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Concanamycin A, a vacuolar type H^+-ATPase inhibitor, induces cell death in activated $CD8^+$ CTL

Ken-ichi Togashi, Takao Kataoka & Kazuo Nagai
Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226, Japan

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Abstract

Concanamycin A (CMA) and concanamycin B (CMB) are specific inhibitors of vacuolar type H^+-ATPase (V-ATPase). In our previous studies, intraperitoneal injection of CMB was shown to suppress the increase in $CD8^+$ CTL population, but not to affect $CD4^+$ and $B220^+$ populations, in mice immunized with allogeneic tumors. To clarify the molecular basis of the selective decrease in the $CD8^+$ CTL population by CMB, we have performed a series of *in vitro* experiments with use of CMA. Cell viability of the $CD8^+$ population prepared from the immunized mice was preferentially decreased by CMA treatment. Moreover, in the $CD8^+$ CTL clone, CMA induced a marked DNA fragmentation and nuclear condensation characteristic of apoptosis. Anti-CD3 or phorbol ester accelerated the CMA-induced reduction in cell viability of the $CD8^+$ CTL clone, but not $CD4^+$ T cell clones. However, this rapid cell death was not accompanied by DNA fragmentation and nuclear condensation. Perforin and granzyme B were unlikely to be involved in such cell death. Thus, our data suggest that V-ATPase activity is essential for survival of $CD8^+$ CTL especially when activated.

Abbreviations: CMA – concanamycin A; CMB – concanamycin B; CTL – cytotoxic T lymphocytes; FCS – fetal calf serum; KLH – keyhole limpet hemocyanin; OVA – ovalbumin; PBS – phosphate-buffered saline; PMA – phorbol 12-myristate 13-acetate; V-ATPase – vacuolar type H^+-ATPase

Introduction

Vacuolar type H^+-ATPase (V-ATPase) supports several cellular functions through the acidification of intracellular organelles such as lysosomes and Golgi apparatus (Mellman *et al*., 1986; Forgac, 1989). These functions are strongly perturbed by specific inhibitors of V-ATPase such as bafilomycins and concanamycins (Bowman *et al*., 1988; Woo *et al*., 1992; Muroi *et al*., 1993; Dröse *et al*., 1993). We have shown that concanamycin A (CMA) increases the internal pH of lytic granules, induces inactivation and degradation of perforin, and thereby blocks the perforin-dependent killing mediated by a variety of $CD8^+$CTL (Kataoka *et al*., 1994; 1996c). Moreover, Fas-based CTL killing has been shown to be insensitive to CMA (Kataoka *et al*., 1996a; 1996b). When concanamycin B (CMB) was injected to mice immunized with allogeneic tumors, the induction of $CD8^+$ CTL population was strongly suppressed but $CD4^+$ and $B220^+$ populations were not affected (Lee *et al*., 1995). In this paper, to address the mechanism of the selective decrease in $CD8^+$ population, we have performed a variety of *in vitro* experiments with use of CMA.

Materials and methods

Mice

C57BL/6, BALB/c and C3H/HeN mice (female, 6 wk-old) were purchased from Charles River Co. Ltd. (Yokohama, Japan). Six to eight wk-old mice were used for experiments.

