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Characterization of the culture filtrate-specific cytotoxic T lymphocyte response induced by bacillus Calmette-Guérin vaccination in H-2^b mice

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Abstract

Although CD8$^+$ T cells are supposed to play an important role in protective immunity to mycobacteria, cytotoxic T lymphocyte (CTL) responses in this infection remain poorly characterized. We previously demonstrated that bacillus Calmette-Guérin (BCG) immunization of H-2^b mice induced CTL able to recognize and kill macrophages incubated with proteins from mycobacterial culture supernatant [culture filtrate (CF) antigens]. In the present study, we have further characterized the lytic activity of these CTL and the processing pathway used for the presentation of CF proteins. We show that they use the degranulation pathway (secretion of perforins and granzymes) as the main lytic mechanism of cytotoxicity and also secrete IFN-γ upon incubation with CF-pulsed macrophages. The *in vitro* presentation of CF proteins to CTL required a processing step inhibited in the cold but insensitive to Brefeldin A. Transporter-associated protein (TAP)-2-deficient RMA-S cells were efficiently recognized and killed by CF-specific CTL, demonstrating the lack of TAP requirement for this presentation. However, recognition of target cells by CTL was abolished when carried out in the presence of chloroquine. These results indicate that a non-classical MHC class I-processing pathway allows the recognition of a CF protein by CTL in BCG-vaccinated H-2^b mice.

Introduction

Tuberculosis remains the leading cause of death among infectious diseases and a major public health problem since the current vaccine, the attenuated *Mycobacterium bovis* bacille Calmette-Guérin (BCG), shows variable efficacy against the pulmonary form of this disease (1,2). Although our knowledge of the immune mechanisms involved in protection against tuberculosis is far from complete, it is well known that generation of protective immunity requires the activation and cooperation of several arms of the immune system. Besides CD4$^+$ T cells and macrophages, some evidence points to a protective role for CD8$^+$ T cells. Studies using CD8$^+$ T cell depletion *in vivo* or gene knockout mice have demonstrated that a lack of CD8$^+$ T cells leads to an increased sensitivity to mycobacterial infections (3,4). In humans, evidence that CD8$^+$ T cells participate in immunity to mycobacteria comes from the immunohistological detection of CD8$^+$ T cells, as well as CD4$^+$ T cells, in lesions of leprosy patients (5) and from the presence of CD8$^+$ T cells in the pleural space of tuberculous patients (6). Activation of CD8$^+$ cytotoxic T lymphocytes (CTL) has been observed after re-stimulation of spleen cells from mycobacteria-infected mice or peripheral blood lymphocytes from purified protein derivative-positive healthy donors with infected macrophages (7,8). Furthermore, some cell lines derived from BCG-immunized mice are able to lyse macrophages infected with BCG *in vitro* (7). In addition to their cytolytic activities, CD8$^+$ T cells secrete large amounts of IFN-γ (9), a cytokine playing an essential role in the control of mycobacteria *in vivo* (10).

Recently, we demonstrated that BCG-immunized H-2^b mice generate CD8$^+$ CTL specific for soluble proteins released in

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the supernatant of mycobacterial cultures [culture filtrate (CF) proteins] (11). These antigens have already been shown to be the main targets of $CD4^+$ T cells and confer, when administered in an appropriate adjuvant, a certain level of protection to mice (12). We showed that these cytotoxic $CD8^+$ T lymphocytes are amplified in bulk culture containing CF and are able to kill, in a MHC class I-restricted way, macrophages exogenously pulsed with CF proteins (11). This activation of CTL and recognition of soluble protein-pulsed target cells is in apparent conflict with the common view on antigen presentation to $CD8^+$ T cells. Indeed, $CD8^+$ T cells recognize short peptides, bound to MHC class I molecules, generated in the cytosol by limited proteolysis of self and foreign proteins. These peptides bind nascent heavy chain of class I molecules and β_2-microglobulin in the endoplasmic reticulum, transit through the Golgi compartment, and finally egress to the cell surface (13). By contrast, exogenous proteins (such as CF proteins) have no access to the cytosol but are degraded in an acidic environment and loaded onto MHC class II molecules leading to an exclusively $CD4^+$ T cell recognition (14). Although various exceptions to these rules have been described (15,16), soluble antigens are commonly considered to be ineffective in the activation of $CD8^+$ T cells.

Therefore, the aim of this study was to provide a further characterization of these CF-specific CTL and to investigate the presentation pathway allowing *in vitro* recognition of CF-pulsed cells by CTL.

