Cr-labeled RMA-S-HHD target cells pulsed with the priming peptide (solid symbols) or incubated with medium alone (open symbols). Results show cumulative data from two to four experiments. Quadrants represent the number of positive mice versus number of tested mice and curves represent means ± SD of responder mice per group. Results obtained after immunization with toxic and detoxified CyaA were not statistically different using a t-test.
specify CTL responses against the Tyr melanoma epitope. These CTL responses were antigen specific, since only peptide-sensitized target cells were killed and CTL activity was not detected on target cells loaded with irrelevant peptides (data not shown). As expected, no significant CTL responses were observed in mice immunized with the wild-type CyaA, showing that the induction of specific CTL responses required in vivo priming by the epitope inserted into recombinant CyaA.

Induction of CTL responses by CyaA-Tyr was then analyzed using different immunization protocols with or without alum as adjuvant. As illustrated in Fig. 2(A and B), two i.p. injections of CyaA-Tyr were enough to induce specific CTL responses against the Tyr epitope, even in the absence of alum. Induction of strong, specific CTL responses was also observed following a single injection of 50 μg of CyaA-Tyr without adjuvant using the i.v. route (Fig. 2C). As expected, these CTL responses were observed only when using peptide-pulsed target cells and splenocytes from mice immunized with the recombinant CyaA-Tyr, showing the specificity of the responses. These results demonstrate the high efficiency of CyaA-Tyr to induce specific CTL responses against the Tyr melanoma epitope.

However, using similar conditions of immunization (two or one i.p. injections with or without alum), no specific CTL response was observed with the recombinant toxin CyaA-GnT-V, indicating that this toxin is less efficient in generating a specific CTL response than the CyaA-Tyr (data not shown). However, using the i.v. route, one injection of CyaA-GnT-V was sufficient to induce a strong CTL response (Fig. 2E).

Finally, we also analyzed CTL responses induced by genetically detoxified mutants of CyaA carrying Tyr or GnTV epitopes that are devoid of CyaA activity following insertion of a dipeptide into the catalytic site. HHD mice immunized with these detoxified molecules developed specific CTL responses against both Tyr and GnTV epitopes (Fig. 2D and F), which were comparable to the responses of mice immunized with the toxic forms of CyaA carrying the corresponding epitope. These results indicate that HLA*0201-restricted CTL induction is independent of the catalytic activity as it was clearly demonstrated for a viral epitope from LCMV in BALB/C mice (18).

Recombinant CyaA-Tyr induces long-lasting memory CTL responses

To analyze the persistence of the CTL responses induced by the recombinant CyaA-bearing melanoma epitope, HHD mice received two i.p. injections of 50 μg of CyaA-Tyr in the presence of alum. At 3 and 5 months after the last injection, splenocytes from immunized mice were stimulated in vitro with the peptide pTyr (solid symbols) or incubated with medium alone (open symbols). Quadrants represent the number of positive mice versus number of tested mice and curves represent means ± SD of responder mice per group from one experiment.

Fig. 3. Immunization of mice with CyaA-Tyr induces long-lasting specific memory CTL activity. HHD mice were immunized i.p. twice on days 0 and 21 with 50 μg wild-type CyaA (circles) or recombinant CyaA-Tyr (squares) in the presence of 1 mg alum. At 3 (A) or 5 (B) months after the last injection, spleens were removed and specific CTL activity was measured after in vitro stimulation as described in Fig. 1 on 51Cr-labeled RMA-S-HHD target cells pulsed with the peptide pTyr (solid symbols) or incubated with medium alone (open symbols). Quadrants represent the number of positive mice versus number of tested mice and curves represent means ± SD of responder mice per group from one experiment.
Human melanoma-specific CTL clones were stimulated by human DC incubated with recombinant CyaA-E5-Tyr or CyaA-E5-GnT-V. Due to the cytotoxicity of CyaA, only detoxified recombinant CyaA was tested in vitro. Immature HLA*0201+ DC derived from human monocytes were incubated with CyaA-E5 (open circles), recombinant CyaA-E5-Tyr (solid circles) (A), CyaA-E5-GnT-V (solid circles) (B) or with the relevant antigenic peptide (solid triangles) (A and B), and were used as APC to stimulate anti-Tyr CTL clone IVS-B (A) or anti-GnT-V CTL clone CMU 579 6/3 (B). The secretion of IFN-γ by the CTL clones was assessed by ELISA. Results are expressed as the mean concentration of IFN-γ released in the supernatants from duplicate wells and are representative of three independent experiments. SEM values are indicated.

Fig. 4. Stimulation of human-specific CTL clones by human DC incubated with recombinant CyaA-E5-Tyr or CyaA-E5-GnT-V. Due to the cytotoxicity of CyaA, only detoxified recombinant CyaA was tested in vitro. Immature HLA*0201+ DC derived from human monocytes were incubated with CyaA-E5 (open circles), recombinant CyaA-E5-Tyr (solid circles) (A), CyaA-E5-GnT-V (solid circles) (B) or with the relevant antigenic peptide (solid triangles) (A and B), and were used as APC to stimulate anti-Tyr CTL clone IVS-B (A) or anti-GnT-V CTL clone CMU 579 6/3 (B). The secretion of IFN-γ by the CTL clones was assessed by ELISA. Results are expressed as the mean concentration of IFN-γ released in the supernatants from duplicate wells and are representative of three independent experiments. SEM values are indicated.

to the dose of CyaA-Tyr up to 30 nM. In our conditions, higher doses appeared to be toxic for the DC, as indicated by the low recognition of the treated DC and by their decreased ability to present the synthetic peptide loaded exogenously (data not shown). In order to assess the relative efficiency of antigen presentation using CyaA as a delivery system, we also produced a titration curve of the Tyr synthetic peptide, which we pulsed on similar DC. As shown in Fig. 4(A), CyaA-Tyr was up to 100 times more efficient than the synthetic peptide in inducing the presentation of the epitope by DC.

