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ORIGINAL ARTICLE

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Suppression of cytotoxic T lymphocyte activity by γ/δ T cells in tumor-bearing mice

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Abstract Spleen cells derived from tumor-bearing mice prove useful for the elucidation of the mechanism determining how tumor cells evade cytotoxic T lymphocytes (CTL) in tumor-bearing hosts. Our data indicate that inactive CTL or precursor CTL specific for tumor antigens are present among lymphocytes of tumor-bearing mice. However, their activity is inhibited by a soluble factor produced by other cells present in the same source. Inhibition of the cytolytic reaction was also detected in the culture supernatant of spleen cells obtained from normal mice, precultured in the presence of tumor cell culture supernatant and interleukin-2 (IL-2). Cell-depletion and cell-purification studies let us conclude that cells that produced the CTL-inhibitory factor (CTL-IF) were γ/δ T cells. The γ/δ T cells that were activated in vivo in tumor bearers were able to produce CTL-IF after isolation and in vitro culture. Maximum activation of γ/δ T cells was achieved by antigenic stimulation and by suppression of cells that interfered with the activation of γ/δ T cells. CTL-IF, which was assayed by use of CTL clones, did not show antigen specificity. Inhibition depended on a relatively heat- and acid-stable, but alkali-labile molecule with a molecular mass of less than 10 kDa. The latter characteristics imply that CTL-IF does not resemble any of the known lymphokines produced by γ/δ T cells. These observations emphasize the crucial role of the γ/δ T cells in the escape of tumor cells from the attack of tumor-specific CTL.

Key words γ/δ T cell · CTL · CTL-inhibitory factor · Tumor escape

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Introduction

The presence of tumor-associated antigens recognized by cytotoxic T lymphocytes has been demonstrated for various experimental tumor cells. However, the tumor cells of mice carrying tumor-associated antigens can be transplanted to normal syngeneic mice. The latter become tumor-bearing mice that apparently lack an immune reaction against proliferating tumor cells. Lymphocytes obtained from cancer patients were expanded by in vitro culture in the presence of interleukin-2 (IL-2) [lymphokine-activated killers (LAK) and tumor-infiltrating lymphocytes (TIL)] and used clinically for treatment of these patients [30, 32]. LAK and TIL contained tumor-specific cytotoxic T lymphocytes (CTL), which were predominantly of the $\alpha/\beta TCR^+$ and $CD8^+$ phenotypes. Therefore CTL precursors or inactive CTL were present among lymphocytes in tumor bearers and were activated and propagated during the in vitro culture. Although these cells exhibited a strong in vitro cytotoxic activity against tumor cells, treatment of patients with cells proliferated in vitro was sometimes ineffective. CTL transfused to tumor-bearing patients tended to cease proliferating and to lose their cytotoxic activity [2, 21, 26, 27, 31, 34]. It seems that either induction of CTL or cytolytic activity of CTL is suppressed in tumor bearers. It is therefore essential to elucidate the mechanism of such suppression.

To explain the suppression of T cell function in tumor bearers, four main mechanisms have been proposed: (a) suppression by soluble factors released by tumor cells [19, 22, 37, 39], (b) clonal expansion of the suppressor cells [9, 16], (c) deletion of tumor-specific T cells [43], and (d) unresponsiveness of T cells caused by reduced expression of major histocompatibility antigens and co-stimulatory molecules on tumor cells [13, 40]. Most of these studies revealed that suppression takes place during induction of the functional

T cells. With regard to the inactivation of CTL in tumor bearers, it was reported that a yet unidentified factor released by tumor cells, which was not transforming growth factor β (TGFβ), inhibited the cytotoxicity reaction by affecting the CD3-associated signal-transduction molecule of the effector cells [19, 23]. These findings, however, have not been fully utilized to abrogate suppression.

We have reported that tumor-associated Q5 antigen, which is a non-classical histocompatibility class I antigen encoded by the Q5 gene located in the Qa-2,3 gene region of the mouse chromosome 17, is expressed on the surface of various experimental tumor cells of mice [35]. The MM2 ascites tumor cell line, derived from C3H/He mice, is a $Q5^+$ immunogenic tumor cell line. Its ascites tumor showed regression simply after removal of the tumor cells with the ascites from the host animals at a certain stage of tumor progression [38]. After the regression the mice acquired immunity to transplanted MM2 cells. As shown in our report, Q5-specific CTL bearing α/β T cell receptor (TCR) and CD8 molecules were induced by in vitro stimulation of lymphocytes obtained from the tumor regressors. A low CTL activity was observed when lymphocytes derived from the tumor bearers were similarly stimulated in vitro. These findings suggested that the activity of Q5-specific CTL was suppressed in tumor-bearing mice while removal of most tumor cells from the host abrogated suppression. The mechanism that causes suppression of the CTL activity, related to this phenomenon, was studied in this report.