Methods

Mice and cell lines

C57BL/6 and BALB.B10 mice (both H-2^b) originated from the Netherlands Cancer Institute and were maintained at the Pasteur Institute by strict brother–sister mating. Female mice were 8–10 weeks old at the beginning of all experiments. EL-4 is a T lymphoma cell line of C57BL/6 origin (H-2^b). The RMA cell line and the transporter-associated protein (TAP)-2 deficient variant RMA-S are derivatives of the Rauscher virus-induced T cell lymphoma cell line RBL-5 of C57BL/6 origin (17,18). These cell lines were maintained in RPMI 1640 medium supplemented with penicillin and streptomycin, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and 10% FCS (complete medium).

Mycobacterial infection and CF antigens

Mice were inoculated i.v. in a lateral tail vein with 0.5 mg (4×10^6 c.f.u.) of freshly prepared *M. bovis* BCG GL2 (Pasteur Institute of Brussels) as described previously (19). They received two injections at intervals of 2 months. CF proteins were obtained from 14-day-old zinc-deficient cultures of *M. bovis* BCG GL2, grown as a surface pellicle culture on synthetic Sauton medium (19). CF was equilibrated with 20 mM phosphate buffer containing 0.45 M NaCl and then applied to a phenyl-Sepharose CL-4B column (Pharmacia, Uppsala, Sweden). Unbound material was concentrated by precipitation with ammonium sulfate (80% saturation), extensively dialysed against PBS and sterilized through a 0.2 μm pore-size filter.

In vitro re-stimulation of CTL

Spleens were removed aseptically 4 weeks after the second BCG injection at the earliest. Spleen cells (2.5×10^6 cells/ml) were cultured for 6 days in RPMI 1640 medium supplemented with penicillin and streptomycin, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 10% FCS, and 25 μg/ml of BCG CF in 24-well plates. Cultures were maintained in 5% CO_2 at 37°C.

In vitro sensitization of target cells for lysis and ^{51}Cr-release assay

In some experiments, target cells were sensitized for CTL recognition by incubation for 16 h with 10 μg/ml of CF. Then, target cells (2×10^6 cells in 500 μl of complete medium) were incubated for 3 h with 200 μCi of ^{51}Cr (Amersham), washed twice and then added to effector cells.

For antigen processing experiments, Brefeldin A (BFA) or chloroquine (Sigma) were added to target cells (2×10^6 cells in 500 μl of complete medium) at concentrations indicated in the figures. After 30 min, CF (25 μg/ml) was added and cells were incubated for 1 h. Then, 200 μCi of ^{51}Cr was added, and cells were incubated for another 2 h and washed twice. Target cells were finally resuspended in medium containing the same concentrations of CF and inhibitors.

For the lytic pathway characterization, $MgCl_2$ (3 mM) and EGTA (Sigma) (at concentrations indicated in the figures) were added to target cells and CTL during their 4 h incubation.

Cell-mediated cytotoxicity was detected in short-term ^{51}Cr-release assays against different targets cells as previously described (20). Briefly, serial dilutions of effector cells (100 μl, in complete medium) were added to ^{51}Cr-labeled targets (100 μl, 2×10^3 cells in complete medium) in round-bottomed wells. After a 4 h incubation at 37°C, 120 μl of supernatant was collected for γ-radiation counting in a Minigamma 1275 counter (LKB Wallac, Turku, Finland). The percentage of specific lysis was calculated as 100×(experimental release – spontaneous release)/(maximum release – spontaneous release). Maximum release was obtained in wells containing 100 μl of target cells and 100 μl of 1 M H_2SO_4. Spontaneously released counts were always <20% of the total counts. Data shown are the mean of triplicate cultures. All experiments were performed at least twice.

Immunofluorescence staining and T cell subset depletion

Double staining for CD4 and CD8 T cells was performed using FITC-labeled rat anti-mouse L3T4 mAb (clone RM4-5; PharMingen, San Diego, CA) and phycoerythrin (PE)-labeled rat anti-mouse CD8b mAb (clone 53-5.8; PharMingen) as already described (11). Fluorescence was analyzed using the CellQuest software on a FACScalibur cytofluorometer (Becton Dickinson, Mountain View, CA) calibrated with CaliBRITE beads (Becton Dickinson). Live lymphocytes were gated on the basis of their characteristic forward and side scatter profile.