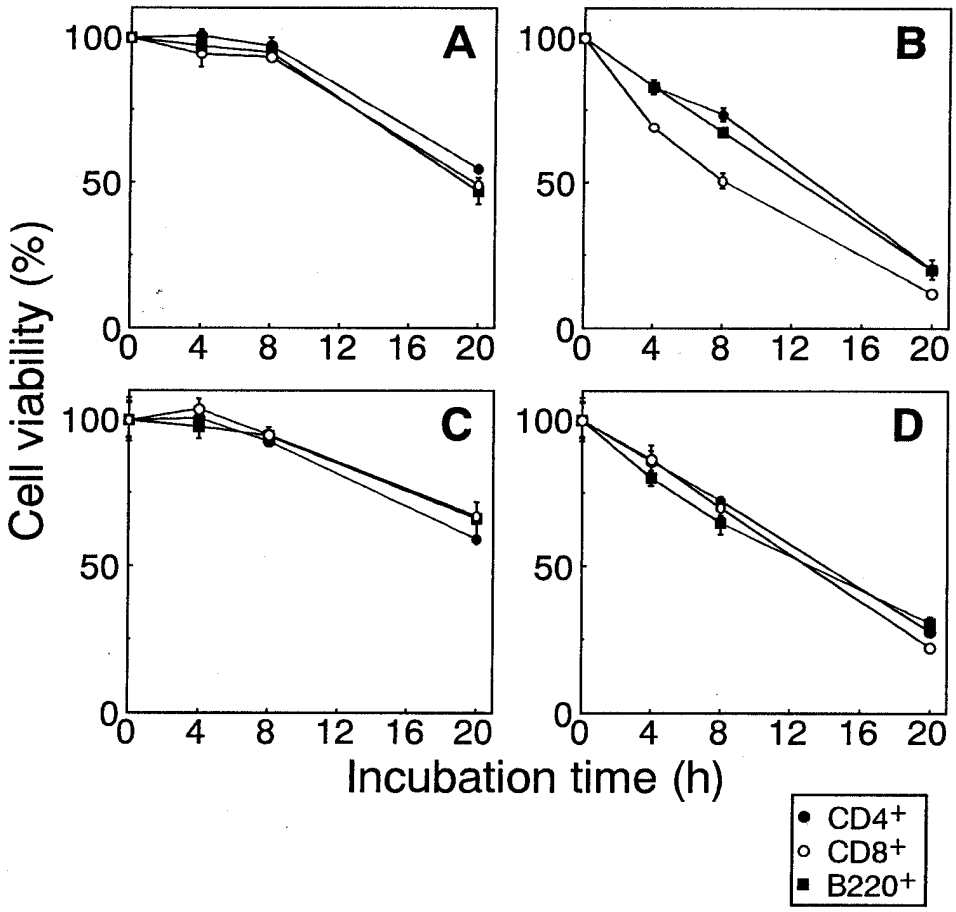

Figure 1. Effect of CMA on cell viability of lymphocyte populations in spleen cells $CD4^+$ (closed circles), $CD8^+$ (open circles) and $B220^+$ (closed squares) populations were obtained from P815-immunized (A, B) and normal (C, D) C57BL/6 mice by the panning method. Each population was cultured in the presence (B, D) or the absence (A, C) of 100 nM CMA. At indicated periods, cell viability was analyzed by MTT method.

Cells

$H\text{-}2^d$-specific $CD8^+$CTL clone OE4 (Staerz *et al.*, 1985), perforin-deficient $H\text{-}2^d$-specific $CD8^+$ CTL clone P0K (Kojima *et al.*, 1994), KLH-specific $I\text{-}E^d$-restricted $CD4^+$ CTL clone BK-1(Shinohara *et al.*, 1991), OVA-specific $I\text{-}A^d$-restricted $CD4^+$ Th1 clone 42-6A (Kakiuchi *et al.*, 1990), and $I\text{-}A^b$-specific $CD4^+$ Th2 clone D10.G4.1 (Kaye *et al.*, 1984) were cultured in the RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 10% (v/v) FCS (Bioserum, Victoria, Australia) and 5% (v/v) culture supernatant of rat spleen cells stimulated with 5 μg ml^{-1} of concanavalin A for 24 h. OE4, P0K, and D10.G4.1 were stimulated with mitomycin C-treated spleen cells from BALB/c, C3H/HeN and C57BL/6 mice every two weeks, respectively. BK-1 and 42-6A were stimulated with mitomycin C-treated BALB/c spleen cells together with 10 μg ml^{-1} of KLH and OVA every two weeks, respectively. These cells were used for experiments at least over 3 wk after the last stimulation. A mastocytoma P815 ($H\text{-}2^d$) was maintained in the RPMI medium plus 10% (v/v) FCS.

Reagents

CMA was kindly provided by Dr. K. Mizoue, Taisho Pharmaceutical Company Ltd. (Tokyo, Japan). Phorbol 12-myristate 13-acetate (PMA) was the commercial product of Wako Pure Chemical Industries Ltd. (Osaka, Japan). Anti-CD4 and anti-CD8 monoclonal antibodies were purchased from Seikagaku Co.