Materials and methods

Mice and tumor cells

C3H/He ($H\text{-}2^k$), C57BL/6 (B6) ($H\text{-}2^b$) and B6.K1 mice were used. B6 ($Qa\text{-}1^-2^+3^+$) and B6.K1 ($Qa\text{-}1^+2^-3^-$) are congenic with respect to Qa antigens [33]. BW5147 is an in vitro cell line derived from a T cell lymphoma of an AKR mouse. MM2, MM46 and MH134 ascites tumor cells were maintained by serial passages in the peritoneal cavities of C3H/He mice. MM2 and MM46 were derived from virally induced mammary carcinoma of C3H/He. MH134 was derived from a chemically induced hepatoma of C3H/He. EL-4 ascites tumor cells, a T cell lymphoma cell line derived from B6, was maintained by passages in B6 mice.

Antibodies, complement and reagents

The monoclonal antibody (mAb) 141-15.8, which is specific for the Qa-2 antigen, was obtained from the American Type Culture Collection, Rockville, Md. This mAb is cross-reactive to the Q5 gene product (the Q5 antigen) [35]. A hamster mAb, H57-597, with specificity for mouse T cell receptors of the α/β type (α/βTCR) and a hamster mAb GL3 with specificity to those of the γ/δ type (γ/δ-TCR) were purchased from PharMingen, Calif. Mouse mAb specific to $H\text{-}2K^b$ and $H\text{-}2D^b$ were purchased from the Meiji Institute of Health Science, Kanagawa. Rat mAb specific to mouse CD3 (KT3), CD4 (YTS191.1) and CD8 (YTS169.4) were purchased from Caltag Laboratories Inc., Calif. An affinity-purified goat antibody specific for hamster IgG and the flourescein isothiocyanate (FITC) derivative of the antibody were purchased from Organon Teknika Corp., Durham. Rabbit complement with low toxicity to mouse lymphocytes was purchased from Cedar Lane Laboratories, Westbury. Human recombinant interleukin-2 (rIL-2) was obtained from Shionogi Inc. Ltd., Osaka. Human recombinant transforming growth factor β (TGFβ) was purchased from Boehringer Mannheim GmbH, Mannheim.

Generation of CTL clones

Anti-Qa-2,3 CTL were induced by immunizing B6.K1 mice with biweekly intraperitoneal inoculations of 2×10^6 B6 lymphocytes. The spleen cells obtained from immunized mice were cultured at a density of 1×10^6 cells/ml in RPMI-1640 medium containing rIL-2 (50 U/ml), 10% fetal calf serum (FCS) and 10% rat concanavalin A supernatant and stimulated every 2 weeks with mitomycin-C-treated B6 lymphocytes at a concentration of 5×10^5 cells/ml. The rat concanavalin A supernatant was prepared by culturing rat spleen cells (2×10^6 cells/ml) in RPMI-1640 medium containing concanavalin A (5 μg/ml) and 10% FCS for 2 days. Four days after the last stimulation, the cells were harvested and used as anti-Qa-2,3 CTL. Cross-reactivity of these CTL to the Q5 antigen has been observed [28]. Q5-specific CTL clones were obtained from the anti-Qa-2,3 CTL by limiting dilution. Cytotoxic activity and the antigen specificity of each clone were determined by cytotoxicity assays using ^{51}Cr-labelled BW5147 target cells ($Q5^+$ and $Qa\text{-}2^-,3^-$) and by inhibiting the CTL activity using a Q5-specific mAb 141-15.8. Four Q5-specific CTL clones with high cytotoxic activity were established. All of these clones were shown to have $CD8^+$ and $\alpha/\beta TCR^+$ phenotypes. Anti-$H\text{-}2^b$ CTL were induced by immunizing C3H/He mice by biweekly repeated intraperitoneal inoculation of 2×10^6 B6 lymphocytes, followed by in vitro stimulation similar to that used for the induction of anti-Qa-2,3 CTL. $H\text{-}2D^b$- and $H\text{-}2K^b$-specific CTL clones were generated by limiting dilution of the anti-$H\text{-}2^b$ CTL. The cytotoxicity and antigen specificity of each clone was determined by the CTL assays using ^{51}Cr-labelled B6 lymphoblasts as the target cells and by inhibiting the cytotoxicity with $H\text{-}2D^b$- or $H\text{-}2K^b$-specific mAb. Two $H\text{-}2D^b$-specific CTL clones and 8 $H\text{-}2K^b$-specific CTL clones were established. All of these clones were shown to have $CD8^+$ and $\alpha/\beta TCR^+$ phenotypes by flourescence-activated cell sorter analysis (FACScan, Beckton Dickinson, California).

Purification of γ/δ T cells

Spleen cells were obtained from normal mice and from tumor-bearing mice 2 weeks after inoculation of MM2 cells. They were hemolysed with 0.17 M ammonium chloride [5] and the dish-adherent cells were removed by incubating the spleen cells in tissue culture dishes for 1 h in a CO_2 incubator. T cells were enriched from the cells not adhering to the dish by passing the cells through a nylonwool column. In order to separate γ/δ T cells, the nylon-wool-nonadherent cells were incubated with affinity-purified anti-(mouse γ/δTCR) hamster mAb GL3 for 30 min at 4 °C. The antibody-coated cells were incubated with anti-(hamster-IgG)-conjugated magnetic beads at a ratio of three beads per cell. Anti-(hamster-IgG)-conjugated magnetic beads were prepared by a standard method using tosyl-activated Dynabeads M-450 (Dynal Inc., Greatneck, N.Y.) and an affinity-purified goat IgG specific for hamster IgG. Cells bound to the magnetic beads were collected by using a magnet and cultured in a CO_2 incubator overnight to separate the cells from the beads.