For T cell depletion, effector cells were purified on Lympholyte-M (Cedarlane, Hornby, Ontario, Canada) and 5×10^6 cells/ml were incubated with mAb RL172 (anti-CD4) or 83.12.5 (anti-CD8) culture supernatants for 30 min at 37°C. Cells were then pelleted and resuspended in Low-Tox rabbit serum (Cedarlane) as a complement source for 30 min at

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37°C. Finally, spleen cells were washed twice before use. Effective T cell depletion was confirmed by immunofluorescence. Remaining $CD4^+$ or $CD8^+$ T cells after depletion were always <2%.

IFN-γ assay

Peritoneal exudate cells (10^5) elicited 1 week before harvesting by i.p. thioglycolate (Difco, Detroit, MI) injection were plated in round-bottomed microplates. CTL (10^6) and CF (10 μg/ml) were added, and 16 h later supernatants were collected. IFN-γ production was measured on serial 2-fold dilutions of the culture supernatants, using a cytopathic effect reduction assay of vesicular stomatitis virus on mouse L929 fibroblastoid cells, as described previously (21). IFN-γ titer was determined as the reciprocal of the last dilution conferring 50% protection against any cytopathic effect. Titers were expressed as mean $\log_2$ laboratory units of duplicate assays. One $\log_2$ unit equals 220 pg/ml compared with the InterTest-γ mouse ELISA (Genzyme). The detection limit of the assay was >50 pg/ml. The antiviral activity could be completely neutralized by the anti-murine IFN-γ mAb F3, ensuring the specificity of the bioassay.

Results

Characterization of the CF-specific CTL response

Splenocytes from BCG-vaccinated mice cultured for 1 week with CF increased their percentage of $CD8^+$ and $CD4^+$ T cells, and decreased in parallel their percentage of $CD4^-$ $CD8^-$ cells. Strikingly, at the end of this culture, the percentage of $CD8^+$ T cells always showed broadly a 4-fold increase as compared to the start of the culture (Fig. 1A). These expansions of $CD4^+$ and $CD8^+$ T cells were not observed when naïve mice were used (not shown). Therefore, CF proteins are able, during this 1 week culture, to activate and amplify, in addition to $CD4^+$ T cells, $CD8^+$ T cells already primed *in vivo* by BCG. Splenocytes from BCG vaccinated mice re-stimulated with CF were next analyzed for their ability to recognize and kill CF-pulsed target cells in a 4 h ^{51}Cr-release assay. Using this assay, we were able to observe a CTL response directed toward CF-pulsed RMA cells (Fig. 1B). CF-pulsed EL-4 cells were killed in the same way by these CTL (data not shown). This response was mediated by $CD8^+$ T cells since it was almost completely abrogated by their depletion. In contrast, $CD4^+$ T cell depletion did not affect the target cell lysis. Therefore, CTL previously detected using a 16 h neutral red uptake assay (10) are also observed using a standard ^{51}Cr-release assay. Moreover, these results show that the ability to present exogenous CF proteins to MHC class I-restricted CTL is not a unique feature of macrophages (10) but can also be observed in non-phagocytic EL-4 and RMA cell lines.

So far, two distinct pathways in $CD8^+$ T cell-mediated cytotoxicity have been described (reviewed in 22). The Fas pathway involves the interaction of the Fas antigen on target cells with a CTL-bound Fas ligand, up-regulated through a TCR activation signal. The other pathway uses the secretion of perforins in combination with granzymes as a lytic effector mechanism. When added during incubation of target cells

