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Perforin is expressed in CTL populations generated in vivo

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1. Summary

Immunization of C57BL/6 mice with syngeneic tumor cells, MBL-2, resulted in the generation of antitumor effector cells in vivo. The immunized C57BL/6 mice permanently rejected viable MBL-2 lymphoma cells, but not B16 melanoma cells. Cytotoxic T cells obtained from MBL-2-immunized mouse peritoneal cells (PEC) showed specific cytotoxicity against MBL-2, but not to YAC-1, RDM-4 and Meth A cells. By sorting with FACStar, the specific CTL were characterized as TCR $\alpha\beta^+$ $CD8^+$ T cells. Moreover, the cytoplasm of in vivo-induced CTL was stained with a monoclonal antibody against perforin. The localization of perforin in cytoplasmic granules of CTL was demonstrated by electron microscope analysis. This experiment presented the first evidence that in vivo-induced $CD8^+$ CTL against syngeneic tumor cells expressed significant amounts of perforin.

2. Introduction

It has been reported that immune T cells play important roles in host's immunological defence against tumors as well as other antitumor cells such as natural killer (NK) cells, lymphokine-activated killer (LAK) cells and macrophages [1 – 5]. Indeed, many reports have demonstrated that the adoptive transfer of immune T cells caused the inhibition of the tumor growth in vivo [6, 7]. However, the mechanisms of tumor destruction by in vivo-induced cytotoxic T lymphocytes (CTL) have been poorly understood.

Recent works have demonstrated that cytolytic molecules such as perforin [8, 9] and serine esterase [10, 11] might be involved in the lysis of tumor cells by CTL and NK cells. This exocytosis model, however, was hypothesized on the basis of the evidence that in vitro-cultured CTL lines expressed high amounts of perforin or serine esterase, which were released by antigenic stimulation [8 – 11]. No direct evidence for the expression of perforin has been demonstrated in the in vivo-primed CTL. Thus, the exact role of perforin in the in vivo-induced CTL has remained unclear.

To address this problem, we investigated the expression of perforin in the FACStar-sorted $CD8^+$ CTL against syngeneic T lymphoma cells using monoclonal antibody (mAb) against mouse perforin [12, 13]. The data described herein shows that the in vivo-induced $CD8^+$ CTL generated against tumor cells expressed significant amounts of perforin.

3. Materials and Methods

3.1. *Animals*

C57BL/6 mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). All mice were female and used at ages of 5 – 8 weeks.

Key words: CTL; Perforin; In vivo; Cytotoxicity

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3.2. *Tumor cells*

Moloney virus-induced MBL-2 lymphoma cells were maintained in ascites form in C57BL/6 mice. B16 melanoma cells were passaged by weekly s.c. injection in C57BL/6 mice. Tumor cells (MBL-2, RDM-4, YAC-1 and Meth A) were maintained in tissue culture by using RPMI1640 medium supplemented with glutamine, penicillin, streptomycin, HEPES buffer, 2-mercaptoethanol and 10% heat-inactivated FCS (UBC).

3.3. *In vivo generation of CTL against MBL-2 tumor cells*

C57BL/6 mice were immunized twice with intraperitoneal (i.p.) injection of mytomycin C (MMC)-treated MBL-2 cells (5×10^6 cells) as reported previously [7]. One week after the last immunization, the peritoneal exudate cells (PEC) were passaged through a nylon-wool column and used as in vivo-induced CTL. In some experiments, the nylon-passed CTL populations were further separated into CD8$^+$ and CD8$^-$ T cells by sorting with FACStar (Becton Dickinson). The detailed

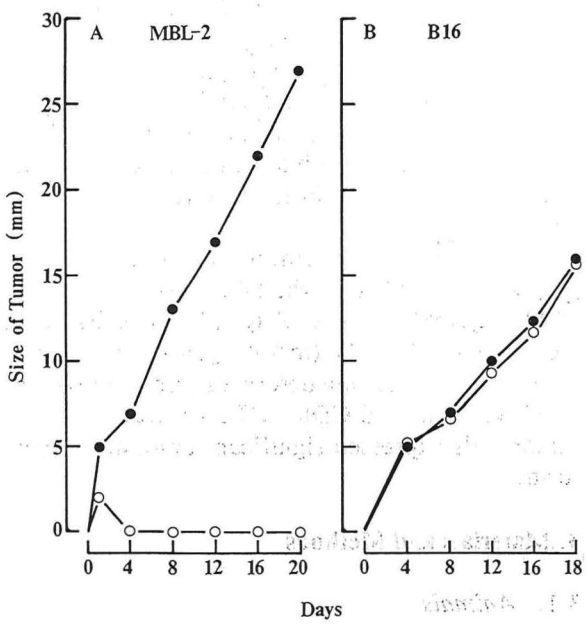

Fig. 1. MBL-2-immunized C57BL/6 mice reject MBL-2 lymphoma cells. Immune (○) and control (●) mice were challenged with 2×10^6 viable MBL-2 lymphoma (A) or B16 melanoma cells (B) by intradermal injection. Tumor growth was measured by two perpendicular diameters. Means of five mice per group were represented.