Assay of cytotoxicity

Cytotoxic activities of the Q5-specific and the H-2K^b-specific CTL clones were assayed by incubating 1×10^4 ^{51}Cr-labelled BW5147 or EL-4 target cells, respectively, with the same number of effector cells in 0.4 ml RPMI-1640 medium supplemented with 10% FCS at 37 °C in a CO_2 incubator for 16 h. Anti-MM2 CTL were induced by culturing spleen cells from the MM2-bearing or the MM2-regressor mice for 5 days in RPMI-1640 medium supplemented with 10% FCS at a concentration of 2×10^6 cells/ml with the addition of mitomycin-C-treated MM2 cells at a concentration of 4×10^5 cells/ml. The activity of the CTL induced was assayed by incubating various numbers of the effector cells with ^{51}Cr-labelled MM2 target cells for 18 h. After the incubation, the radioactivity released into the medium and that remaining in the cells was determined. The percentage specific lysis was calculated as previously described [18].

Preparation of culture supernatant with CTL inhibitory activity

MM2, MM46, MH134 or EL-4 tumor cells were harvested freshly from the ascites. The cells were washed three times with Dulbecco's minimal essential medium (DMEM) using centrifugation at 1000 rpm for 10 s. The harvested cells contained more than 99% pure tumor cells. The cells were incubated overnight in DMEM supplemented with 10% FCS at a concentration of 2×10^6 cells/ml in a CO_2 incubator. The tumor cell culture supernatants (TCCS) were obtained by removing the cells through centrifugation at 1500 rpm for 5 min. The culture supernatant of MM2 cells was used as TCCS unless otherwise stated. Lymphocytes obtained from normal C3H/He mice were cultured for 10 days at a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with rIL-2 (50 U/ml), 10% FCS and in the presence or absence of 50% TCCS. The cultured cells were washed three times with DMEM and then recultured at a concentration of 2×10^6 cells/ml in RPMI-1640 medium supplemented with rIL-2 (50 U/ml) and 10% FCS for 3 days. The culture supernatants obtained from the recultures were used to determine the inhibitory activity in a cytotoxicity assay. The culture supernatants were designated as NSCS(10d) and NSCS(TCCS, 10d), indicating that they are culture supernatants of normal splenocytes precultured for 10 days in normal culture medium or in the presence of TCCS. The inhibition assay was carried out by adding the supernatant to the reaction mixture of the cytotoxicity assay of a Q5-specific CTL clone unless otherwise stated. When the γ/δ T cells separated from spleen cells of normal or MM2-bearing C3H/He were used, they were cultured either for 4 days or for 10 days in RPMI-1640 medium supplemented with rIL-2 (50 U/ml), 10% FCS with or without addition of 50% TCCS either in culture dishes coated with CD3-specific mAb (KT3) or in non-treated dishes. Mitomycin-C-treated spleen cells of C3H/He mice were added as feeder cells. Cultured cells were collected by centrifugation, washed and recultured for 1 day at a concentration of 4×10^5 cells/ml in the medium described above. The following culture supernatants were used: TBγ/δCS(4d), culture supernatant of tumor-bearer γ/δ T cells after preculture for 4 days in normal culture medium; TBγ/δCS(TCCS-CD3,4d), that of tumor-bearer γ/δ T cells after preculture for 4 days in the presence of TCCS and CD3 stimulation: Nγ/δCS(TCCS,10d), that of normal γ/δ T cells precultured in the presence of TCCS for 10 days, etc.

Characterization of the effective components in the culture supernatants

The approximate molecular masses of the supernatants's active components were estimated by ultrafiltration on a Microcon apparatus (Amicon, Mass). The expected cut-off molecular masses of YM3, YM10, YM30 and YM100 membranes are 3, 10, 30 and 100 kDa, respectively. The stability of the components in acidic and alkaline conditions was tested by adjusting the pH of the culture supernatants to 3 with 0.154 M hydrochloric acid or to 10 with 0.154 M sodium hydroxide. The solutions were kept at room temperature for 30 min and were then neutralized using sodium hydroxide and hydrochloric acid of the same concentration. The heat stability of the components was tested by treating the culture supernatants either at 50 °C for 30 min or at 100 °C for 5 min.

Results

CTL suppression detected in assays using MM2-induced effector cells derived from tumor-bearer spleens

Spleen cells, obtained from non-immunized C3H/He mice following tumor regression and cultured in vitro in the presence of mitomycin-C-treated MM2 cells as stimulation, elicited considerable cytolytic activity. Cytotoxicity was observed at all effector-to-target (E/T) ratios examined (Fig. 1B). The cytotoxic activity is supposed to be produced by CTL because it was inhibited strongly by an α/βTCR-specific mAb (H57-597) (data not shown). When the spleen cells, obtained from MM2-bearing C3H/He, were cultured similarly and used as effector cells in the cytotoxicity assays the CTL activity was observed only at low E/T ratios (Fig. 1A). In accordance with the increase of the number of effector spleen cells in an assay mixture, the activity decreased to an undetectable level. The experiments, which were highly reproducible, revealed two results. First, CTL can be induced from lymphocytes of the tumor bearers. Second, cultured spleen cells used as the