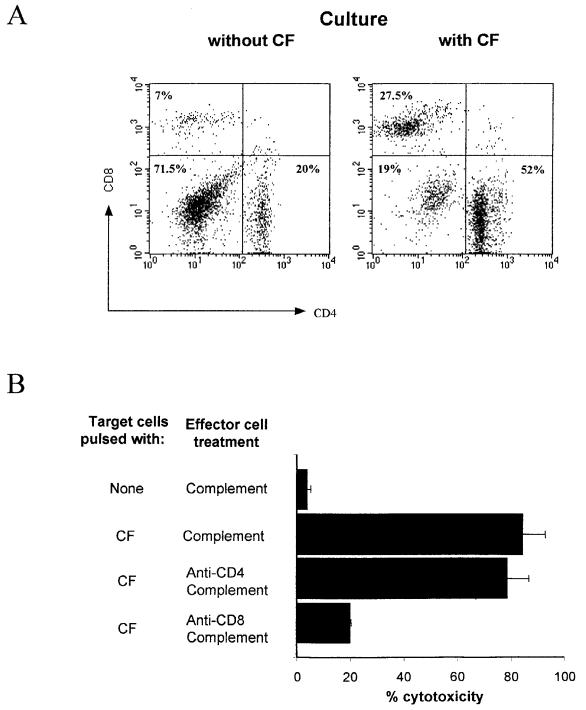

Fig. 1. (A) Spleen cells from BCG-immunized mice cultured in CF increase their number of $CD4^+$ and $CD8^+$ T cells. Spleen cells from C57BL/6 mice immunized 12 weeks previously with BCG were cultured with or without CF. Seven days later, double staining for $CD4^+$ and $CD8^+$ T cells was performed, and the percentage of positive cells was analyzed by flow cytometry. (B) CF-specific CTL are $CD8^+$ T cells. Spleen cells from BALB.B10 mice immunized 10 weeks previously were cultured for 6 days with CF. Effector cells were treated with complement alone or depleted of $CD4^+$ or $CD8^+$ T cells. Targets were RMA cells untreated or incubated for 16 h with CF (10 μg/ml). After labeling with ^{51}Cr, they were added to effector cells at the E:T ratio indicated. Cytotoxicity was detected in a standard 4 h ^{51}Cr-release assay as described in Methods.

with CTL, the drug EGTA can block the perforin/granzyme-mediated cytotoxicity pathway without affecting the Fas-dependent pathway (22,23). Therefore, we analyzed the effect of EGTA on CF-specific CTL. As shown in Fig. 2, target cell lysis was totally abolished in the presence of an optimal concentration of EGTA (2 mg/ml) while CTL were almost unaffected when a suboptimal dose (0.5 mg/ml) was used demonstrating that target killing involved the action of perforins and granzymes. The response obtained using 3 h-pulsed target cells was constantly lower as compared with overnight-pulsed target cells, indicating the requirement for a longer time to maximally process and present the antigens to CTL. Another widely recognized characteristic of CTL, in addition to their ability to recognize and destroy target cells, is their ability to secrete IFN-γ (9). Since this cytokine plays a crucial role in acquired protection against mycobacteria (10) we analyzed whether presentation of CF to CTL led to a synthesis of IFN-γ. For this purpose, CTL were cultured with unpulsed or CF-pulsed macrophages and IFN-γ level was measured in supernatants 16 h later. As shown in Table 1, a high IFN-γ production was detected in re-stimulated cells from immune mice cultured with macrophages and CF proteins. A lower

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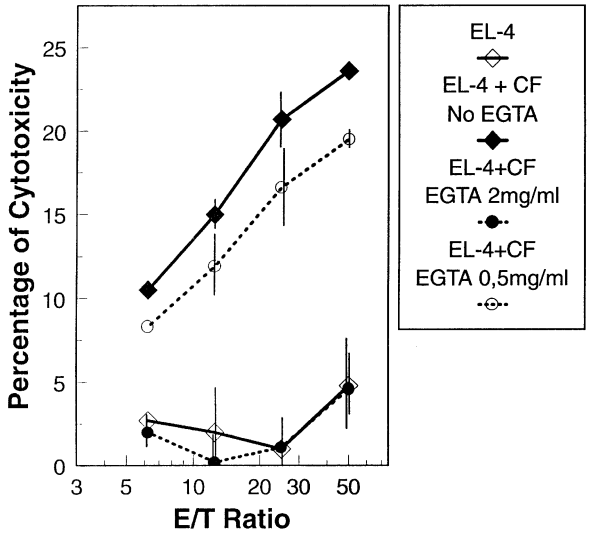

Fig. 2. Killing of target cells by CTL generated in BCG-infected mice is perforin/granzyme dependent. Effector cells from BCG-immunized C57BL/6 mice were generated 6 weeks after secondary immunization. ^{51}Cr-labeled EL-4 cells, incubated with or without CF (25 μg/ml) for 3 h, were washed and diluted in medium with or without 4 mM $MgCl_2$ and 2 or 0.5 mg/ml EGTA. Next, target cells were added to CTL at the indicated E:T ratios and cytotoxicity was analyzed after 4 h of co-culture.