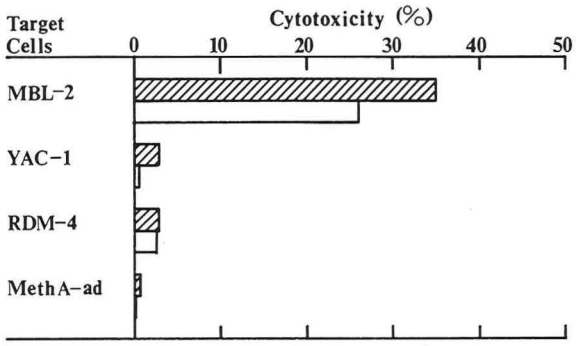

Fig. 2. Target specificity of in vivo-induced CTL against MBL-2. CTL were prepared from MBL-2-immunized mice PEC. The cytotoxicity was determined by 4 h ^{51}Cr-release assay. E/T ratio was 20:1 (hatched) or 10:1 (open).

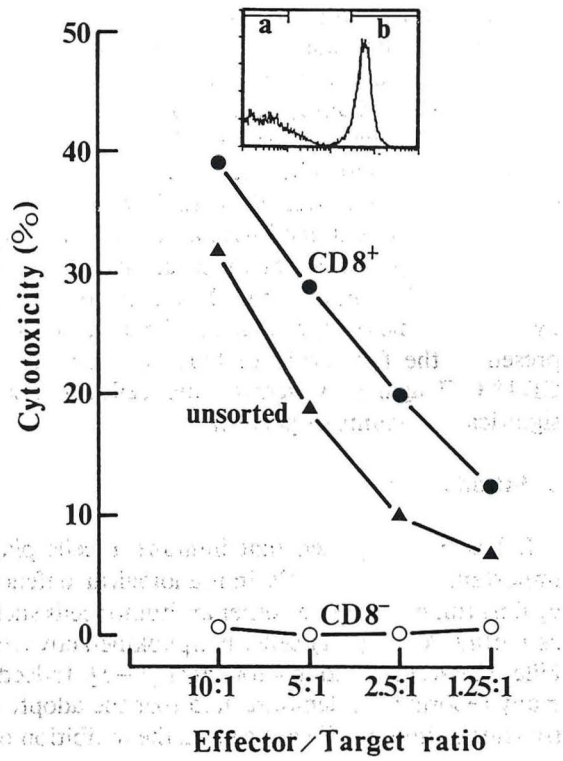

Fig. 3. CD8$^+$ T cells were responsible for the in vivo-induced CTL against MBL-2. CTL obtained from MBL-2-immunized mice were separated into CD8$^+$ and CD8$^-$ T cells. The cytotoxicity of unsorted (▲), CD8$^-$ (range a, ○), or CD8$^+$ (range b, ●) T cells was determined. Target: MBL-2 cells.