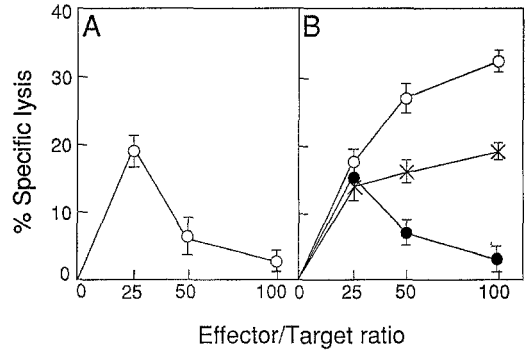

Fig. 1A, B Effect of effector/target (*E/T*) ratio on MM2-lytic activity induced by mouse spleen cells cultured in vitro in the presence of mitomycin-C-treated MM2 cells. Spleen cells were obtained from MM2-bearing (**A**) or MM2-regressor (**B**) C3H/He mice. Cytotoxic activities at various E/T ratios are shown. ○ Cytotoxic activity of the effector cells obtained by standard culture; ● serum from MM2-bearing C3H/He mice was added to the culture medium for induction of cytotoxic T lymphocytes (CTL) at a concentration of 10%; × serum from normal C3H/He mice was added similarly. Data are expressed as means ± SE of the results of duplicate experiments

source of the effector cells contained cells that were inhibitory to cytolytic reactions by the CTL. The ratio of the cytotoxic cells to the inhibitory cells in the effector cell mixture was identical, irrespective of the E/T ratios. Therefore a similar extent of suppression at various E/T ratios, and hence an increase of the cytotoxicity in accordance with the increase of E/T ratio, may be expected if the suppressive cells interact directly with the effector cells. Stronger inhibition at higher E/T ratios would be expected if the inhibition were mediated by a soluble factor (or factors) produced by the suppressive cells during the culture for the cytotoxicity assays. The reaction mixture at higher E/T ratios contains larger numbers of the suppressive cells. This would result in a higher concentration of the suppressive mediator and the mediator would exert the activity in a concentration-dependent manner. Since such a pattern of cytotoxicity was only observed when spleen cells from the tumor bearers were used, it is conceivable that the cells showing inhibitory activity were generated under the influence of tumor cells. Suppressive cells might have been activated by direct interaction with the tumor cells or by a soluble factor(s) produced in the tumor-bearing mice. The latter possibility seems more likely because effector cells exhibited a similar pattern of cytotoxic activity with stronger suppression in the presence of a larger number of the effector spleen cells when serum from an MM2-bearing C3H/He mouse was added to the culture of the MM2-regressor spleen cells to induce CTL (Fig. 1B). In a control experiment, addition of normal mouse serum to the culture resulted in a slight decrease of the cytotoxic activity even though strong suppression at high E/T ratios was not observed. These results suggest that a component (or components) in the tumor-bearer serum, which may be produced by tumor cells or by other cells under the influence of tumor cells, directly or indirectly activated the suppressive cells.

Production of the CTL-inhibitory factor (CTL-IF) by normal spleen cells cultured in the presence of tumor cell culture supernatant

We examined whether the component in the tumor-bearer serum that activated the suppressive cells was directly or indirectly produced by tumor cells. To this end, spleen cells obtained from normal mice were precultured in the presence of TCCS and recultured in its absence. The harvested culture supernatant [NSCS(TCCS,10d)] was added to the CTL assays. Q5-specific CTL clones and H-2K^b-specific CTL clones, all bearing α/βTCR, were used as the effector cells and Q5$^+$ BW5147 lymphoma cells and EL-4 (H-2^b) cells were used as the target cells. Inhibition of the activity of two representative CTL clones by NSCS(TCCS,10d) is shown in Fig. 2. The CTL activity of these clones and of other clones was inhibited in a dose-dependent manner. Activities of the Q5-specific CTL clones and the H-2K^b-specific CTL clones were inhibited to a similar extent, showing that the inhibition is not antigen-specific. Inhibition of the CTL activity was not detected in the reculture supernatant of 4-day spleen cell cultures, even though TCCS was added to the cultures. Maximum activity was only detected in 10-day cultures. The requirement for long-term culture to achieve activation implies that a cascade of events eventually leads to the final activation of the suppressive cells. Alternatively, effective suppression will only be detected if the suppressive cells expand. Spleen cells cultured for 10 days in the absence of TCCS did not mount a detectable CTL-inhibitory activity. Even the addition of TCCS directly to the CTL assays did not yield an inhibition (data not shown).