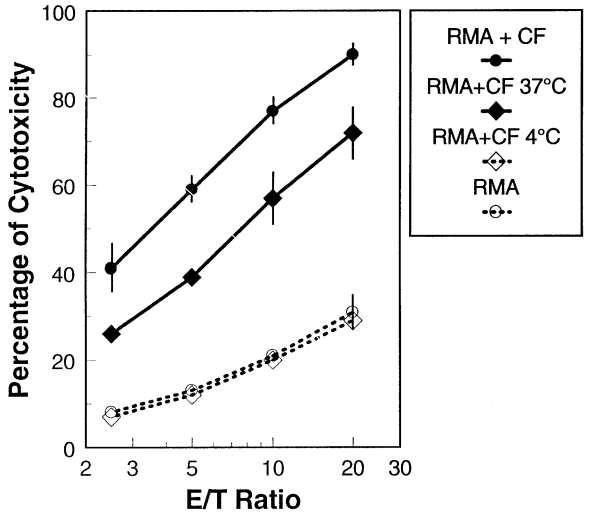

Fig. 3. Presentation of exogenous CF proteins requires a processing step which is inhibited at 4°C. Spleen cells from C57BL/6 mice immunized twice with BCG were cultured for 6 days with CF. RMA cells were incubated with ^{51}Cr for 1 h, then CF (100 μg/ml) was added and the cells were incubated either at 37°C (RMA + CF 37°C) or at 4°C (RMA + CF 4°C) for 2 h before addition to CTL. As positive and negative controls, RMA cells were cultured overnight with (RMA + CF) or without CF (RMA) respectively before being labeled with ^{51}Cr as described in Methods. Target cells were added to effector cells at the indicated E:T ratios and ^{51}Cr release was measured after 4 h of culture.

Table 1. IFN-γ production by spleen cells from H-2^b mice vaccinated with BCG restimulated *in vitro* with CF

Strain	IFN-γ (pg/ml/10^6 cells) on	
	Unpulsed	CF-pulsed PEC
BALB.B10 (nonimmune)	275 (33)*	247 (33)
BALB.B10		
Untreated	1600 (209)	15108 (1089)
C-treated	ND†	14135 (1045)
CD4-depleted	ND	3190 (418)
CD8-depleted	ND	5500 (1375)
CD4+CD8-depleted	ND	270 (60)
PEC without effector cells	319 (77)	324 (28)

Spleen cells from naïve or BCG vaccinated mice were restimulated for one week with CF. CTLs were left untreated, treated with complement only or depleted of CD4 and/or CD8 T cells and then incubated in round bottom microplates with peritoneal exudate cells (PEC) pulsed or not with CF. Supernatants were removed 16 hours later and IFN-γ content was determined using a cytopathic reduction assay of vesicular stomatitis virus on L929 cells.

*=Mean and SD of triplicates determinations.

†=Not determined.

IFN-γ release was also constantly observed upon culture with unpulsed macrophages; this low secretion probably reflected prior CTL activation during the bulk culture. Finally, T cell subset depletion clearly demonstrated that CF-specific $CD8^+$ T cells in addition to $CD4^+$ T cells contributed to this IFN-γ secretion since its complete inhibition required depletion of both $CD4^+$ and $CD8^+$ T cell subsets.

Analysis of the processing pathway involved in the CTL recognition of CF-pulsed target cells

CF-specific CTL are efficiently amplified in bulk culture supplemented with soluble CF proteins. Moreover, these CTL recognize and destroy target cells incubated with soluble CF proteins. In order to demonstrate that peptides contaminating our CF preparation were not responsible for this CTL activation, RMA cells were labeled with ^{51}Cr and then incubated for 2 h with a high concentration (100 μg/ml) of CF either at 37 or 4°C before their addition to CTL. As shown in Fig. 3, RMA cells pulsed with CF at 4°C were not lysed by CTL while RMA cells pulsed at 37°C were efficiently recognized. Therefore, it is unlikely that peptides able to directly bind MHC class I molecules were present in our CF and responsible for the CTL activation. To determine whether presentation of soluble CF proteins to CTL involved the classical MHC class I pathway we first tried to block this presentation with BFA. BFA inhibits exocytosis of proteins from the endoplasmic reticulum and Golgi complex, and thus prevents newly assembled peptide–MHC class I complexes from reaching the cell surface (24). RMA cells were pulsed with exogenous CF proteins in the presence of concentrations of BFA ranging from 20 to 5 μg/ml, before their addition to CTL. Although these concentrations are commonly reported to completely block the conventional MHC class I pathway (25,26), they failed to inhibit the presentation of CF proteins and the lysis of target cells (Fig. 4).