Human DC incubated with CyaA-E5-GnT-V induced a weak, but reproducible, production of IFN-γ by the GnT-V-specific CTL clone, as compared with DC incubated with the peptide pGnT-V (Fig. 4B). This result indicates that human DC are able to present the GnT-V epitope inserted into CyaA, although with a moderate efficiency.

Altogether, our results clearly demonstrate the capacity of human DC to process and present human epitopes inserted into CyaA.

Discussion

The CyaA of B. pertussis represents a new delivery system able to specifically stimulate CD8+ T lymphocytes leading to protective antiviral and antitumoral immunity in mice (16,17). This indicates that CyaA is a powerful non-replicating vector for induction of adaptive immunity and might potentially be used for vaccine design. However, demonstration that the inserted epitopes can be processed and presented in association with human MHC molecules is an indispensable prerequisite for the use of this vector in humans. In this report, using recombinant CyaA in which human melanoma epitopes were inserted, we showed that strong and long-lasting melanoma-specific CTL responses could be induced in HHD mice expressing the human HLA*0201 class I molecule. Similar results were obtained with recombinant detoxified CyaA devoid of CyaA activity. CyaA represents an efficient vector to induce specific CTL responses in vivo since >80% of immunized HHD mice respond to the Tyr epitope inserted into CyaA following one i.v. injection without adjuvant, while only 26% of HHD mice respond to this epitope following one injection of 100 µg of peptide in the presence of incomplete Freund’s adjuvant [(26) and data not shown]. We also showed that human DC efficiently processed these recombinant molecules for antigenic peptide presentation to human CTL. Strikingly, the recombinant CyaA-Tyr was much more efficient than the synthetic peptide in delivering the Tyr epitope to DC. Alternative antigen delivery systems, e.g. based on recombinant viruses, usually result in an in vitro presentation efficiency that is lower than the synthetic peptide. Our results from in vivo and in vitro experiments therefore underline the power of CyaA as a delivery system, and suggest that CTL responses could be obtained in humans after immunization with recombinant CyaA and thus that efficient immunotherapy could be achieved with this vector. However, the immunogenicities of the two recombinant CyaA tested in this study were quite different. Indeed, strong CTL responses in HHD mice were induced with only one i.p. injection of CyaA-Tyr in the absence of adjuvant, while three i.p. injections of CyaA-GnT-V in the presence of alum were required to generate specific CTL responses. The weak efficiency of CyaA-GnT-V to deliver GnT-V melanoma epitope was also evidenced in vitro, since human DC incubated with this vector poorly stimulated an anti-GnT-V CTL clone as compared to CyaA-Tyr, which stimulated efficiently a specific anti-Tyr CTL clone. This difference could be explained by the fact that the GnT-V peptide grafted into CyaA-GnT-V was poorly processed as compared to the Tyr peptide inserted into CyaA-Tyr. Indeed, flanking regions of a given epitope are known to influence the proteolytic generation of the mature peptide (27-29), particularly for subdominant and/or cryptic epitopes (30). Thus, we can expect that modification of the molecular context of GnT-V epitope in CyaA could enhance the efficiency of processing of this epitope by APC. On the contrary, the sequence flanking the Tyr epitope in CyaA-Tyr appears to allow its efficient processing.

Furthermore, we observed that CyaA-Tyr is very efficient in activating HLA*0201-restricted CD8+ T cells in vivo since a
single i.v. immunization or two i.p. injections without adjuvant were sufficient to generate strong specific CTL responses. This could be explained by the fact that CyaA targets specifically CD11b+ DC, the most potent APC to induce the primary response, as a result of its interaction with the αβ integrin expressed by these cells (14). Thus, CyaA has the exceptional property of being able to specifically deliver antigens to the cytosolic antigen class I-presentation pathway of professional APC.

Further improvements of the CyaA recombinant strategy are conceivable. First, multiple insertion of CD8+ T cell epitopes into the same recombinant molecule has already been successfully achieved. Indeed immunization of mice with recombinant CyaA carrying three different epitopes, including a LCMV epitope, led to the induction of specific CTL responses for each of the three epitopes, as well as protection against a lethal LCMV challenge (31). Detoxified CyaA carrying multiple melanoma epitopes are under evaluation and such recombinant molecules could constitute a good alternative to induce multispecific CTL responses. Furthermore, additional insertion of CD4+ T cell epitopic peptides is also considered. Although the implication of CD8+ T cells in eradication of established tumors has been clearly demonstrated (32), Tc1 cells could be also required to induce efficient antitumoral responses (33–35). We have recently demonstrated that recombinant CyaA can also deliver epitopes into the MHC class II-processing pathway (36), and are able to induce in vivo both specific Tc1 and CTL responses (37). This characteristic is of great interest for vaccination strategies where both kinds of T cell responses have to be induced, noticeably in the context of cancer immunotherapy.

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Abbreviations

APC antigen-presenting cell
CTL cytotoxic T lymphocyte
CyaA adenylate cyclase of Bordetella pertussis
DC dendritic cell
GM-CSF granulocyte macrophage colony stimulating factor
GnT-V N-acetylglucosaminyl-transferase V
PBMC peripheral blood mononuclear cell
Tyr tyrosinase

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