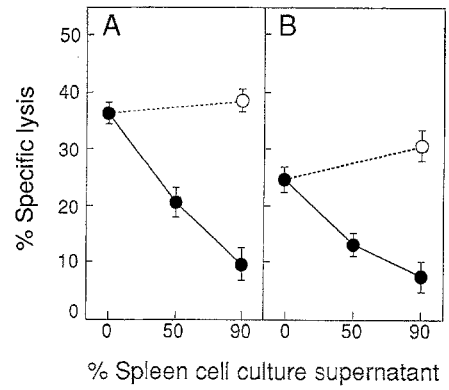

Fig. 2A, B Inhibition of cytotoxic activity by normal splenocyte culture supernatants cultured for 10 days in the presence of tumor cell culture supernatants [NSCS(TCCS,10d)]. The activity of a Q5-specific CTL clone (**A**) and that of an H-2K^b-specific CTL clone (**B**) was assayed using BW5147 and EL-4 target cells respectively. NSCS(TCCS,10d) (●) or NSCS(10d) (○) was added to the reaction mixtures of the cytotoxicity assays at various concentrations. The CTL assays were carried out at E/T = 1

We then examined the capability of various tumor cells to produce the CTL-IF-inducing material (Table 1). When ascites tumor cells (MM2, MM46, MH134 and EL4), maintained by in vivo passages, were freshly harvested from the hosts and cultured, the culture supernatant showed the CTL-IF-inducing activity. Suppression of CTL activity therefore seems to be a common phenomenon in tumor bearers. In contrast, supernatants of tumor cells cultured in vitro, such as BW5147, lacked the activity. Moreover, TGFβ did not replace the CTL-IF-inducing activity (data not shown).

As shown in Fig. 3, effector cells that had been pretreated with NSCS(TCCS,10d) showed a decrease of activity. Pretreatment of target cells did not have any effect. CTL-IF in the spleen cell culture supernatants therefore seems to exert its activity by affecting the effector cells rather than blocking or protecting the target cells.

Table 1 The cytotoxic-T-lymphocyte-inhibitory factor (CTL-IF)-inducing activity in the culture supernatants of various tumor cells. Fresh ascites tumor cells obtained from the respective tumor-bearing mice and purified by centrifugation were cultured for 24 h in order to produce tumor cell culture supernatants (*TCCS*). Normal splenocyte culture supernatant cultured for 10 days in the presence of TCCS[NSCS(TCCS,10d)] was prepared using each TCCS and assayed for the CTL-inhibitory activity. A Q5-specific CTL clone and BW5147 target cells were used for CTL assays (E/T = 1). NSCS(10d) or NSCS(TCCS,10d) was added to the assays at a concentration of 80%. Percentage inhibition was calculated from the percentage specific lysis detected in the presence of NSCS(TCCS,10d) and the control CTL activity in the presence of NSCS(10d). The values are means ± SE of the results of two experiments for each TCCS. The control CTL activities ranged between 50.6% and 82.8%

Tumor cells used to obtain TCCS	Inhibition (%)
MM2	65.6 ± 5.9
MM46	45.9 ± 4.3
MH134	43.1 ± 6.3
EL-4	38.0 ± 3.9
BW5147	1.6 ± 3.0

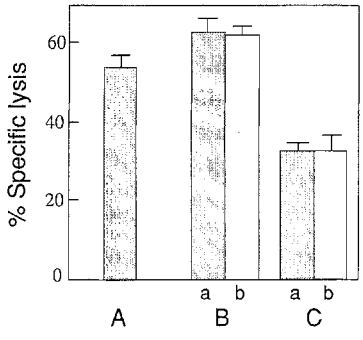

Fig. 3 Effect of pretreatment of effector or target cells with NSCS(TCCS,10d). A Q5-specific CTL clone and BW5147 target cells were used for the CTL assays (E/T = 1). *A* Control cytotoxic activity (neither effector nor target cells were pretreated), *B* the target cells were pretreated, and *C* the effector cells were pretreated. Pretreatment was carried out by culturing the cells either for 1 h (*a*) or for 6 h (*b*) in a medium containing NSCS(TCCS,10d) at a concentration of 80%. Cultured cells were washed three times with Dulbecco's minimal essential medium and used for the cytotoxicity assays

Identification of the cells that produce the CTL-IF

In order to analyze the phenotypes of the cells that produce the CTL-IF, we harvested spleen cells cultured in the presence of TCCS and treated the cells with various mAb and complement. The latter cells were recultured and the supernatant was tested for its ability to inhibit the cytotoxicity. Treatment of the cells with a CD3-specific mAb and complement or with a γ/δTCR-specific mAb and complement caused a significant decrease of the inhibitory activity. The production of the inhibitory factor persisted after treatment of

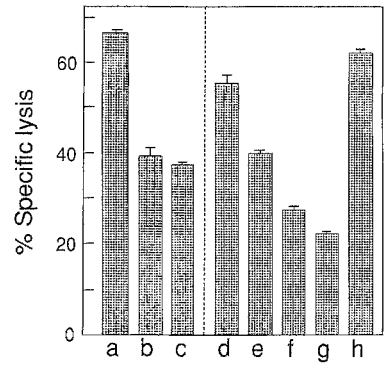

Fig. 4 Characteristics of the cells that produce the CTL-inhibitory factor (CTL-IF). Spleen cells obtained from normal C3H/He mice were precultured in the presence of TCCS for 10 days, harvested, treated with various mAb and complement and recultured for 3 days. The CTL-inhibitory activity was examined by adding culture supernatant to the CTL assay at a concentration of 80%. A Q5-specific CTL clone and BW5147 target cells were used for the CTL assays (E/T = 1). The culture supernatants added were as follows: *a* none (the control CTL activity), *b* culture supernatant of non-treated precultured cells, *c* culture supernatant of cells treated with complement only, *d* that of cells treated with CD3-specific mAb and complement, *e* that of cells treated with CD4-specific mAb and complement, *f* that of cells treated with CD8-specific mAb and complement, *g* that of cells treated with α/β T cell receptor(TCR)-specific mAb and complement, *h* that of cells treated with γ/δ TCR-specific mAb and complement