We also determined whether presentation of exogenous CF proteins required functional TAP molecules. Indeed, one hallmark of the conventional MHC class I presentation pathway

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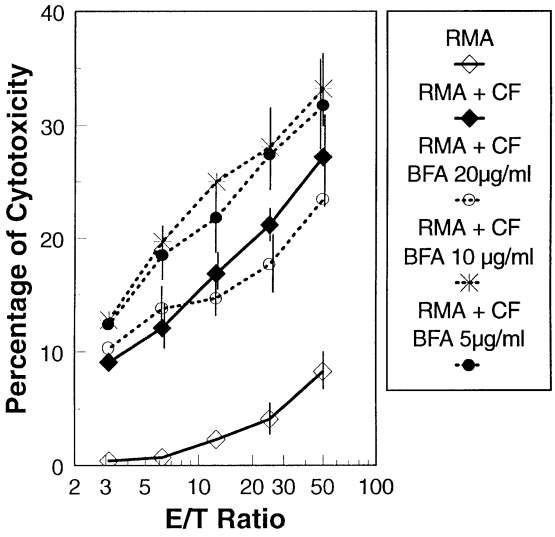

Fig. 4. Presentation of exogenous CF proteins to CTL is not inhibited by BFA. Spleen cells from BALB.B10 mice were obtained 12 weeks after secondary immunization with BCG and amplified with CF. Effector cells were harvested from bulk culture 6 days later. 2×10^6 target RMA cells were incubated with 20, 10 or 5 µg/ml of BFA for 30 min. CF (25 µg/ml) and ^{51}Cr (200 µCi) were added, and target cells were incubated for 3 h in the presence of BFA. Target cells were washed in medium containing the same concentrations of CF and inhibitor, added to CTL at the E:T ratios indicated, and cytotoxicity was analyzed after 4 h of culture.

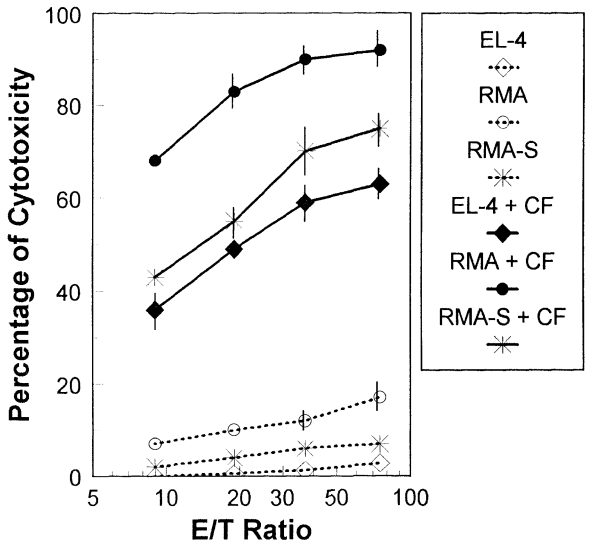

Fig. 5. RMA-S cells efficiently present exogenous CF proteins to CTL. C57BL/6 mice were immunized twice with BCG. Fourteen weeks later, spleen cells were cultured for 6 days with CF. ^{51}Cr-labeled RMA and RMA-S cells, incubated with or without CF (10 µg/ml) for 16 h, were used as target cells for CF-specific CTL recognition. Target cells were added to effector cells at the indicated E:T ratios and ^{51}Cr release was measured after 4 h of culture.

is the requirement of TAP-1 and TAP-2 molecules to transport peptides produced in the cytoplasm into the lumen of the endoplasmic reticulum, where they can associate with nascent MHC class I molecules. We compared the ability of EL-4 cells, RMA cells and TAP-2-deficient variant RMA-S cells to present CF to CTL. As shown in Fig. 5, CF-pulsed RMA and EL-4 cells were recognized and lysed by CTL. Moreover, TAP-deficient RMA-S cells were almost as efficiently lysed as TAP-positive RMA cells, clearly demonstrating that functional TAP proteins are not necessary to present exogenous CF proteins to CTL. The lower overall response observed with RMA-S cells compared to RMA cells could probably be explained by the lower level of MHC class I molecule expression on the cell surface which is only increased after stabilization with peptides (18).