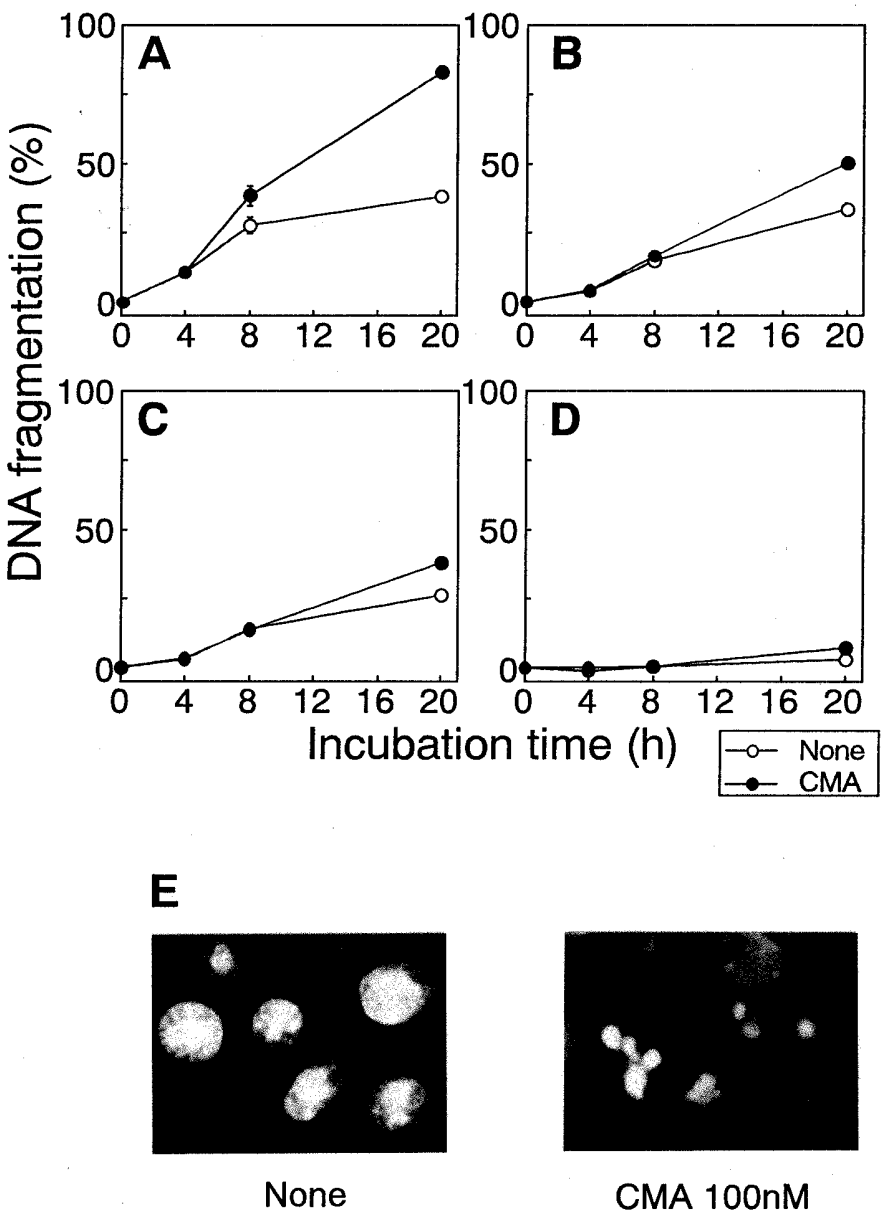

Figure 2. Effect of CMA on DNA fragmentation in $CD4^+$ and $CD8^+$ T cell clones [^{3}H]thymidine-labeled OE4 (A) , BK-1 (B), 42-6A (C), D10.G4.1 (D), were incubated in the presence (closed circles) or the absence (open circles) of 100 nM CMA for the indicated time, and DNA fragmentation (%) was estimated. (E) OE4 was treated with or without 100 nM CMA for 20 h, and then stained with Hoechst 33342. The cells were examined under fluorescent microscopy.

(Tokyo, Japan) and anti-mouse Immunoglobulin polyclonal antibody was obtained from Cedarlane Laboratories Ltd. (Ontario, Canada).

Preparation of lymphocyte populations

C57BL/6 mice were injected intraperitoneally with P815 (2×10^7 cells/mice). Spleen cells were removed from the mice on day 11. After adherent cells were removed, spleen cells (1.5×10^7 cells) were incubated in antibody-coated culture dishes (35 mm diam-

