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ORIGINAL ARTICLE

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Cytotoxic effector T cells elicited by the killed tumor vaccine differ significantly from the effectors generated during active growth of a murine B-cell lymphoma

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Abstract Active specific immunotherapy of neoplastic diseases is an elusive goal. Using a murine B lymphoma 2C3, we showed that vaccination with the killed tumor cells effectively induces protective immunity and a cytotoxic T cell (CTL) response. Similar protection, however, is rarely observed in mice bearing live tumor cells. These animals usually succumb to the progressively growing tumor. In this study, we inquired whether the splenic CTL induced during tumor progression in mice differ from those evoked by the killed tumor cells. Here we demonstrate that the CTL generated following vaccination are significantly different from those induced in the tumor-bearing hosts. Adding to the complexity, the CTL from the early tumor bearers also differ significantly from those induced at the late stages. These differences are based on their cytotoxic activity, MHC allele specificity, mitogen responsiveness, cytokine secretion profile and T cell receptor Vβ gene expression. The results clearly indicate that passive immunization with killed tumor is most effective, possibly because the CTL induced are not subject to the same regulatory pressure as those induced during active tumor growth. This decreasing effectiveness of CTL could be due to greater variability in antigenic stimulus, less involvement of innate immunity, changes in cytokine milieu and/or costimulatory factors.

Key words Cytotoxic T cells · Vaccination · Cytokine profile · B lymphoma · TCR Vβ expression

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Introduction

Immune responses to progressively growing tumors involve a complex array of effector cells and biological responses modifiers. The success or failure of such responses is often dictated by antitumor cytotoxic T cells (CTL) that may or may not be induced to an effective level in the tumor-bearing animals. Nevertheless, antitumor CTL represent one of the most beneficial components of the immune response provoked in any preventive vaccination and adoptive immunotherapy protocols [3, 5, 10, 14, 15, 20]. In many studies involving animal models, tumor-specific CTL proved effective in preventing tumor growth and providing long-term protection [5, 6, 14, 20, 24]. Usually these therapeutic T cells are induced by immunization of naïve animals with the killed or modified tumor vaccines. They are also obtained from the tumor-bearers' infiltrating lymphocytes (TIL) following in vitro activation and cultivation [5, 10, 27]. The latter approach provides large numbers of syngeneic or autologous antitumor lymphocytes suitable for the treatment of patients in clinical settings [8].

Thus, the eradication of tumors by immunoprophylaxis seems a feasible concept. However, success has been limited to a few experimental cases [15, 21, 25, 27]. One possible reason is the lack of a clear understanding of the immunological processes associated with tumor progression. A recalcitrant or a metastatic tumor escaping radiotherapy, chemotherapy or surgical manipulations significantly increases the risk of recurrence and, under such circumstances, vaccination of these tumor-bearing hosts with a modified or attenuated tumor may prove detrimental or ineffective. The presence of active and viable tumors still remaining after regular therapy is likely to influence the course of immunological responses differently from what usually occurs following prophylactic immunizations in the absence of any viable tumor [15, 25]. This raises the question of whether the CTL populations from naive immunized hosts are functionally and phenotypically different from

those arising in tumor-bearing hosts, as has been noted in some earlier studies [8, 21, 27, 33].

Moreover, not all tumors, such as autochthonous tumors that arise spontaneously, are immunogenic and, even for some tumors that are antigenic, CTL activation does not occur in a predictable fashion particularly at the late stages of tumor growth [11, 35]. Nevertheless, at this late stage there is a clear evidence of concomitant immunity when the primary tumor grows progressively, and yet vaccination leads to a specific antitumor response, provoking rejection of a fresh challenge with the same tumor at a second site [28]. In this instance, tumor-specific CTL seem to function effectively against the incipient tumor, but not against the established primary tumor.