Since the classical MHC class I-processing pathway could not account for the presentation of CF to CTL and since some non-classical endosomal MHC class I-processing pathways have been described (15,16), we tried to inhibit the presentation of CF proteins with chloroquine. Chloroquine is a weak base which raises the pH of the distal acidic vesicles (27). As a result, it inhibits the hydrolysis of proteins to oligopeptides by acid-optimal protease in the endocytic compartment. Target EL-4 cells were pulsed with CF proteins, in the presence of chloroquine, before addition to CTL. Figure 6 shows that lysis of target cells was completely inhibited when optimal concentrations of chloroquine (100 and 50 µg/ml) were used while this response was almost unaffected by a suboptimal concentration (10 µg/ml). Our results therefore indicate that the presentation of these exogenous CF proteins to CTL does not follow the general scheme of MHC class I presentation.

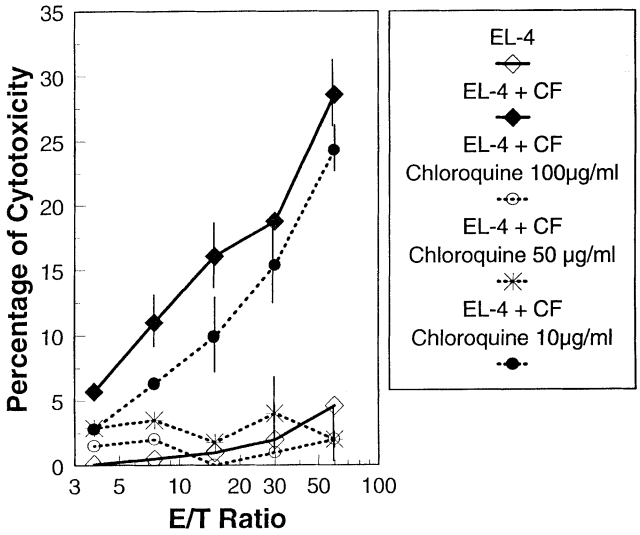

Fig. 6. Presentation of exogenous CF proteins to CTL is inhibited by chloroquine. Effector cells from BCG-immunized C57BL/6 mice were generated 6 weeks after secondary immunization. 2×10^6 target EL-4 cells were incubated with 100, 50 or 10 µg/ml of chloroquine for 30 min. Cells were then incubated for 3 h with CF (25 µg/ml) and with ^{51}Cr (200 µCi) in the continuous presence of chloroquine. Target cells were washed in medium containing the same concentrations of CF and inhibitor, added to CTL at the indicated E:T ratios, and cytotoxicity was analyzed after 4 h of culture.

Indeed, recognized peptides do not seem to be generated in the cell cytoplasm but in a chloroquine-sensitive acidic compartment and the loading of these peptides to MHC class I molecules does not appear to involve TAP molecules.

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Discussion

Mycobacteria are facultative intracellular pathogens which replicate in the macrophage phagosome and it has long been believed that T_h1 $CD4^+$ T cells secreting IFN-γ were the only protective population during infection with these pathogens. However, numerous reports have provided strong evidence for a definite role of $CD8^+$ in protective immune response in both humans and mice (5–8). Recently, we demonstrated that mice vaccinated with BCG can mount an effective $CD8^+$-mediated CTL response specific for CF proteins. CTL amplified in a 1 week culture with exogenous soluble CF proteins were capable of lysing macrophages incubated with hydrophilic antigens found in CF from various mycobacteria (11). This sensitization of target cells with exogenous soluble antigens seems in apparent conflict with an activation of MHC class I-restricted CTL since soluble antigens are normally pinocytosed and degraded upon encounter with lysosomes, leading to an exclusively MHC class II-restricted presentation (13,14). Therefore, we decided to further characterize this CTL response and the processing pathway involved in the MHC class I presentation of exogenous CF proteins to $CD8^+$ T cells. We now demonstrate that these CTL characteristically use the perforin/granzyme pathway as a lytic mechanism. However, we do not know whether they are also able to use the Fas-mediated pathway since our target cells (EL-4, RMA and RMA-S) showed a very low expression of Fas and were insensitive to the lysis induced by the anti-Fas JO-2 antibody (not shown).