the cells with either the α/βTCR-specific mAb or the CD4-specific mAb or the CD8-specific mAb and complement. The results were reproducible and a representative example of them is shown in Fig. 4. These data suggest that the production of the inhibitory factor is dependent on $CD4^-$, $CD8^-$ T cells bearing γ/δTCR. This finding was confirmed by isolating γ/δ T cells from the spleen cells using a hamster mAb specific for mouse γ/δTCR and magnetic beads conjugated with a mouse anti-(hamster IgG) antibody. After the purification, more than 95% of the cells were of the $\gamma/\delta TCR^+$ phenotype and fewer than 3% were of the $\alpha/\beta TCR^+$ phenotype, as shown in Fig. 5. The recovery was 2×10^6 and 1×10^6 γ/δ T cells from 1×10^8 original spleen cells isolated from the MM2-bearing mice and normal mice, respectively. After culture of purified cells derived from the MM2-bearing mice, the suppressive activity was detected in the culture supernatants [TBγ/δCS(4d) and TBγ/δCS(TCCS,4d)]. The 4-day culture in the presence or absence of TCCS and reculture for 1 day in its absence was sufficient to produce a detectable CTL-inhibitory activity (Fig. 6A). These results show that the γ/δ T cells are activated in tumor-bearing mice and that they are capable of releasing CTL-IF. When the initial cultures were carried out in dishes coated with the CD3-specific mAb KT3, the inhibitory activities in the reculture supernatants [TBγ/δCS(CD3,4d) and TBγ/δCS(TCCS-CD3,4d)] were significantly enhanced, indicating that an antigenic stimulation of the γ/δ T cells via TCR is necessary

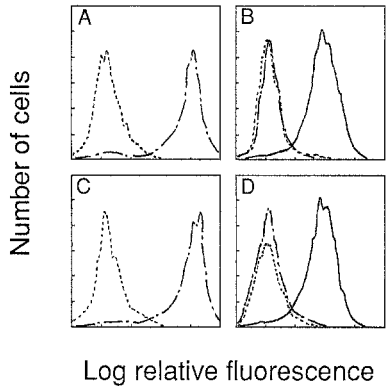

Fig. 5A–D Fluorescence-activated cell sorting (FACS) patterns of the γ/δ T cell fractions obtained from spleen cells of MM2-bearing C3H/He and normal C3H/He mice. The T-cell-enriched fraction obtained by passing cells not adhering to the dish through a nylon-wool column was subjected to a purification of γ/δ T cells using magnetic beads. **A** T-cell-enriched fraction of spleen cells from MM2-bearing C3H/He, **C** the fraction from normal C3H/He, **B** γ/δ T cell fraction from T-cell-enriched spleen cells of MM2-bearing C3H/He, **D** the fraction from T-cell-enriched spleen cells of normal C3H/He. The cells were stained using an α/βTCR-specific mAb (—·—) or a γ/δTCR-specific mAb (——) as the first antibody. A control without treatment with the first antibody is also shown (– – –). Fluorescein-isothiocyanate-conjugated goat anti-(hamster IgG) was used as second antibody

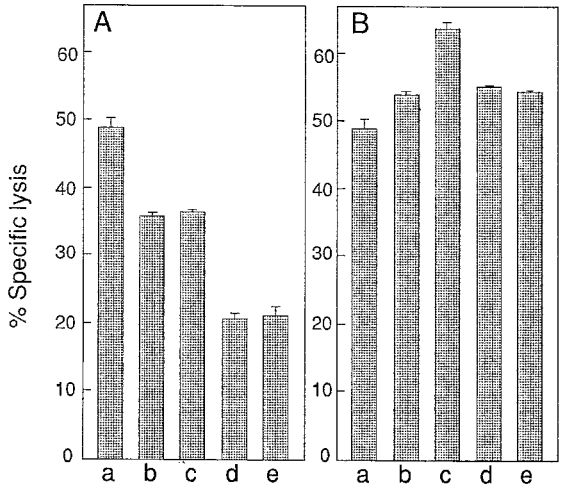

Fig. 6A, B Effect of the culture supernatants of γ/δ T cells purified from spleen cells of the tumor bearing or normal mice on CTL activity. The following culture supernatants were added to the CTL assays at a concentration of 80%. **A** *a* none (the control CTL activity), *b* TBγ/δCS(4d), *c* TBγ/δCS(TCCS,4d), *d* TBγ/δCS(CD3,4d), *e* TBγ/δCS(TCCS-CD3,4d). **B** *a* None, *b* Nγ/δCS(4d), *c* Nγ/δCS (TCCS,4d), *d* Nγ/δCS(CD3,4d), *e* Nγ/δCS(TCCS-CD3,4d). A Q5-specific CTL clone and BW5147 target cells were used for the CTL assays (E/T = 1)

for the maximal production of CTL-IF. However, culture supernatants of the purified γ/δ T cells obtained from normal spleen cells did not show any inhibitory activity as long as the precultures were carried out for