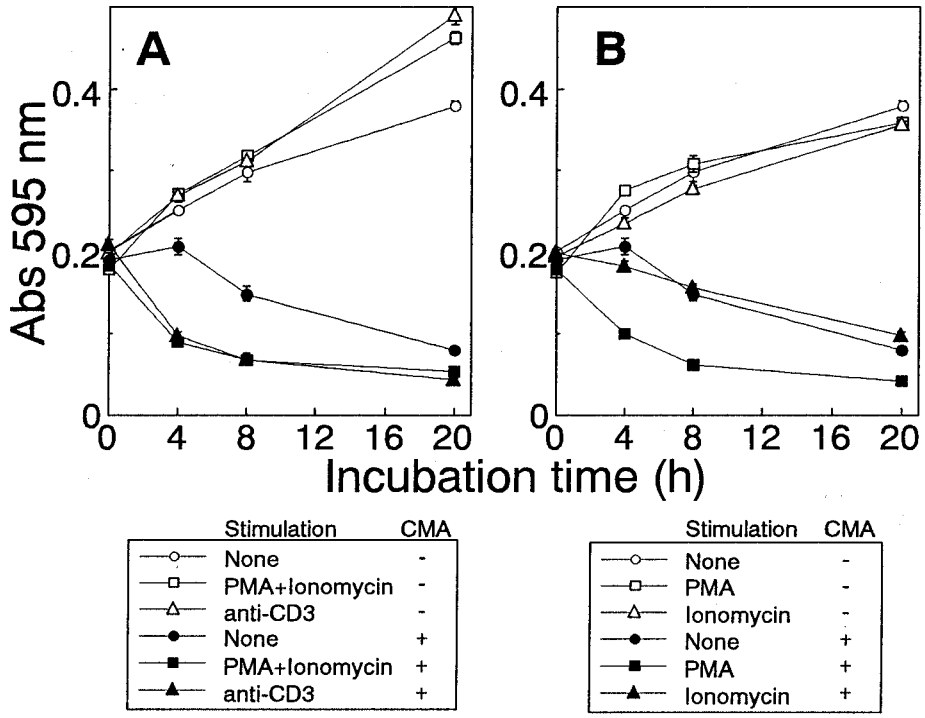

Figure 3. Effect of activation signals on CMA-induced cell death in $CD8^+$ CTL clone (A) OE4 was treated with anti-CD3 (triangles), 100 nM PMA plus 100 nM ionomycin (squares), or without stimuli (circles), in the presence (closed symbols) or the absence (opened symbols) of 100 nM CMA. (B) OE4 was treated with 100 nM PMA (squares), 100 nM ionomycin (triangles), or without stimuli (circles), in the presence (closed symbols) or the absence (opened symbols) of 100 nM CMA. At indicated periods, cell viability was analyzed by MTT method.

eter) for 30 min on ice. Non-adherent cells were removed by washing three times with PBS, and then antibody-bound cells were collected. Each population was shown to be >90% purity by flow cytometer.

Measurement of cell viability

Cells (2×10^4 cells/well) were cultured in microtiter plates for indicated periods. For the last 3 h, the cells were incubated with 500 μg ml^{-1} of 3-(4,5-dimethlthazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO). One hundred microliters of 0.04 N HCl in isopropanol were added to each well, and mixed vigorously. Absorbance at 595 nm was measured. The experiments were carried out in triplicate cultures.

Detection of DNA fragmentation

[^{3}H]thymidine-labeled T cell clones (2×10^4 cells/well) were treated with drugs in microtiter plates as indicated, and then the cells were lysed by the addition of 0.01% Triton X-100. Intact DNA was precipitated by centrifugation ($3000 \times g$, 3 min), and then the supernatants were recovered. Radioactivity in the supernatants was measured. Percentage of DNA fragmentation was calculated by using following formula: (experimental release – spontaneous release)/(maximum release – spontaneous release) $\times$ 100. The experiments were carried out in triplicate cultures.

Hoechst 33342 staining

OE4 was treated with drugs for indicated periods. Then the cells were treated with 200 μg ml^{-1} of Hoechst 33342 and immediately examined under fluorescent microscopy.

Measurement of granzyme B activity

OE4 (5×10^6 cells) was washed three times with PBS and suspended in 1 ml of 250 mM sucrose, 1 mM HEPES, and 4 mM EGTA (pH 7.4). The cells were disrupted through a nitrogen cavitation (450 psi, 4 °C,

