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Direct Delivery of the *Bordetella pertussis* Adenylate Cyclase Toxin to the MHC Class I Antigen Presentation Pathway[1]

Pierre Guermonprez,* Daniel Ladant,† Gouzel Karimova,† Agnes Ullmann,† and Claude Leclerc[2]*

Among bacterial toxins, the adenylate cyclase toxin of *Bordetella pertussis* (CyaA) has a unique mechanism of entry that consists in the direct translocation of its catalytic domain across the plasma membrane of target cell, a mechanism supposed to be independent of any endocytic pathway. Here, we report that the CyaA toxin is delivered to the cytosolic pathway for MHC class I-restricted Ag presentation. Using peritoneal macrophages as APC, we show that the OVA 257–264 $CD8^+$ epitope genetically inserted into a detoxified CyaA (CyaA-OVA E5) is presented to $CD8^+$ T cells by a mechanism requiring 1) proteasome processing, 2) TAP, and 3) neosynthesis of MHC class I. We demonstrate that the presentation of CyaA-OVA E5, like the translocation of CyaA into eukaryotic cells, is dependent on extracellular Ca^{2+} and independent of vacuolar acidification. Moreover, inhibitors of the phagocytic and macropinocytic endocytic pathways do not affect the CyaA-OVA E5 presentation. The absence of specific cellular receptors for CyaA correlates with the ability of various APC to present the recombinant CyaA toxin, including dendritic cells, macrophages, splenocytes, and lymphoid tumoral lines. Taken together, our results show that the CyaA presentation pathway is not cell type specific and is unrelated to a defined type of endocytic mechanism. Thus, it represents a new and unconventional delivery of an exogenous Ag into the conventional cytosolic pathway. *The Journal of Immunology*, **1999, 162: 1910–1916.**

Cytotoxic T lymphocytes recognize antigenic peptides from 8 to 10 amino acids derived from endogenous cytosolic Ag complexed with MHC class I molecules at the surface of APCs. After degradation of Ag by the proteasome, the processed peptides are transported to the lumen of the endoplasmic reticulum (ER)[3] by the ATP-dependent transporters associated with Ag presentation, TAP1 and TAP2. Then, peptides associate with nascent MHC class I molecules and β_2-microglobulin. The trimeric complexes reach the surface using the conventional secretory pathway via the Golgi network (reviewed in Ref. 1). As a consequence, immunization with an exogenous Ag does not induce, in most cases, a CTL response.

However, it has been well established that in some cases, exogenous Ag can be processed and presented to $CD8^+$ T cells (reviewed in Refs. 2–4). Schematically, these pathways have been classified as either cytosolic or noncytosolic. Noncytosolic pathways are related to the endocytic processing and involve loading of peptides on post-Golgi MHC class I molecules and are independent of the TAP system (2, 3). Cytosolic pathways constitute a nonconventional access of exogenous Ag to the cytosol, resulting in its introduction into the classic presentation pathway for endogenous Ag described above. This has been clearly demonstrated for particulate Ag internalized by phagocytosis in a restricted subset of macrophages (5–7) and for soluble Ag internalized by macropinocytosis in both activated macrophages (8) and dendritic cells (9, 10). In both cases, a defined endocytic mechanism (phagocytosis or macropinocytosis) was involved, and a membrane-disruption mechanism was suggested to explain the introduction of exogenous Ag into the cytosolic pathway.

Several bacterial toxins exert their toxic effect by modifying cytosolic components. Therefore, they are equipped with a dedicated translocating capability, and as such, they constitute attractive vectors for the delivery of heterologous CTL epitopes into the endogenous pathway. Some toxins, such as the diphtheria toxin, are internalized after receptor-mediated endocytosis in an acidic compartment where they translocate to the cytosol (11). Other toxins use a receptor-mediated anterograde transport to the ER via the Golgi network, where they translocate to the cytosol (reviewed in Ref. 12). The adenylate cyclase toxin of *Bordetella pertussis* (CyaA) is able to invade numerous mammalian cell types, in which, upon activation by the endogenous calmodulin, it catalyzes the production of supraphysiological levels of cAMP (13–19). The invasivity of CyaA seems to be independent of any known cellular receptor: it does not involve any acidic compartment and can take place in erythrocytes devoid of vesicular transport (20–27). The precise mechanism of translocation is not fully understood but might occur in two steps. After binding of CyaA to the eukaryotic cell surface mediated by the C-terminal domain (residues 400-1706), the N-terminal catalytic domain (residues 1–400) is directly translocated through the plasma membrane into the cell.

We showed previously that a CyaA toxin genetically tagged with a CTL epitope from the nucleoprotein of lymphocytic choriomeningitis virus (LCMV) into its catalytic domain remained

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[3] Abbreviations used in this paper: ER, endoplasmic reticulum; CyaA, adenylate cyclase toxin of *Bordetella pertussis*; CyaA-OVA E5, genetically detoxified CyaA carrying the OVA CTL epitope; LCMV, lymphocytic choriomeningitis virus; CyaA-LCMV E5, genetically detoxified CyaA carrying the lymphocytic choriomeningitis virus CTL epitope; BMM, bone marrow-derived macrophage; BMDC, bone marrow-derived dendritic cell; Thio-PEC, thioglycollate-induced peritoneal exudate cells; CM, complete medium; M-CSF, macrophage CSF; GM-CSF, granulocyte-macrophage CSF; CCB, cytochalasine B; DMA, dimethyl amiloride; BFA, brefeldin A; LLnL, *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal; LF, lethal factor.