In a previous study, we showed that prophylactic immunization of naïve mice with the killed B lymphoma 2C3 induces both a tumor-specific CTL response and long-term protective immunity against the tumor [5, 27]. However, such protection is rarely observed after surgical removal of the palpable tumor if the host harbors actively growing tumor cells, and yet, under these circumstances, antitumor CTL may still be detectable. In tumor-bearing mice, specific CTL are induced initially but they tend to decline significantly with tumor progression, and these CTL from mice bearing early 2C3 tumors are therapeutically effective [27]. In contrast, those from the late stages of tumor growth show limited effectiveness even in conjunction with interleukin-4 (IL-4) [27].

Thus, in the majority of cases, active specific immunization does not necessarily eliminate the tumor or retard its growth in tumor-bearing hosts, and CTL induction, in the presence of actively growing tumors, is not invariably indicative of protective immunity [10, 15, 21, 25, 33]. It therefore seems worthwhile to ascertain the therapeutic effectiveness of the CTL harvested from tumor-bearing hosts. Using the 2C3 tumor model, we inquired whether CTL linked to protective immunity are different from those induced during tumor progression in vivo. The study shows that, on the basis of their specificity, mitogen and cytokine responsiveness, cytokine dependence and T cell receptor (TCR) Vβ diversity, the splenic CTL induced by vaccination differ significantly from those generated in tumor-bearing hosts. Furthermore, when a similar analysis is performed with the CTL from the mice bearing early and late tumors, a trend is clearly evident that reveals increasing heterogeneity but decreasing therapeutic efficacy among the effector populations.

Materials and methods

Animals

BALB/c mice from Harlan-Sprague-Dawley Inc. (Indianapolis) were bred and maintained in the animal facility of Indiana State University. Each experiment was performed following protocols written on the principles of laboratory animal care [23] and approved by the Institutional Animal Care and Use Committee of Indiana State University.

Cell lines and cell culture

A B cell hybridoma 2C3 (2C3E1) of BALB/c origin was used as the tumor model in this study [5, 10, 27]. This tumor is a plasmablast producing both membrane and secreted forms of phthalate-specific Ig (γ_1, κ) and expressing a unique idiotype as previously described [11]. The following cell lines were used as controls: 1BF7 [9], a syngeneic B cell hybridoma that secretes estriol-specific antibody that is idiotypically different but of the same isotype as that of 2C3 (γ_1, κ).

CTL line

In this study, we used a 2C3-idiotype-specific CTL line A102 that was isolated from the spleens of mice receiving repeated immunizations with irradiated 2C3 cells [5]. This long-term antitumor CTL line was maintained by cultivation at a density of 5×10^5 cells/well in the presence of 20 units IL-2, and 5×10^4 killed 2C3 cells as stimulators. If the line was not in use, it was stored frozen in liquid nitrogen after a few passages in vitro.

Monoclonal antibodies

Syngeneic hybridoma 2F7 producing anti-(2C3 Id) mAb was kindly provided by Dr. R. B. Bankert, Roswell Park Memorial Institute (Buffalo, N.Y.). This antibody, 2F7 (IgG1), specifically recognizes the idiotypic determinants of 2C3 Ig. We previously used this antibody both as hybridoma supernatants and as affinity-purified anti-(2C3 Ig) idiotype antibody to assess humoral and cellular anti-Id responses of mice to 2C3 tumor [5]. The mAb produced by 1BF7 (IgG1), described above, served as the control.

The following monoclonal-antibody-producing hybridomas, with specificity for anti-(T cell receptor αβ) anti-(TCR αβ) (HB 218, H57-597), anti-CD3 (ATCC CRL 1975), anti-Lyt2 (CD8) (TIB105, 53-6.72), anti-L3T4 (CD4) (TIB207, GK1.5), anti-K^d (HB159), and anti-D^d (HB102), anti-L^d (30-5-7), were obtained from American Type Culture Collection (Rockville, Md.). Anti-TCR hybridoma is a hamster cell line that recognizes all murine αβ receptors. Murine recombinant IL-4 and IL-10 were obtained from the Biological Response Modifiers Program, National Cancer Institute, Frederick, Md. Mouse interferon γ(IFNγ) and anti-IFNγ antibodies were obtained from NIAID Repository, Md. The cytokine assay kits were purchased from Endogen Inc. (Cambridge, Mass.).