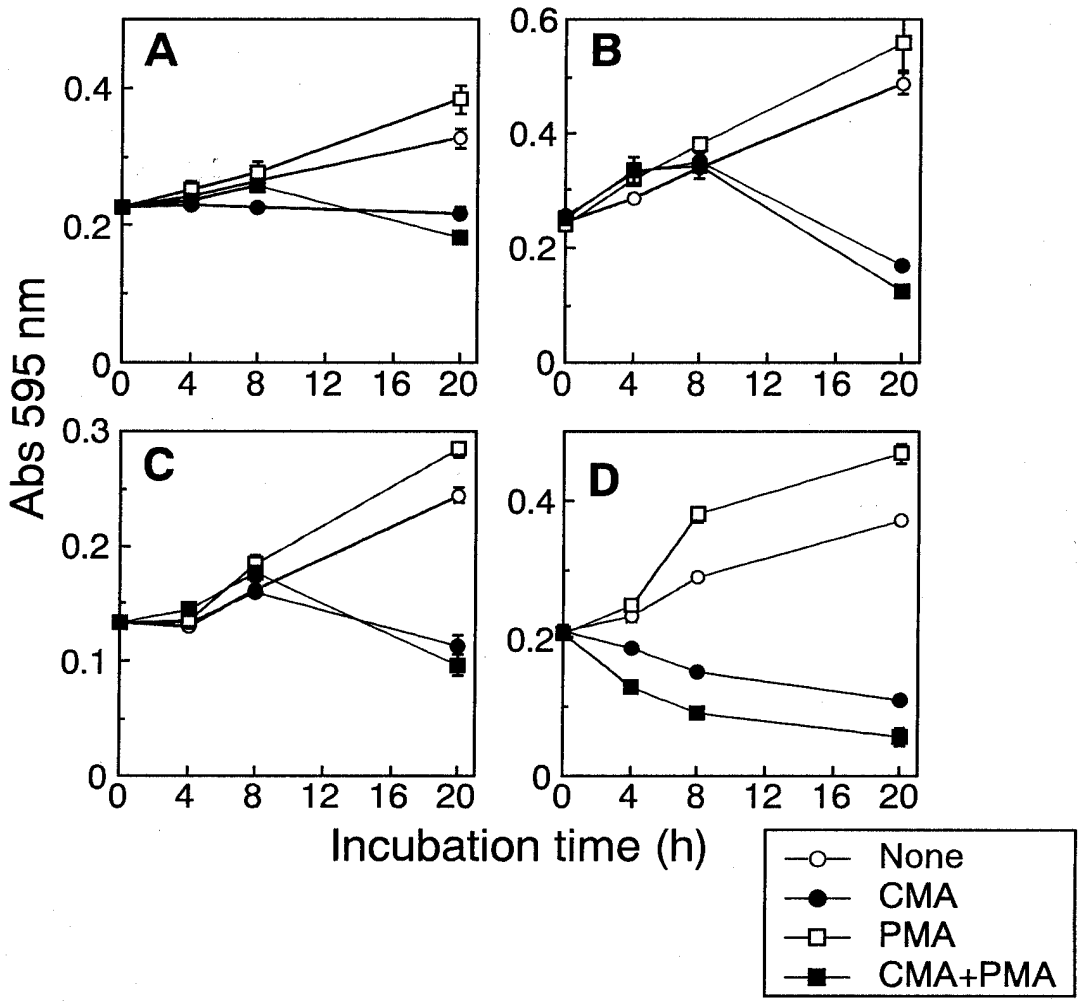

Figure 4. Effect of CMA and PMA on cell viability of $CD4^+$ and perforin-deficient $CD8^+$ T cell clones BK-1 (A), 42-6A (B), D10.G4.1 (C) and P0K (D) were treated with 100 nM CMA (closed symbols) or without (open symbols) in the presence (squares) or the absence (circles) of 100 nM PMA. At indicated times, cell viability was analyzed by MTT method.

20 min), and the resultant lysates were ultracentrifuged (100 000 × *g*, 1 h) to prepare supernatants as cytosolic fractions. Twenty micrograms of supernatants were incubated in 200 μl of 200 μM Boc-Ala-Ala-Asp-thiobenzylester (Enzyme Systems Products, Dublin, CA) and 220 μM 5,5'-dithio-bis-(2-nitrobenzoic acid) in PBS at 37 °C for 30 min. Absorbance at 415 nm was measured.

Results

Selective cell death of $CD8^+$ CTL by CMA in vitro

To clarify the molecular mechanism of the CMB-induced selective decrease in $CD8^+$ population *in vivo*, we prepared lymphocyte populations by panning method, and treated these cells with CMA, a close analog of CMB *in vitro*. All lymphocyte populations of both normal and the immunized mice retained their viability until 8 h, and then gradually lost their viability (Fig. 1A and 1C). The addition of CMA accelerated the decrease in cell viability of all populations (Fig. 1B and 1D). It should be noted, however, that cell viabil-