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cell invasive (28). Moreover, this toxin was able to generate, in vivo, a protective CTL response against LCMV (29–30). In this report, we asked whether the CyaA toxin, taken as an exogenous Ag, can be delivered to the cytosolic pathway for MHC class I presentation without the contribution of any endocytic mechanisms. To address this question, we have analyzed, in vitro, the pathway leading to the presentation of the K^b-restricted $CD8^+$ OVA epitope from OVA, which was inserted in a genetically detoxified form of CyaA (CyaA-OVA E5). We used the stimulation of a specific $CD8^+$ T cell hybridoma to monitor the appearance of K^b/OVA complexes on the surface of APC.

Materials and Methods

Mice

Female C57BL/6 (H-2^b) mice from Iffa Credo (L'Arbresle, France) were used between 6 and 8 wk of age. Female TAP1 knockout mice (31) bred onto a C57BL/6 background were a kind gift from Dr. A. Bandeira (Institut Pasteur, Paris, France).

Recombinant adenylate cyclase toxins and peptide

The synthetic peptide SIINFEKL corresponding to the $CD8^+$ T cell epitope encompassing the OVA residues 257–264 recognized by the B3Z $CD8^+$ T cell hybridoma was synthesized by Neosystem (Strasbourg, France) and stored in PBS.

The wild-type CyaA toxin was produced in *Escherichia coli* from an expression plasmid, pCACT3, which carries the *cya C* gene required for the activation of the pro-CyaA toxin and the modified *cya A* gene under the transcriptional control of the *lac* UV5 promoter (28).

CyaA-OVA E5 is a genetically detoxified CyaA toxin carrying the OVA epitope. It differs from the wild-type CyaA toxin by 1) the insertion between Arg^{224} and Ala^{225} of the amino acid sequence, PASIINFEKLGT (the amino acid sequence of the OVA epitope is underlined), and 2) the insertion between Asp^{188} and Ile^{189} of the dipeptide LQ. This latter modification abolishes the adenylate cyclase activity without affecting the cell-invasive activity (32). Details of the construction can be provided upon request. The genetically detoxified CyaA toxin carrying the LCMV CTL epitope (CyaA-LCMV E5) (due to the same LQ insertion between Asp^{188} and Ile^{189}) carrying the 118–132 $CD8^+$ T cell (H-2^d restricted) epitope from the LCMV nucleoprotein has been previously described (29). The CyaA toxins, overproduced in the *E. coli* BLR strain, accumulated as inclusion bodies. After solubilization in 8 M urea, 20 mM HEPES-sodium (pH 7.5) they were purified to >95% homogeneity (as judged by SDS-gel analysis, not shown) by two sequential chromatographies on DEAE-Sepharose and phenyl-Sepharose. Toxin concentrations were determined spectrophotometrically from the absorption at 280 nm using a molecular extinction coefficient of 142,000 $M^{-1} \cdot cm^{-1}$.

Cell lines

B3Z (33), a $CD8^+$ T cell hybridoma specific for the OVA 257–264 peptide (SIINFEKL) in the context of K^b, was a generous gift from Dr. N. Shastri (University of California, Berkeley, CA). The LB27.4 B cell lymphoma and the EL4 thymoma were obtained from American Type Culture Collection (Manassas, VA). The immortalized immature dendritic cell line FSDC derived from fetal skin (34) was a kind gift from Dr. P. Ricciardi-Castagnoli (Consiglio Nazionale delle Ricerche, Milan, Italy).

Preparation of thioglycollate-induced peritoneal exudate cells (Thio-PEC), bone marrow macrophages (BMM), enriched populations of bone marrow dendritic cells (BMDC), and splenocytes

C57BL/6 mice were injected i.p. with 2 ml of thioglycollate (Sanofi Diagnostic Pasteur, Paris, France). Four to seven days later, mice were sacrificed and the cells infiltrating the peritoneal cavity were harvested by washing with 10–15 ml of RPMI 1640 containing antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). The cells were then pelleted, resuspended in complete medium (CM, consisting of RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-ME), and used for presentation experiments.

BMM were obtained after a 5-day culture of bone marrow cells harvested on naive C57BL/6 mouse femurs in CM supplemented with 10% of macrophage CSF (M-CSF) containing supernatant of L929 cell culture. Cultures were realized in bacteriology petri dishes (Becton Dickinson, Mountain View, CA). At the end of the culture period, nonadherent cells were removed, and adherent cells were harvested with 2 mM trypsin-EDTA (NEN Life Science, Boston, MA).

BMDC were obtained with a protocol adapted from Inaba et al. (35). Briefly, bone marrow cells were washed twice and cultured for 2 days in DC medium (CM supplemented with recombinant granulocyte-macrophage CSF (GM-CSF; Peprotech, Rocky Hill, NJ) at 40 ng/ml) at 10^6 cells/ml in 100-mm culture-treated petri dishes. Then, nonadherent cells were removed, and fresh DC medium was added. After a total of 5–6 days of culture, BMDC were visible as clumps of loosely adherent cells. The nonadherent cells were harvested by vigorous pipetting, washed, and used for presentation experiments.