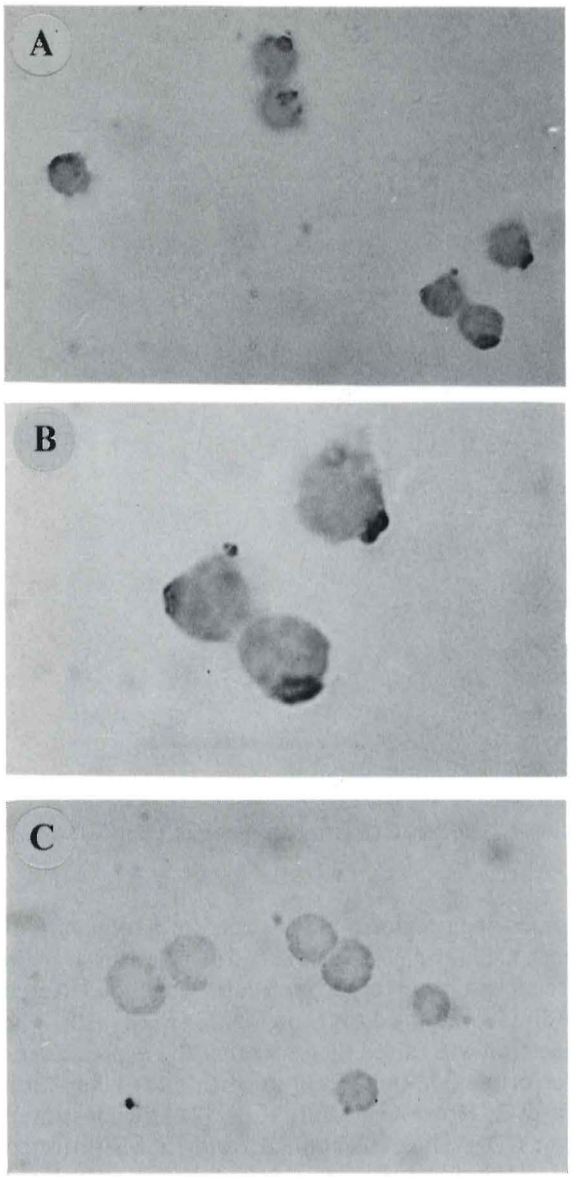

Fig. 4. Immunoperoxidase staining of $CD8^+$ populations with the mAb anti-perforin P1 – 8. (A) $CD8^+$ T cell populations. ×250; (B) $CD8^+$ T cell populations (enlargement of Fig. 4A). ×500; (C) $CD8^-$ T cell populations. ×250.

procedure for cell staining and sorting were described in a previous paper [20].

3.4. *Cytotoxicity assay*

The cytotoxicity was determined by 4 h ^{51}Cr release assay as described previously [11].

Percent cytotoxicity + (^{51}Cr release with effector cells – spontaneous ^{51}Cr release)/(maximum ^{51}Cr release with 0.1 M HCl – spontaneous ^{51}Cr release) × 100.

3.5. *Detection of cytoplasmic perforin with monoclonal antibody*

The characteristics of mAb against mouse perforin (P1 – 8) were described in previous papers [12, 13]. Single cell suspensions of CTL (10^5/100 μl) were deposited onto a glass slide by Cytospin 2 (Shardon Southern Products, U.K.) and dried in air. The slides were fixed with cold acetone for 3 min and then rinsed in PBS. After incubation with 10% sheep serum for 30 min, the slides were exposed to the anti-perforin mAb P1 – 8 for 1 h. After washing with PBS, the slides were incubated with peroxidase-conjugated sheep anti-rat IgG (Amersham) for 1 h. Finally, after extensive rinsing, the slides were developed with diaminobenzidine tetrahydrochloride (DAB 0.2 mg/ml). Electron microscopic analysis was performed using a JEM-100C (JEOL, Tokyo, Japan) as described previously [15]. The proportion of perforin positive cells was determined by counting positively stained cells per 500 cells.

4. Results

C57BL/6 mice immunized with MMC-treated MBL-2 cells were inoculated one week later with MBL-2 lymphoma or B16 melanoma cells. The MBL-2 lymphoma cells were rejected by the mice while B16 melanoma cells grew (Fig. 1). These results demonstrated the development of specific immunity against MBL-2 cells.

The nylon-passaged peritoneal exudate T cells obtained from immunized mice exhibited cytotoxic activity against MBL-2 but not against other tumor cells including the NK-sensitive YAC-1 (Fig. 2). To clarify the characteristics of the killer cells, we further separated the population into $CD8^+$ T cells and $CD8^-$ cells. The separated $CD8^+$ T cells showed higher cytotoxicity compared to unfractionated cells (Fig. 3). The $CD8^-$ cell populations had no significant cytotoxicity. The sorted $CD8^+$ T