Inoculation of tumor in mice

Mice were each injected intraperitoneally with 5×10^6 2C3 or control tumor cells in 0.5 ml phosphate-buffered saline. A group of at least five to ten mice was used in each experiment, as mentioned in the legends.

Preparation of splenocytes and in vitro stimulation of the effector cells

This was done as described previously [27]. Briefly, 1×10^7 splenocytes from normal or tumor-bearing mice were stimulated with 1×10^6 mitomycin-C-treated 2C3 tumor cells in 60-mm tissue-culture petri dishes (Falcon, Becton-Dickinson, Mountain View, Calif.) containing RPMI-1640 medium (Life Technologies, Grand Island, N.Y.) and 10% fetal bovine serum (Hyclone, Logan, Utah). The cells in the petri dishes were incubated at 37 °C in a modular incubator containing a gas mixture of 10% CO_2, 7% O_2 and the balance of N_2. Effector cells were then harvested from the culture on the 6th day of incubation, and washed twice by suspending in fresh RPMI-1640 medium containing 10% fetal bovine serum followed by low-speed centrifugation at 300 *g* for 10 min. After checking for viability, the cells were counted, adjusted to specific

cell number and then used in a ^{51}Cr-release cytotoxicity assay. Maximal cytotoxicity was observed following a 5-day stimulation with killed 2C3 cells.

Separation of T cell subsets from splenocytes by panning

This was done following the method described by Mage [18]. Brieflya, 100-mm sterile anti-(mouse Ig)-coated polystyrene tissue-culture plates (Falcon no. 3003) were used to pan splenocytes (2×10^8) in 5 ml RPMI-1640 medium. The non-adherent (Ig^-) splenocytes collected in tubes were then treated with a 1:10 dilution of anti-CD4 hybridoma culture supernatant on ice for 30 min. The cells were then washed and resuspended in RPMI-1640 medium at a concentration of 1×10^7 cells/ml, and added to an anti-Ig-coated petri dish. The process was repeated once more. Panned CD4 T cells were recovered by forceful pipetting. $CD8^+$ T cells were isolated likewise from the unbound splenocytes, using anti-CD8 monoclonal antibody.

^{51}Cr-release cytotoxicity assay

This assay was performed as described earlier [27]. Briefly, target tumor cells were labeled with 150 mCi ^{51}Cr (New England Nuclear Corp., Boston, Mass.) for 1 h at 37 °C in 5% CO_2. The washed labeled target cells and different amounts of the effector cells, in triplicate, were mixed in a total volume of 200 µl in 96-well, U-bottomed tissue-culture plates (Falcon) and then incubated for 4 h at 37 °C in 5% CO_2. The radioactivity of the supernatants was measured in a Beckman 5500 gamma counter. To measure the spontaneous release of ^{51}Cr, the target cells were incubated with 100 µl of medium only and, for maximum release, with 100 µl 1% NP-40 in PBS. Spontaneous release in each experiment was around 15%–20% of the maximum release. The percentage specific cytotoxicity was calculated as:

$$\frac{(\text{experimental release} - \text{spontaneous release}) \times 100}{\text{maximum release} - \text{spontaneous release}}$$

Cytokine ELISA assay

Cytokine assays were done using enzyme-linked immunosorbent assay (ELISA) kits for IL-2, IL-4, IFNγ and IL-10 from Endogen Inc., as described [27]. Briefly, the supernatants (50 µl) from $CD8^+$ T cells obtained after stimulation with mitogens, concanavalin A (ConA; Sigma, St. Louis, Mo.), or immobilized anti-CD3 (hybridoma 145-2C11) were mixed in 96-well plates with 50 µl the plate reagent (provided with the kits). After a 2-h incubation at either 37 °C or 4 °C, the wells were washed five times with wash buffer. Then the antibody conjugate was added to the wells, which were then incubated and washed. 3,3′,5,5′-Tetramethyl benzidine substrate (100 µl) was added to each well and the plate was incubated in the dark for 30 min. Then 100 µl stop solution was added and the absorbance was measured at 450–550 nm.