For the preparation of splenocytes, spleen cells from naive C57BL/6 mice were washed once with RPMI 1640 supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), resuspended in CM, and used for presentation assays.

Ag presentation assays

The stimulation of B3Z cells (10^5 cells/well) was monitored by IL-2 release in the supernatants of 24-h cultures in the presence of APC in 96-well culture plates. In some experiments (see legends to Figs. 1 and 7), the APC (10^5 cells/well except for FSDC, 10^4 cells/well) were cocultured with B3Z in the presence of Ag. In other experiments, APC were first pulsed with Ag for 5 h and then fixed with glutaraldehyde 0.05% (2 min at 37°C) and washed twice before being cocultured with B3Z. The B3Z stimulation was done in 96-well culture microplates in a 0.2-ml final volume in the presence of 0.25 μM indomethacin (Sigma, St. Louis, MO). The Ag concentrations used in each experiment are indicated in the figure legends. After 24 h, the supernatants were harvested and frozen for at least 2 h at −70°C. Then, 10^4 cells/well of the CTLL cell line, which proliferates specifically in response to IL-2, were cultured with 100 μl of supernatant in 0.2-ml final volume. Two days later, [^{3}H]thymidine (NEN Life Science) was added, and the cells were harvested 18 h later with an automated cell harvester (Skatron, Lier, Norway). Incorporated thymidine was detected by scintillation counting. In all experiments, each point was done at least in duplicate and more often in triplicate. Results are expressed in cpm or Δcpm (cpm in the presence of Ag − cpm in the absence of Ag).

Inhibition studies

For inhibition studies, Thio-PEC APC were first incubated in 0.1 ml with the drugs for 1 h at the indicated final concentration. Then, Ag diluted in 0.1 ml were added to the wells (0.2-ml final volume) at the final concentration indicated, in the continuous presence of the inhibitor for 5 h. APC were then fixed with glutaraldehyde 0.05% (2 min at 37°C) and washed twice, and the B3Z T cells were added to the wells and cultured for 18 h in a 0.2-ml final volume as indicated above. Lactacystin (Tebu, Le Perray-en-Yvelines, France) was dissolved in PBS at 1 mg/ml, brefeldin A (BFA; Sigma) was dissolved in methanol at 0.5 mg/ml, dimethyl amiloride (DMA, Sigma) was dissolved in DMSO at 10 mM, and cytochalasine B (CCB) and *N*-acetyl-L-leucinyl-L-leucinal-L-norleucinal (LLnL, Sigma) were dissolved in DMSO at 10 mg/ml. Cycloheximide (Sigma) was dissolved in water at 2 mg/ml. Chloroquine (Sigma) was dissolved in CM at 10 mM.

Results

Formation of K^b-SIINFEKL complexes after CyaA-OVA E5 processing by peritoneal macrophages

Using thioglycollate-induced peritoneal macrophages (Thio-PEC) from C57BL/6 (H-2^b) mice as APC, we monitored the formation of K^b-SIINFEKL complexes by the stimulation of B3Z, a $CD8^+$ T cell hybridoma specific for the 257–264 SIINFEKL peptide of chicken OVA (33). Fig. 1 shows that B3Z cells cocultured for 18 h with Thio-PEC were efficiently stimulated in the presence of CyaA-OVA E5, a genetically inactivated CyaA toxin that carries the SIINFEKL epitope. In contrast, a detoxified toxin carrying an irrelevant epitope (CyaA-LCMV E5) or the nonrecombinant CyaA was unable to stimulate the B3Z T cells. As expected, the stimulation of B3Z was specifically blocked by an anti-K^b mAb and was not observed with MHC-mismatched Thio-PEC as APC (data not shown), confirming that stimulation was due to the specific recognition of K^b/SIINFEKL complexes. Moreover, the presentation of CyaA-OVA E5 required processing, since the stimulation of

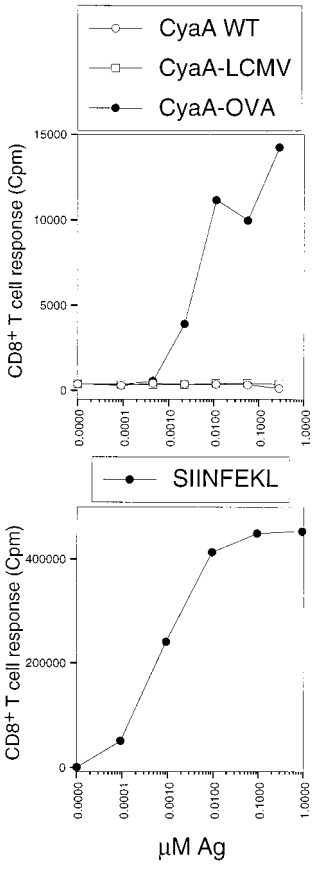

FIGURE 1. Presentation of CyaA-OVA E5, a detoxified recombinant CyaA carrying the OVA epitope to the anti-OVA B3Z CD8$^+$ T cell hybridoma by Thio-PEC. Thio-PEC from C57BL/6 mice were cocultured for 18 h with the anti-OVA CD8$^+$ T cell hybridoma in the presence of wild-type CyaA, CyaA-OVA E5, or CyaA-LCMV E5 (*A*) or the SIINFEKL synthetic peptide (*B*) at various concentrations. The IL-2 release by B3Z was measured in a CTLL proliferation assay. Results are expressed in cpm.