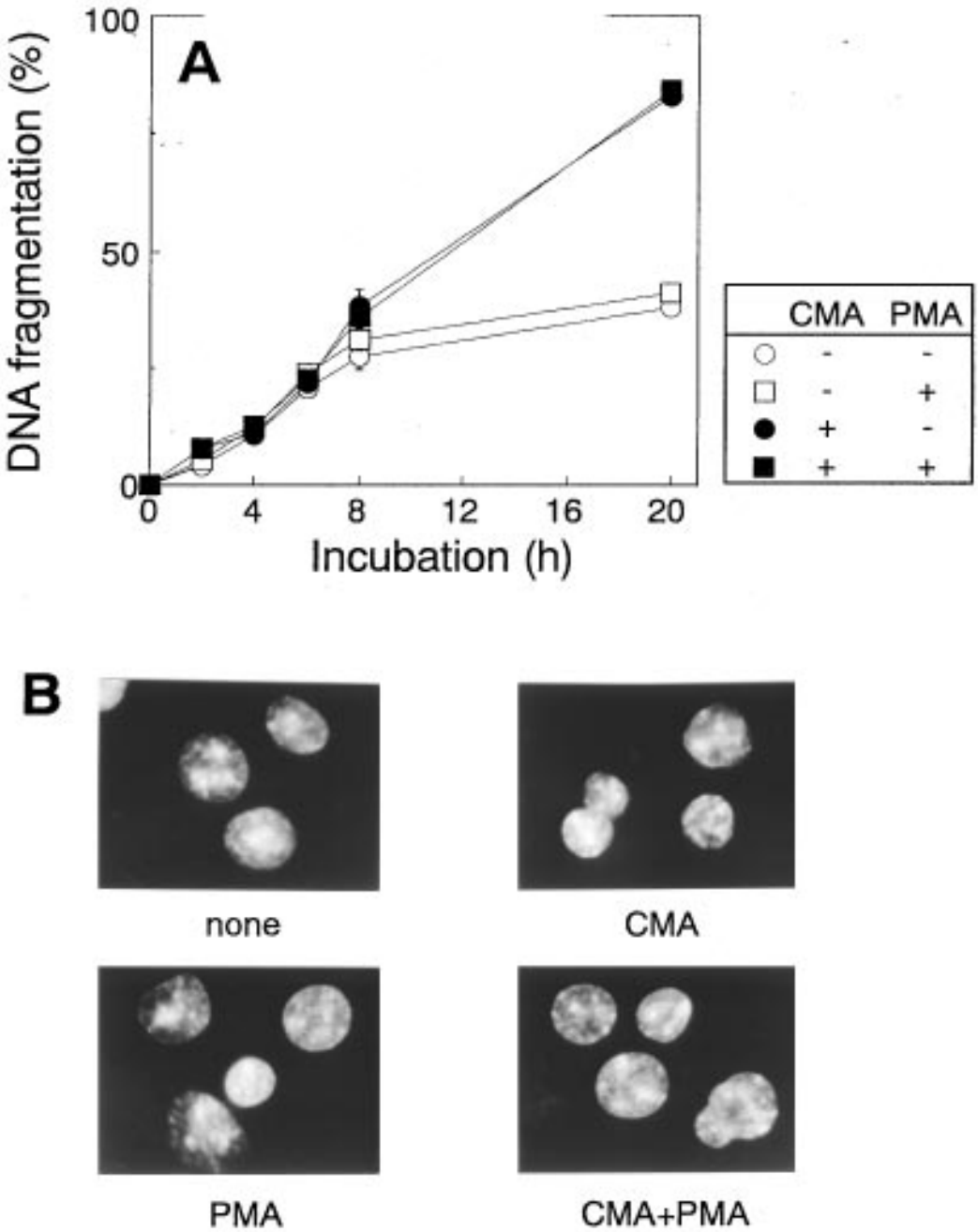

Figure 5. Absence of DNA fragmentation and nuclear condensation in CMA- plus PMA-induced acute cell death in $CD8^+$ CTL (A) OE4 was treated with (closed symbols) or without (open symbols) 100 nM CMA in the presence (squares) or the absence (circles) of 100 nM PMA, and percentage of DNA fragmentation was estimated. (B) OE4 was treated with 100 nM CMA and/or 100 nM PMA for 4 h, and then stained with Hoechst 33342. The cells were examined under a fluorescent microscope.

ity of $CD8^+$ population prepared from the immunized mice was preferentially decreased by CMA treatment (Fig. 1B). A certain group(s) of $CD8^+$ population in the immunized mice might be highly susceptible to CMA.