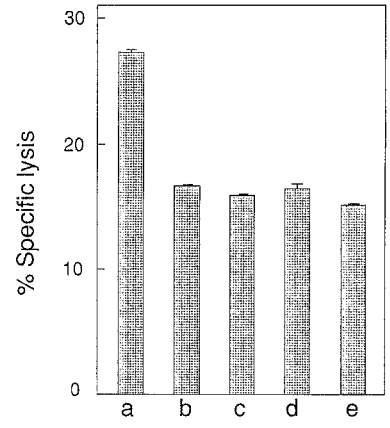

Fig. 7 Detection of the CTL-inhibitory activity in the culture supernatants of γ/δ T cells purified from spleen cells of normal mice and precultured for 10 days. The following culture supernatants were added to the CTL assays at a concentration of 80%: *a* none (control CTL activity), *b* Nγ/δCS(10d), *c* Nγ/δCS(TCCS,10d), *d* Nγ/δCS(CD3,10d), *e* Nγ/δCS(TCCS-CD3,10d). A Q5-specific CTL clone and BW5147 target cells were used for the CTL assays (E/T = 1)

4 days. Even culturing in the presence of TCCS and in dishes coated with mAb KT3 [Nγ/δCS(CD3,4d) and Nγ/δCS(TCCS-CD3,4d)] did not raise any inhibitory activity (Fig. 6B). Prolonged preculture, however, of the γ/δ T cells derived from normal spleen cells in the presence of rIL-2 but in the absence of TCCS and CD3 stimulation, resulted in a suppressive activity in the reculture supernatants [Nγ/δCS(CD3,10d) and Nγ/δCS(TCCS-CD3,10d)] (Fig. 7). Addition of TCCS to the culture medium did not have any effect in this experiment either. Compared to the results obtained from unfractionated normal spleen cells (Fig. 2), this finding suggests that the effect of the tumor-derived factor on the γ/δ T cells may be indirect and mediated by cells other than γ/δ T cells. We examined the ratio of γ/δ T cells by FACS analyses in the T cell fraction of normal spleen cells under CTL-IF-producing TCCS culture conditions. The ratio before culture was 1:19.6 and that after the 10-day culture was 1:28.5. The ratio in the T cell fraction obtained from tumor-bearer spleen cells was 1:11.9. Although the number of γ/δ T cells increased 1.6 times in tumor-bearing mice, in vitro propagation of the γ/δ T cells was not necessary for the maximal induction of the inhibitory activity. Therefore a cascade of activation events, including cells other than γ/δ T cells in the unfractionated spleen cells, may take place in the prolonged culture.

Characteristics of the CTL-IF

As shown in Table 2, some chemical characteristics of the CTL-IF were examined. The approximate molecular mass of CTL-IF in NSCS(TCCS,10d) was

Table 2 Chemical characteristics of CTL-IF. A Q5-specific CTL clone and BW5147 target cells were used to assay the CTL activity. NSCS(TCCS,10d) fractionated or treated as above was added to the CTL assays at a concentration of 80% as the source of CTL-IF. The values are means ±SE of the results of duplicate experiments. The control CTL activities ranged between 37.2% and 82.9%

Addition	Suppression (%)
NSCS(TCCS,10d) fractionated according to molecular size	
Unfractionated	79.2 ± 1.9
YM100-retained	5.6 ± 4.8
YM100-passed and YM30-retained	4.9 ± 1.9
YM30-passed and YM10-retained	13.9 ± 5.8
YM10-passed and YM3-retained	72.9 ± 2.1
YM3-passed	83.3 ± 0.5
NSCS(TCCS,10d) after treatment with acid or alkali	
Nontreated	79.2 ± 1.6
pH 3 at room temperature for 30 min	89.6 ± 0.5
pH 10 at room temperature for 30 min	27.8 ± 1.9
NSCS(TCCS,10d) after heat treatment	
Nontreated	65.2 ± 1.2
100 °C for 5 min	− 2.2 ± 1.1
50 °C for 30 min	74.8 ± 3.9

estimated by ultrafiltration. The activity was associated with materials that passed through YM100, YM30 and YM10 membranes. The activity was also detected in both YM3-passed and YM3-retained fractions. We conclude from these results that the molecular mass of the CTL-IF is below 10 kDa. The factor was stable at pH 3 but labile at pH 10. Although the factor resisted heat treatment at 50 °C, it lost its activity completely after treatment at 100 °C for 5 min. In addition, similar experiments were carried out with regard to the CTL-IF-inducing activity detected in TCCS. The molecular mass of the tumor-cell-derived factor seemed to be higher than 30 kDa. The activity was lost after exposure to either pH 3 or pH 10 or 50 °C (data not shown).