B3Z by CyaA-OVA E5 was observed only with live APC but not with prefixed APC (data not shown).

CyaA-OVA E5 processing requires proteasome activity

We next characterized the degradation steps involved in the generation of the SIINFEKL CD8$^+$ T cell epitope from the recombinant CyaA-OVA E5. It is now well established that the processing of cytosolic Ag for loading on MHC class I molecules requires the proteolytic activities of the proteasome (36–38). As shown in Fig. 2, LLnL and lactacystin, two inhibitors of the proteasome activity (37, 38), totally inhibited the presentation of CyaA-OVA E5 (Fig. 2*A*) without altering the presentation of the SIINFEKL synthetic peptide (Fig. 2*B*). We conclude that the proteolytic processing of CyaA-OVA E5 to yield the SIINFEKL peptide is dependent on the proteasome function.

The CyaA-OVA E5 presentation pathway is dependent on TAP

The intracytoplasmic location of the proteasome-mediated degradation of CyaA-OVA E5 raised the question of the translocation of the processed peptides toward a compartment containing MHC class I molecules. To test the intervention of the TAP system in the presentation pathway of CyaA-OVA E5, we compared the presentation of this Ag by Thio-PEC obtained from C57BL/6 wild-type mice (+/+) and from C57BL/6 TAP1 knockout mice (−/−), which do not have a functional TAP system (31). Fig. 3 shows

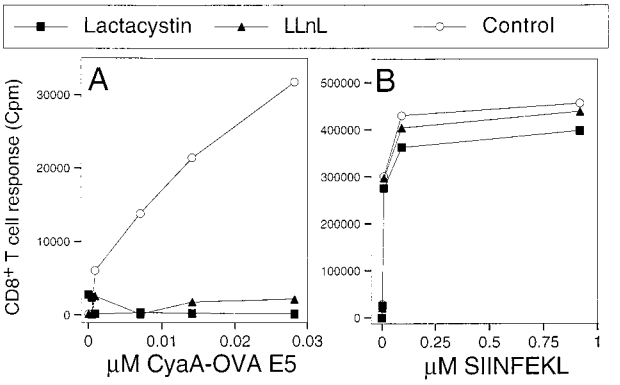

FIGURE 2. The processing of CyaA-OVA E5 by Thio-PEC requires the proteasome proteolytic activity. Thio-PEC from C57BL/6 mice were preincubated for 1 h in the absence (control) or in the presence of 30 μM LLnL or 10 μM lactacystin. Then, CyaA-OVA E5 (*A*) or the SIINFEKL synthetic peptide (*B*) was added at the indicated final concentrations for a 5-h pulse in the continuous presence of the drugs. After glutaraldehyde fixation of Thio-PEC, B3Z CD8$^+$ T cells were added for an 18-h culture. The IL-2 release by B3Z was measured in a CTLL proliferation assay. Results are expressed in cpm.

that both APC populations have a similar ability to present the SIINFEKL synthetic peptide to B3Z, whereas only APC from wild-type mice presented CyaA-OVA E5 to B3Z. Thus, it can be concluded that the TAP system is required for the transport of the processed OVA peptide from the cytosol to the lumen of the ER.

The CyaA-OVA E5 presentation pathway is sensitive to Golgi disruption by BFA and protein synthesis inhibition by cycloheximide

The TAP requirement for the presentation of CyaA-OVA E5 to B3Z suggests that the SIINFEKL peptide generated from the recombinant toxin might be loaded on newly synthesized MHC class I molecules in the lumen of the ER. To test this possibility, we disrupted the conventional secretory pathway with the drug BFA, which has been shown to inhibit the export of newly synthesized MHC class I-peptide complexes from the ER to the cellular membrane (39). BFA completely inhibited the presentation of

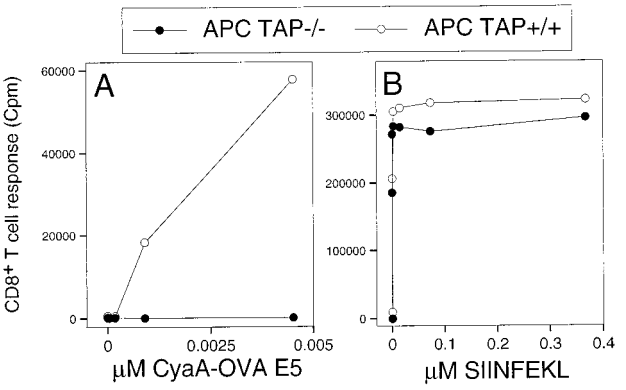

FIGURE 3. The presentation pathway of CyaA-OVA E5 is TAP dependent. Thio-PEC derived from TAP1 knockout mice bred on a C57BL/6 genetic background (APC TAP −/−) or from control C57BL/6 mice (APC TAP +/+) were pulsed for 5 h with various concentrations of the CyaA-OVA E5 molecule (*A*) or the SIINFEKL synthetic peptide (*B*). After glutaraldehyde fixation of Thio-PEC, B3Z CD8$^+$ T cells were added for an 18-h culture. The IL-2 release by B3Z was measured in a CTLL proliferation assay. Results are expressed in cpm.