We also show that these CF-specific CTL are able to secrete IFN-γ. Therefore, during a mycobacterial infection, they could participate in the protective immune response in two distinct ways. First, IFN-γ secreted by CTL could increase the bactericidal activity of macrophages. The essential role of this cytokine in anti-mycobacterial immunity has clearly been shown in IFN-γ knockout mice (10). Second, these CTL could induce programmed cell death of macrophages chronically infected by mycobacteria and unable to control their growth. Indeed, apoptosis of infected macrophages *in vitro* was shown to result in a 60–70% loss in viability of intracellular bacilli (28). *In vivo*, the relative importance of perforin and granzyme-mediated cytotoxicity remains controversial since perforin and granzyme knockout mice are able to control the early replication of mycobacteria (29,30). However, these knockout mice appeared to have a compensatory activation of cytokine genes, including IFN-γ and IL-12, even before infection. Moreover, other secreted molecules, apart from perforin and granzyme, could also directly act on mycobacteria viability. So, the precise mechanism of action of $CD8^+$ T cells remains unclear and participation of cell lysis in the control of mycobacterial growth *in vivo* cannot be ruled out.

In order to analyze the mechanism responsible for the presentation of CF proteins to CTL, inhibition studies using BFA or chloroquine were performed. We found that presentation of CF to CTL *in vitro* was inhibited by chloroquine but not by BFA. Moreover, RMA-S cells, deficient for TAP-2 molecules, were able to present exogenous CF to CTL. These results clearly demonstrate that the processing of CF does not involve the transfer of antigens from the endosome to the cytosol. They rather suggest that antigens are degraded in a low pH environment, probably in late endosomes or early lysosomes. The exact nature of this pathway remains to be determined, but it is worth noting that other reports have also described some TAP-independent processing of non-particulate exogenous antigens (25,31,32). For instance, a non-phagocytic MHC class I presentation pathway has been demonstrated for inactivated virus, virus particles and glycopeptides. This pathway is rapid, requires low doses of antigens, is resistant to BFA and TAP independent, but is blocked by lipophilic amines (reviewed in 15). Another alternative MHC class I-processing pathway involves the transfer of antigens from the phagosome to the cytosol. This pathway is BFA-sensitive and is observed after phagocytosis of particulate antigens such as bacteria or beads coupled to proteins (16). Interestingly, *M. tuberculosis* has been shown to facilitate the MHC class I presentation of soluble ovalbumin by a TAP-dependent mechanism (33). It remains to be determined whether mycobacteria, which normally reside in phagosomes, could use both of these pathways *in vivo*.

As mentioned earlier, the CF-specific CTL response seems to be directed against a low number of antigens (11). Indeed, CF-specific CTL were observed exclusively in $H\text{-}2^b$ mice and presentation of CF was exclusively restricted to D^b molecules (11 and unpublished results). Therefore it is possible that a single peptide could be the target of these CTL but it is unlikely that a small peptide contaminating our CF preparation directly mediated this response because presentation of CF antigens required a processing step which was inhibited when the target cells were cultured with CF proteins in the cold. Presentation of CF was also inhibited when target cells were incubated with chloroquine. Moreover, the target-sensitizing activity was recovered in the exclusion volume when CF was subjected to gel filtration chromatography on a Sephadex G-25 column (11). Therefore, we propose that some acid optimal proteases, located in the endosome or even at the cell surface, could release a peptide containing a D^b-binding motif from a CF protein. This peptide could thereafter bind D^b molecules once again inside the endosome or at the cell surface and be recognized by CTL as has been already described previously with other antigens (31,32).

It is clear that identification of antigens recognized by $CD8^+$ T cells could be helpful in the development of new protective strategies against tuberculosis. However, data concerning the nature of mycobacterial antigens recognized by CTL and the mechanisms of protein release by mycobacteria into the MHC class I-processing pathway are scarce. Strong CTL responses have been observed after vaccination with naked DNA encoding Hsp60 (34) or the Ag85, a major component of the CF (35). CTL specific for the 38 kDa glycolipoprotein (36) and the Hsp60 (37) have also been reported to be activated during infection with *M. tuberculosis* H37Rv and BCG respectively. However, their generation required several rounds of *in vitro* re-stimulation, indicating low precursor frequencies for these CTL. In contrast, a single bulk culture in the presence of CF strongly increased the percentage of $CD8^+$ T cells and readily allowed the detection of CF-specific CTL using a ^{51}Cr assay or an IFN-γ-secretion assay. Accordingly, these CF-specific CTL seem to recognize an immunodominant antigen which could play an important role in the protective response to mycobacterial infection.

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Identification of this antigen, beside its potential utility as a vaccine candidate, could also provide new information on the mechanisms of CTL activation by mycobacteria *in vivo*.

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Abbreviations

BCG	*Mycobacterium bovis* bacille Calmette-Guérin
BFA	Brefeldin A
CF	culture filtrate
CTL	cytotoxic T lymphocytes
TAP	transporter-associated protein

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