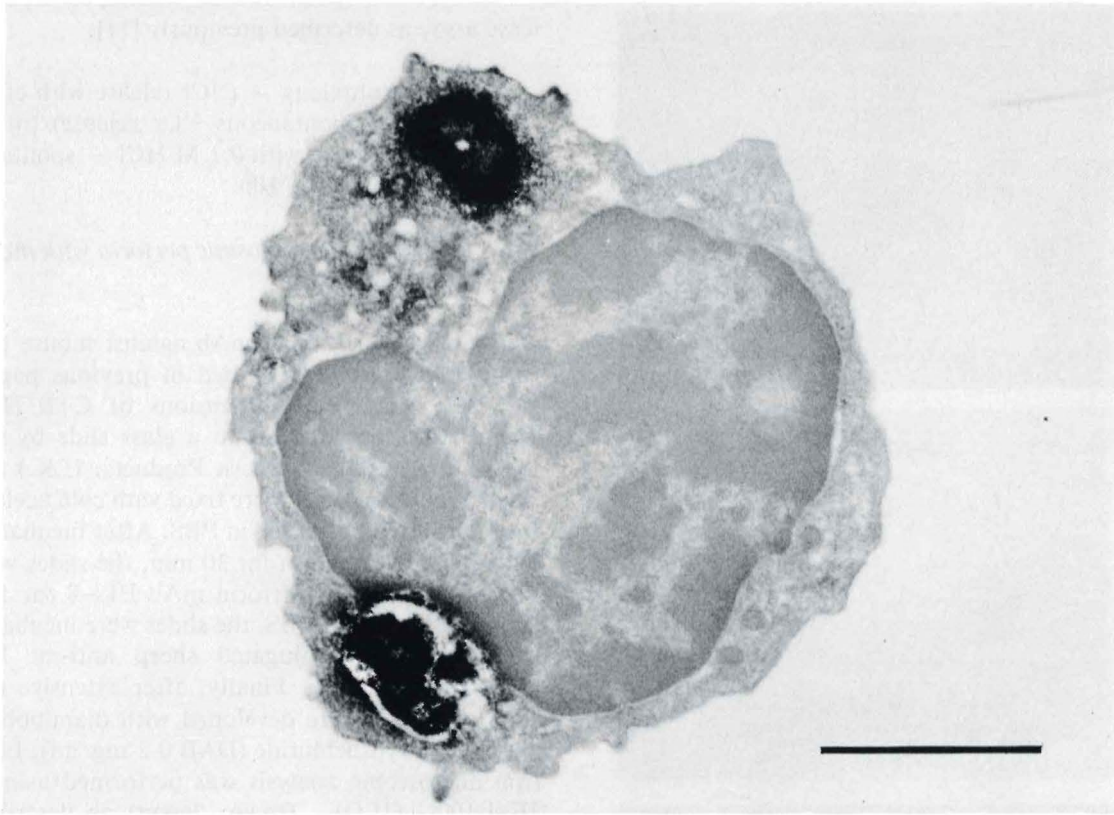

Fig. 5. Electron microscopic analysis of perforin expression in the in vivo-induced CD8$^+$ CTL. The bar represents 2 μm. $\times$15000.

cells were proven to be TCR $\alpha\beta^+$ CD3$^+$ by flow cytometry (data not shown).

Finally, we investigated whether the CTL expressed detectable amounts of perforin in the cytoplasm. Almost all of the CD8$^+$, but not the CD8$^-$ T cells, reacted with perforin specific antibody (Fig. 4). Data from three experiments demonstrated that over 98% of CD8$^+$ T cells were perforin positive, while the CD8$^-$ population contained less than 5% of perforin positive cells (data not shown). The electron microscopic analysis of the stained CTL demonstrated that the stained granules were localized in the cytoplasm, but not on the cell membrane (Fig. 5).

5. Discussion

We provide the first evidence that CD8$^+$ CTL generated in vivo against syngeneic tumor cells express perforin in their cytoplasms. It has been assumed that perforin plays a role in cytotoxicity of both CTL and NK cells [8, 9, 16, 17]. However, in recent results, cytolytic molecules were not detected in in vivo-induced CTL populations [18 – 20]. The question was raised therefore that the expression of perforin might only occur in vitro, due to induction by IL-2. However, Young et al. [21] demonstrated that CD8$^+$ T cells obtained from LCMV-infected mice expressed perforin, detected with polyclonal antibodies against perforin. Moreover, Nagler-Anderson et al. [22] reported that in vivo-primed CTL populations generated against alloantigens expressed the message for perforin. These results suggested that perforin might be an important cytolytic molecule, expressed in CTL populations generated in in vivo immunization systems. However, they could not show the relationship between the perforin expression and the cytotoxic function.

We induced specific CTL populations in vivo against syngeneic tumor cells. The CTL population

was represented by $CD8^+$ T cells and these cells expressed low but significant levels of perforin. Since P1 – 8 anti-perforin mAb reacts only with denatured perforin [12, 13], the cytotoxic activity of CTL was not blocked by it (data not shown). Therefore, a direct demonstration of whether the perforin in the CTL populations is involved in this function is absent.

Acknowledgements

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