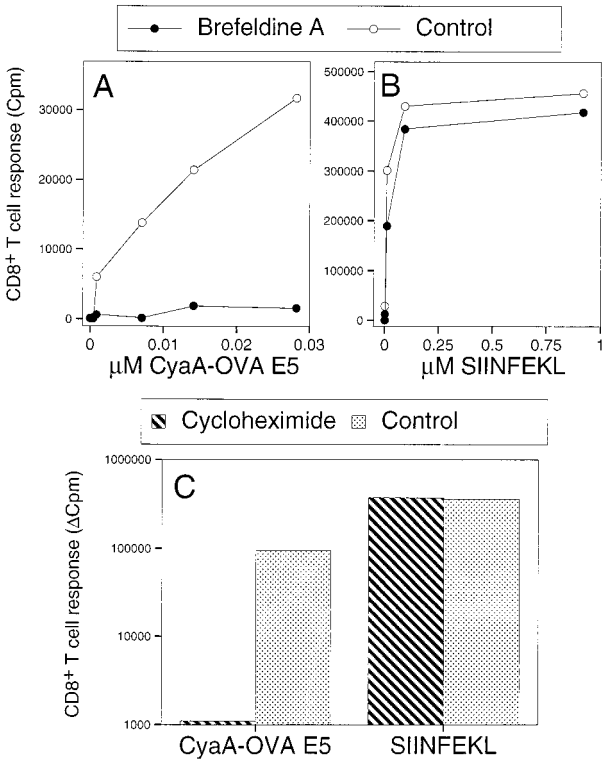

FIGURE 4. The presentation pathway of CyaA-OVA E5 is sensitive to BFA and cycloheximide. Thio-PEC from C57BL/6 mice were preincubated for 1 h in the absence (control) or in the presence of 5 μg/ml BFA or 4 μg/ml cycloheximide. Then, the CyaA-OVA E5 molecule (*A* and *C*) or the SIINFEKL synthetic peptide (*B* and *C*) was added for 5 h at the indicated final concentration (*A* and *B*) and at 6 and 100 nM (*C*) for CyaA-OVA E5 and the SIINFEKL peptide, respectively. Incubations were performed in the continuous presence of the drugs. After glutaraldehyde fixation of Thio-PEC, B3Z CD8$^+$ T cells were added for an 18-h culture. The IL-2 release by B3Z was measured in a CTLL proliferation assay. Results are expressed in cpm in *A* and *B* and in Δcpm (cpm in the presence of Ag − cpm in the absence of Ag) in *C*.

CyaA-OVA E5 to B3Z (Fig. 4*A*), whereas it did not modify the presentation of the SIINFEKL synthetic peptide (Fig. 4*B*). Cycloheximide, a potent inhibitor of protein synthesis, had a similar effect (Fig. 4*C*). These results indicate that the processed peptides bind to nascent MHC class I molecules in the lumen of the ER and that the complexes formed are exported to the cell surface via the Golgi apparatus by the conventional secretory pathway.

Altogether, these results demonstrate that CyaA-OVA E5 is processed like a cytosolic Ag, and this implies that the catalytic domain carrying the OVA epitope has to be delivered to the cytosol of APC.

The presentation of CyaA-OVA E5 requires extracellular Ca^{2+}

The translocation of the CyaA catalytic domain through the plasma membrane of eukaryotic cells is critically dependent upon extracellular Ca^{2+} (20). To test whether the CyaA-OVA E5 presentation by Thio-PEC is mediated by a Ca^{2+}-dependent internalization mechanism, we performed experiments in the presence of EGTA to deplete extracellular Ca^{2+} (Fig. 5). EGTA was used in a 15-min Ag pulse followed by a 5-h chase in the absence of EGTA and Ag (Fig. 5). Under these conditions, EGTA completely inhibited the CyaA-OVA E5 presentation without affecting the presentation of the SIINFEKL synthetic peptide. This suggests that the Ca^{2+}-dependent direct translocation of the catalytic domain of CyaA-OVA E5 into APC is the prerequisite for presentation.

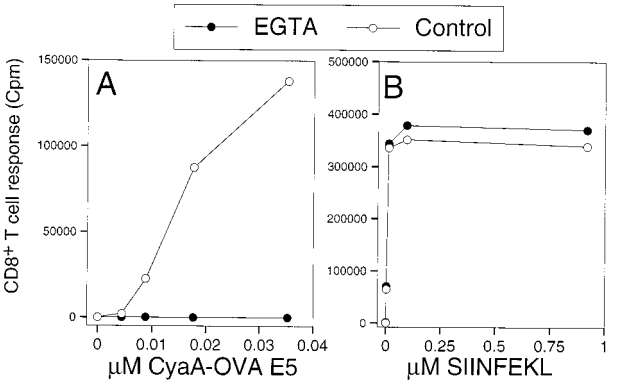

FIGURE 5. The presentation of CyaA-OVA E5 is Ca^{2+} dependent. Thio-PEC from C57BL/6 mice were incubated for 15 min with various concentrations of the CyaA-OVA E5 molecule (*A*) or the synthetic peptide SIINFEKL (*B*) in the absence (control) or in the presence of EGTA at the final concentration of 5 mM. After one wash with CM, the cells were incubated for 5 h in CM without EGTA and Ag. After glutaraldehyde fixation, the B3Z CD8$^+$ T cells were added for an 18-h culture. The IL-2 release by B3Z was measured in a CTLL proliferation assay. Results are expressed in cpm.