Above data prompted us to examine the effect of CMA on various $CD8^+$ and $CD4^+$ T cell clones (Fig. 2). CMA induced a significant DNA fragmentation in a $CD8^+$ CTL clone OE4 in 8 h to 20 h (Fig. 2A). Moreover, nuclear condensation was also observed in CMA-treated OE4 (Fig. 2E), suggesting that CMA induces apoptosis. However, CMA only marginally increased DNA fragmentation in $CD4^+$ T cell clones (Fig. 2B to 2D). Although CMA is reported to induce apoptotic cell death to the several cultured cell lines (Nishihara *et al*., 1995), $CD8^+$ CTL clone seems to be most sensitive to CMA (See discussion).

Induction of acute cell death by CMA in activated $CD8^+$ CTL

A part of $CD8^+$ population in mice immunized with allogeneic tumors might repeatedly receive activation signals from neighboring cells in the spleen and become more sensitive to CMB. To examine this possibility, $CD8^+$ and $CD4^+$ T cell clones were treated with CMA in the presence of stimuli such as immobilized anti-CD3 or combination of PMA and ionomycin (Figs. 3 and 4). Although CMA itself gradually decreased the number of viable cells, CMA in combination with these stimuli dramatically decreased the viable cells within 4 h after the treatment (Fig. 3A). PMA alone, but not ionomycin, was efficient to induce such acute cell death in the presence of CMA (Fig. 3B). Similar results were obtained by dye exclusion assay using trypan blue (data not shown). In contrast, PMA failed to affect the CMA-induced cell death of $CD4^+$ T cell clones (Fig. 4A to 4C). These results suggest that the activation through T cell receptors, especially PKC activation, increases the sensitivity of $CD8^+$ CTL to CMA.

Acute cell death of $CD8^+$ CTL induced by CMA and PMA is not apoptosis

To address whether CMA- and PMA-induced rapid cell death is apoptosis, two typical characteristics usually associated with apoptosis were examined (Fig. 5). PMA did not accelerate the DNA fragmentation (Fig. 5A). Moreover, condensed nuclei were undetectable in CMA- and PMA-treated cells (Fig. 5B). These data imply that acute cell death of $CD8^+$ CTL induced by CMA in the presence of PMA is not apoptosis.

Acute cell death of $CD8^+$ CTL induced by CMA in combination with PMA is independent of perforin and granzyme B

$CD8^+$ CTL harbors lytic granules which contain cytotoxic factors (e.g. perforin and granzymes) responsible for target cell lysis (Henkart, 1994; Podack *et al*., 1991). To examined the involvement of perforin in the process of acute cell death, perforin-deficient $CD8^+$ CTL clone P0K was treated with CMA and/or PMA, and the number of viable cells was measured (Fig. 4D). CMA plus PMA treatment dramatically induced cell death of P0K, similar to OE4. This result clearly suggests that CMA-induced acute cell death is independent of perforin.

In the perforin-mediated cell cytotoxicity, granzyme B is a major protease which enters the cytosol of target cells and triggers the death signal (Heusel *et al*., 1994). Thus, a leakage of cytotoxic molecules like granzyme B from the lytic granules into cytosol can be lethal for host killer cells. Since CMA induces the drastic morphologic changes of lytic granules (Kataoka *et al*., 1994, 1996a), we tried to check this possibility. CMA increased the granzyme B activity in cytosol only at 20 h treatment, irrespective of the presence of PMA (Fig. 6). Thus, it is unlikely that granzyme B is involved in the CMA- and PMA-induced acute cell death of $CD8^+$ CTL.

Discussion

$CD8^+$ population from P815-immunized mice showed higher sensitivity to CMA as compared with other lymphocyte populations from naive and immunized mice. Moreover, $CD8^+$ CTL clones, but not $CD4^+$CTL clones, were extremely sensitive to CMA, and exhibited two kinds of cell death: (1) apoptosis in the long-term culture and (2) rapid cell death in the short-term culture in the presence of PMA, which apparently lacked DNA fragmentation and nuclear condensation.

It is reported that CMA induces apoptotic cell death to several cultured cell lines (Nishihara *et al*., 1995). However, CMA induces only about 30% of apoptosis in B lymphoma by 24 h treatment (Nishihara *et al*., 1995). In our present data, CMA induced 90% of DNA fragmentation in OE4 by 20 h treatment, and CMA plus PMA induced rapid cell death within 4 h. Thus, $CD8^+$ CTL clones seem to be most sensitive to CMA among various cell types so far tested.