Discussion

It has been reported that the ratio of γ/δ T cells to lymphocytes in tumorous tissues is sometimes higher than in peripheral blood lymphocytes [17]. The increase of γ/δ T cells has been interpreted as an indication of an antitumor immune response because the γ/δ T cells that were recovered from the tumorous tissues and cultured in vitro in the presence of IL-2 killed tumor cells [1, 17, 44]. Concerning recognition specificities of the γ/δ T cells, it has been shown that γ/δTCR can recognize non-classical histocompatibility class I antigens such as murine Qa, TL and CD1c, directly or as restriction molecules [3, 4, 8, 12, 28, 29, 42] as well as heat-shock proteins presented by class I antigens [10, 15, 20, 41]. Antigen recognition by the γ/δ T cells either leads to proliferation of the γ/δ T cells and IL-2 production or lysis of the target cells. We have reported that the Q5 antigen, a member of the Qa antigens, is expressed on the surface of certain murine tumor cells as a tumor-associated antigen. Cytotoxic lymphocytes activated by immunopotentiators contain γ/δ T cells with specificity for the Q5 antigen [28, 35, 38]. Peptides derived from heat-shock proteins and presented by class I molecules on the surface of tumor cells are also recognized as tumor antigens by the γ/δ T cells. This observation suggests that some of the γ/δ T cells can recognize tumor cells and respond to them. In addition it was reported that γ/δ T cells play a role as immunomodulators by producing cytokines [6, 11, 24, 25]. The cytokines produced by γ/δ T cells include IL-2, tumor necrosis factor α, interferon γ and granulocyte macrophage-colony-stimulating factor. Moreover, Kaufmann et al. reported that in vivo depletion of γ/δ T cells by γ/δTCR-specific mAb caused vigorous proliferation and functional activation of α/β T cells in vitro [14]. The so-called cross-talk between α/β T cells and γ/δ T cells, implies a role for γ/δ T cells in the regulation of the α/β T cell functions. In the present report, we demonstrated that γ/δ T cells obtained from tumor-bearing mice produced a soluble material, CTL-IF, which regulated the activity of cytotoxic α/β T cells. The low molecular mass and relative heat- and acid-stability of this factor imply that the factor is different from any lymphokine known to be produced by γ/δ T cells. The enhancing effect of TCCS on the production of the CTL-IF was only observed with unfractionated spleen cells. The purified γ/δ T cells, however, were not affected by TCCS. The observation that the effect of the tumor-derived factor on the γ/δ T cells is indirect suggests that cells other than γ/δ T cells contained in the spleen cells suppress the CTL-IF release of γ/δ T cells while the activity of the suppressive cells is abrogated by the effect of the tumor-derived factor. Activation of γ/δ T cells in normal spleen cells may involve a series of events. Foremost, the activity of the cells that are inhibitory to γ/δ T cells would be abrogated, then the γ/δ T cells would recover from the suppression and eventually become activated. The sequence of these events may account for the time-consuming activation of the γ/δ T cells. With regard to the γ/δ T cells of tumor bearers, the suppression is inhibited in vivo under the influence of tumor cells. The activation of purified γ/δ T cells isolated from their spleen cells therefore proceeded faster. Though a 4-day culture in the presence of rIL-2 was sufficient to detect the production of the CTL-IF, activation of γ/δ T cells was accelerated by stimulation with CD3-specific mAb. If γ/δ T cells purified from normal spleen cells are cultured, further processes might be necessary for the activation. The 4-day culture period seemed too short to observe effects of these processes. After cells had been cultured for 10 days in the presence of rIL-2, production of the CTL-IF was detected. The prolonged

culture period appeared to result in maximal activation. Additional activation by stimulation with CD3-specific mAb was not observed.

Spaner et al. produced a line of TCR γ/δ double transgenic SCID mice [36]. The mice lack α/β T cells and have only γ/δ T cells with a known antigen specificity. When the mice were stimulated with the antigen, γ/δ T cells responded excessively but most of them subsequently died by apoptosis. Such a phenomenon was not observed after stimulation of a normal mouse, having both α/β T and γ/δ T cells, with the antigen. It is conceivable that the response of γ/δ T cells is suppressed in the presence of α/β T cells, and the cells that suppress the production of CTL-IF by γ/δ T cells might be α/β T cells. It seems that cross-talk between γ/δ T cells and α/β T cells mutually regulates their activity.

Stimulation of γ/δ T cells obtained from tumor bearers with a CD3-specific mAb accelerated the activation of the cells to produce CTL-IF. The activation of cells by antigenic stimulation through the TCR raises the possibility that these cells recognize specific tumor antigens. Indeed certain γ/δ T cells, mostly those with in vitro cytotoxic activity are tumor-antigen-specific. At present it is unclear whether the tumor-antigen-specific γ/δ T cells with the tumor-specific lytic activity are identical to those with CTL-IF-producing activity. It was reported that mature $CD8^+$ cytolytic cells, when activated in the presence of IL-4, switched to $CD8^-$, $CD4^-$ cells with T_H2 activity [7]. Likewise, it might be possible to convert the phenotype of γ/δ T cells that produce CTL-IF in tumor bearers to the phenotype of in vitro cultured and IL-2-supplemented cytolytic γ/δ T cells that display a recognition specificity similar to that of γ/δ T cells stimulated in vivo by immunopotentiators. The culture supernatant of the γ/δ T cells that inhibited the lytic activity of CTL clones also inhibited growth of the CTL clones (data not shown). Whether or not the two activities are the properties of a single factor remains to be solved by further chemical characterization of the CTL-IF.

Our present results indicate the involvement of γ/δ T cells in tumor-induced suppression of the immune responsiveness in tumor bearers. The real value of this observation is that the depletion of γ/δ T cells in vivo is a means to overcome suppression and to improve the efficacy of anticancer immunotherapy. At present, trials to test this possibility are in progress.

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