The presentation pathway of CyaA-OVA E5 does not require vacuolar acidification and is independent of the macropinocytic and phagocytic pathways

The internalization of CyaA has already been shown to be independent of vacuolar acidification (23). However, it would be possible that an endocytic acid-optimal proteolysis event could participate in the processing of CyaA-OVA E5 to give rise to the SIINFEKL epitope. To investigate this possibility, we tested the effect of the weak base chloroquine on the presentation of CyaA-OVA E5. As shown in Fig. 6, chloroquine that inhibits the endolysosomal proteolysis and interferes with vesicular transport affected neither the presentation of CyaA-OVA E5 nor the presentation of the SIINFEKL synthetic peptide. EGTA was included in this experiment as a negative control.

Phagocytosis has been shown to be a means to deliver particulate exogenous Ag into the cytosolic pathway of macrophages (5–7). To test whether the soluble CyaA-OVA E5 molecule could be delivered to the cytosolic presentation pathway through the phagocytic pathway, we analyzed the effect of CCB, an inhibitor of actin polymerization and phagocytosis, on the presentation of CyaA-OVA E5. As expected, CCB did not inhibit the presentation of CyaA-OVA E5.

Macropinocytosis is a recently described form of endocytosis distinct from the clathrin-mediated endocytosis associated with membrane-ruffling activity. The uptake of soluble Ag by macropinocytosis can result in delivery of the Ags to the cytosol and their subsequent introduction in the endogenous pathway for MHC class I-restricted Ag presentation. Macropinocytosis has been shown to be inducible in macrophages and constitutive in dendritic cells (8–10). To determine whether the delivery of CyaA-OVA E5 in the cytosolic pathway was related to the macropinocytic route, we tested the effect of DMA, a potent inhibitor of the macropinocytic pathway, on the presentation of CyaA-OVA E5. As shown in Fig. 6, DMA was without effect on CyaA-OVA E5 presentation. This demonstrates that the CyaA-OVA E5 pathway represents an original mechanism for the presentation of exogenous Ag to CD8$^+$ T

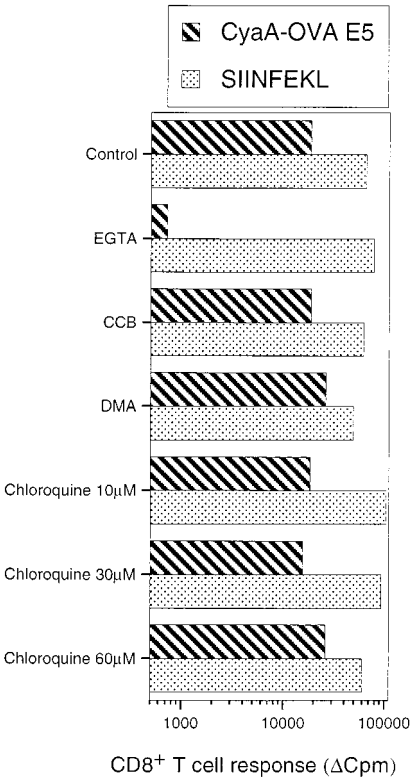

FIGURE 6. The presentation of CyaA-OVA E5 is independent of phagocytosis, macropinocytosis, and vacuolar acidification. Thio-PEC from C57BL/6 mice were preincubated for 1 h in the absence (control) or in the presence of 5 mM EGTA or 10 μg/ml CCB or 150 μM DMA or 10, 30, and 60 μM chloroquine. Then, the CyaA-OVA E5 molecule at 6 nM or the SIINFEKL synthetic peptide at 100 nM final concentration was added for a 5-h pulse in the continuous presence of the drugs. After glutaraldehyde fixation of Thio-PEC, B3Z $CD8^+$ T cells were added for an 18-h culture. The IL-2 release by B3Z was measured in a CTLL proliferation assay. Results are expressed in Δcpm (cpm in the presence of Ag − cpm in the absence of Ag).

cells through the direct delivery of epitope into the cytosol through the plasma membrane of APC.