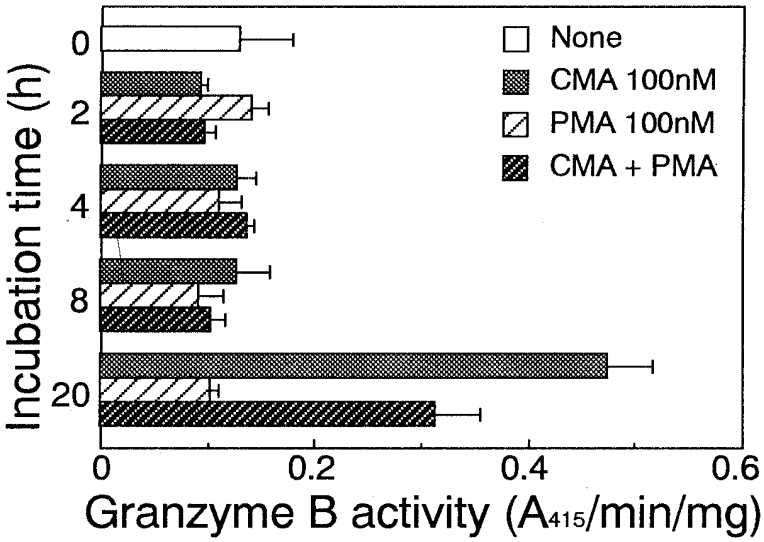

Figure 6. Leakage of granzyme B activity into cytosol by CMA-treated $CD8^+$ CTL clone OE4 was cultured with 100 nM CMA and/or 100 nM PMA for indicated periods and disrupted through N_2 cavitation. Then the resultant cell lysates were ultracentrifuged to prepare cytosol fractions. Twenty micrograms of the fractions were applied for the measurement of granzyme B activity.

Interestingly, CMA treatment induced two kinds of cell death in OE4. In the long-term incubation with CMA, OE4 exhibited DMA fragmentation and nuclear condensation. However, in the short-term incubation with CMA, together with PMA or anti-CD3, OE4 was destined to the rapid death. This acute cell death is unlikely to be apoptosis, since DNA fragmentation and nuclear condensation were totally absent.

Why are $CD8^+$ CTL clones most susceptible to CMA? As far as the CMA-induced apoptosis is concerned, one possibility is the involvement of apoptosis-inducing effector molecules (e.g. granzyme B) present in the lytic granules. Since CMA induced drastic morphologic changes of the lytic granules such as vacuolation (Kataoka *et al.*, 1994, 1996a), granular content(s) might leak from the granules into cytosol, thereby inducing apoptosis. Granzyme B activity was detected only in the cytosol fractions of OE4 undergoing apoptosis, thus it seems likely that granular content(s) such as granzyme B play a role.

What mechanism(s) is involved in the rapid cell death of the $CD8^+$ CTL clones in the presence of CMA and PMA (or anti-CD3)? Inhibition of acidification seems to be irrelevant, since monensin and chloroquine which neutralize the internal pH in acidic organelles had negligible effects (data not shown). The intracellular acidification is reported to proceed to apoptotic cell death in various systems (Gottlieb *et al.*, 1995; Li *et al.*, 1996; Pérez-Sala *et al.*, 1995; Gottlieb *et al.*, 1996). In this regulation, V-ATPase seems to play an essential role, because up-regulation of V-ATPase activity delays the programmed cell death (Brisseau *et al.*, 1996; Gottlieb *et al.*, 1995). $CD8^+$ CTL clones might contain a large number of V-ATPase, in that these cells harbor a number of the lytic granules. If this is the case, $CD8^+$ CTL clones might depend heavily on V-ATPase activity for cell survival. It is reported that PMA increases V-ATPase activity (Nanda *et al.*, 1992; Heming *et al.*, 1995). Thus, it is possible that the activation of V-ATPase-regulating functions, such as the maintenance of the intracellular pH, is one of the important factors for the survival of PMA-activated $CD8^+$ CTL clones, therefore, PMA treatment in the presence of CMA results in the severe unbalance of cellular conditions which causes rapid cell death. However, at present, besides unusual intracellular pH, possible contribution of other factors such as ecto-type ATPase sensitive to CMA and/or PMA-induced gene expression to cell survival can not be excluded.

Which kind of the cell death, or both, are actually involved in the CMB-induced selective decrease in $CD8^+$ population in the immunized mice has not been answered. In the spleen of the immunized mice, $CD8^+$ CTL might be repeatedly stimulated by cognate interaction with antigen-presenting cells or soluble factor(s). If this is the case, the rapid cell death might be dominantly involved in the elimination of activated $CD8^+$ T cells.

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Address for correspondence: Kazuo Nagai, Department of Bioengineering, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226, Japan.