CyaA-OVA E5 presentation by various types of APC

Previously described pathways for the presentation of exogenous Ag by MHC-class I molecules have been shown to be restricted to defined cell types, mainly macrophages and dendritic cells. For example, phagocytic and macropinocytic pathways clearly exhibit a cell-type specificity linked to the endocytic mechanism underlying the access of Ag to the cytoplasm. As CyaA is able to enter into various eukaryotic cell types independently of receptor-mediated endocytosis (20, 22–27), processing and presentation of CyaA-OVA E5 should be conducted by different cell types, the possible consequence being that the presentation of CyaA-OVA E5 might take place in multiple cell types. To test this hypothesis, we examined the presentation of CyaA-OVA E5 by different types of APC. Macrophages derived from bone marrow by culture with M-CSF presented CyaA-OVA E5 with a slightly reduced efficiency compared with macrophages derived from the peritoneal cavity after thioglycollate injection (Thio-PEC). Dendritic cells derived from bone marrow by culture in GM-CSF were able to present CyaA-OVA E5 with an efficiency comparable with that of Thio-PEC (Fig. 7*A*). To exclude the possibility that granulocytes contaminating the BMDC preparation contributed to the presentation of CyaA-OVA E5, we performed experiments with the FSDC dendritic cell line. The FSDC cell line was able to present CyaA-OVA E5 to B3Z (Fig. 7, *A* and *B*). The weak efficiency of presentation is probably linked to the immature phenotype of this cell line. Unfractionated splenocytes, as well as the EL4 thymoma and the LBZ7.4 B cell lymphoma, were also able to present the CyaA-OVA E5 to B3Z T cells (Fig. 7*C*).

Thus, taken together, our results establish that, despite differences in efficiency, several APC of both myeloid and lymphoid origin were able to present the CyaA-OVA E5 to B3Z $CD8^+$ T cells.

Discussion

In the present study, we established that the OVA $CD8^+$ epitope inserted in a genetically detoxified adenylate cyclase toxin from *B. pertussis* (CyaA-OVA E5) is efficiently presented to a specific $CD8^+$ T cell hybridoma by thioglycollate-induced peritoneal macrophages. The presentation of CyaA-OVA E5 in peritoneal macrophages retains all the hallmarks of the classic cytosolic pathway for the presentation of endogenous Ag. Although other proteolytic machinery may participate in the generation of the appropriate N terminus of the antigenic peptide from the whole protein (40), the proteasome activity is essential for the presentation of CyaA-OVA E5 as demonstrated by lactacystin inhibition. Moreover, the presentation of CyaA-OVA E5 is dependent on the TAP system, which is involved in the transport of the processed peptides from the cytosol to the ER, and on a population of neosynthesized pre-Golgi MHC class I molecules. The presentation pathway of CyaA-OVA E5, like the invasive activity of wild-type CyaA, is 1) dependent on extracellular Ca^{2+} and 2) independent of vacuolar acidification as shown by resistance to chloroquine. This pathway is independent of the endocytic pathways implicated in the presentation of exogenous Ag (5–7). The presentation of CyaA-OVA21 E5 does not depend on phagocytosis or macropinocytosis, since it is resistant to CCB and DMA inhibition, respectively.

The use of recombinant toxins for the delivery into the cytosol of CTL epitopes gives rise to an increasing interest. Besides CyaA, two other systems have recently been developed using the *Pseudomonas aeruginosa* exotoxin (41, 42) and the lethal factor (LF) of *Bacillus anthracis* in the presence of the protective Ag component of the toxin (43–45). In both cases, sensibilization of target cells was effective in vitro, but the recombinant LF of *B. anthracis* was also shown to elicit a CTL response in vivo against the inserted epitope (44).

The presentation of the recombinant *P. aeruginosa* exotoxin toxin was found unexpectedly to be independent of cytosol translocation. Moreover, unlike CyaA-OVA E5, its processing retains the characteristics of the noncytosolic pathway due to its TAP independence (42). Fusion proteins between LF and HIV gp120 were found to be presented in vitro to specific $CD8^+$ cells in a protective Ag-dependent way. Lactacystin inhibition demonstrated that this presentation pathway involves proteasome processing (45). Although the TAP dependence and the sensitivity to BFA inhibition were not established, it was speculated that the recombinant toxin is delivered into the cytosolic pathway.

In contrast to the adenylate cyclase of *B. anthracis* (a toxin different from LF but internalized in a similar mechanism), the adenylate cyclase of *B. pertussis*, CyaA, is internalized by a mechanism that does not require vacuolar acidification (22, 23). Our results concerning the resistance of the CyaA-OVA E5 presentation to chloroquine are consistent with these earlier observations. This result excludes a putative endolysosomal degradation of CyaA-OVA E5 as a source of SIINFEKL peptide.

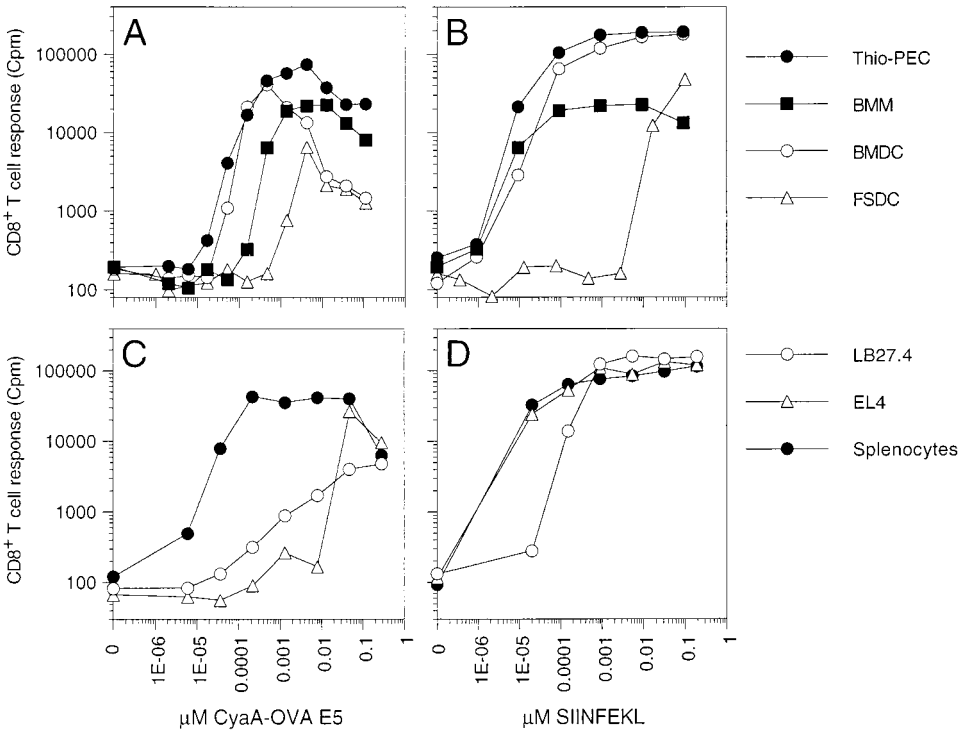

FIGURE 7. Presentation of CyaA-OVA E5 by different APC. Different APC (Thio-PEC, BMM, BMDC, FSDC, splenocytes, EL4, and LB27.4) were cocultured with B3Z CD8$^+$ T cell hybridoma in the presence of the CyaA-OVA E5 molecule (*A* and *C*) or the SIINFEKL synthetic peptide (*B* and *D*). The IL-2 release by B3Z was measured in a CTLL proliferation assay. Results are expressed in cpm.

Compared with other toxins, the originality of CyaA stems from its translocation mechanism, apparently independent of receptor-mediated endocytosis. The translocation of CyaA occurs in two steps (16, 21, 25). First, the C-terminal domain of CyaA binds to the external surface of plasma membrane with a mechanism requiring the posttranslational palmitoylation of Lys^{983} (46) and possibly through interaction with gangliosides (22). Second, the catalytic domain is translocated across the plasma membrane in a Ca^{2+}-dependent manner (25). We show, in the present study, that the presentation of CyaA-OVA E5 is also dependent on Ca^{2+} concentration. This last point establishes that delivery of CyaA-OVA E5 in the cytosolic pathway for MHC I presentation depends on the same mechanism of membrane translocation as the delivery of the catalytic domain of wild-type CyaA in target cells.

The strong immunogenicity of CyaA-LCMV E5 in vivo (29, 30) suggests that this molecule could be presented by professional APC and especially by dendritic cells that are thought to be the main APC population involved in the priming of naive T cells in vivo (47). Indeed, we found that GM-CSF-induced BMDC and the FSDC line are able to present efficiently CyaA-OVA E5 to B3Z CD8$^+$ T cells. The phenotypic heterogeneity of macrophage populations does not seem to limit the presentation of CyaA-OVA E5 to B3Z, since both peritoneal macrophages recruited by thioglycollate-induced inflammation of the peritoneal cavity (Thio-PEC) and macrophages derived from bone marrow precursors by culture in the presence of M-CSF are able to present CyaA-OVA E5 to B3Z T cells. Moreover, unfractionated splenocytes are also able to present CyaA-OVA E5 with a comparable efficiency. The LB27.4 B cell lymphoma and the EL4 thymoma cell lines, as well as the LK^b fibroblastic cell line (Fig. 7 and data not shown), also present CyaA-OVA E5 but with a reduced efficiency for reasons that remain to be clarified. Thus, taken together, our results demonstrate that the presentation of CyaA-OVA E5 to CD8$^+$ T cells is not restricted to professional APC. If the variety of cell types intoxicable in vitro by wild-type CyaA (16) is well established, little is known, to our knowledge, on the cell types targeted by CyaA in vivo during the *B. pertussis* infection or after an immunization with the recombinant CyaA toxin. Experiments are currently being performed to clarify the role of the different cell types (from lymphoid and myeloid origin) involved in the presentation of detoxified recombinant CyaA both in vitro and in vivo. However, the lack of cellular specificity distinguishes unequivocally the CyaA presentation pathway from the previously described pathways for the presentation of exogenous Ags and is consistent with the supposed absence of cellular receptor for this toxin.

In the mouse model, CyaA has been described to be a major virulence factor during the initial phases of *B. pertussis* infection (48, 49). Moreover, it was demonstrated that immunization with purified CyaA can protect mice from *B. pertussis* infection (50, 51). Our results strongly suggest that intoxication of cells with wild-type CyaA toxin could result in the activation of MHC class I-restricted CD8$^+$ T cells specific for the toxin itself. Mills et al. (52) have shown that the transfer of CD8$^+$ T cells from immune mice to immunosuppressed naive recipient increases the bacterial load in a respiratory model of infection, whereas CD4$^+$ T cells confer protection. Thus, the exact role of CD8$^+$ T cells in the *B. pertussis* infection remains to be clarified.

In conclusion, our results show that the presentation of recombinant CyaA to CD8$^+$ T cells constitutes a new and unconventional access of an exogenous Ag to the cytosolic pathway. These findings are relevant to the rational design of recombinant CD8$^+$ T cell vaccines based on the invasivity of the CyaA molecule and to the understanding of the immune response raised against CyaA during the *B. pertussis* infection.

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