Mitogen-induced proliferation

A tritiated-thymidine-uptake assay was used to study the proliferative responses of splenic lymphocytes. Briefly, isolated lymphocytes (10^5 cells/well were stimulated in vitro with 10^4 mitomycin-C-treated 2C3 cells in a 96-well tissue-culture plate. After stimulation for 3 days, each well was treated with 5 µg/ml Con A. In some experiments, 96-well plates were coated with 10 µg/ml anti-CD3 antibody and blocked before the addition of 10^5 splenic T cells/well. After 48 h incubation, 1 µCi [^{3}H]dT (New England Nuclear) was added to each well and incubated for an additional 16 h at 37 °C in 5% CO_2. Subsequently cells were harvested on glass filters using a Skatron cell harvester, and the radioactivity was counted in a liquid scintillation counter.

Isolation of total cellular RNA and first-strand cDNA synthesis

Total RNA was isolated from $CD8^+$ CTL, using Trizol reagent (Life Technologies, Grand Island, N.Y.) and following the manufacturer's instructions. First-strand DNA synthesis was done as follows: 1 µg CTL-derived total RNA was heated to 70 °C for 5 min, chilled on ice and then treated for 1 h at 37 °C in a total volume of 30 µl with the following reagents: 0.5 mM all four dNTP, 100 pM random hexamer, 0.5 µl Rnasin (RNase inhibitor from Promega, Madison, Wis.), 200 units Superscript II reverse transcriptase (RT; Life Technologies, Grand Island, N.Y.), 5× RT buffer (250 mM TRIS/HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) and 0.1 M dithiothreitol. The synthesized cDNA was stored at −20 °C until assayed.

Labeling of oligonucleotide primer specific for TCR Cβ

The oligonucleotide primers for both constant and variable regions, described previously [1], were synthesized and purified at the Biopolymer facility of Roswell Park Memorial Institute, Buffalo, N.Y. The radiolabeling of the Cβ was done as described [1] using 1 mCi [γ-^{32}P]ATP (specific activity 7000 Ci/mmol; ICN Biochemicals, Costa Mesa, Calif.) and 100 pmol Cβ primer CTT GGG TGG AGT CAC ATT TCT C in a reaction containing 10 units T4 polynucleotide kinase (Promega, Madison, Wis.) and 10× T4 kinase buffer. After 30 min incubation at 37 °C, the reaction was stopped by heating at 90 °C, diluted to 1 pmol/µl in water and stored at −20 °C. The polymerase chain reaction (PCR) was then performed with the cDNA as the template, radiolabeled Cβ and 23 different Vβ primers as described [1]. Amplification was carried out for 35 cycles with a denaturation step of 1 min at 95 °C, annealing at 64 °C for 1 min and synthesis at 72 °C for 2 min.

Electrophoresis and auto-radiography

Samples of 5 µl PCR products were mixed the loading buffer (95% formamide, 0.25% bromophenol blue, 0.25% xylene cyanol) and were electrophoresed on a 40 × 34-cm 6% polyacrylamide/8 M urea gel in 1 × TRIS/borate buffer, pH 8.3, till xylene cyanol reached 3/4 of the gel length. The gel was then dried and exposed to autoradiographic film (Kodak, Rochester, N.Y.).

Statistical analysis

Statistical analysis of the data was performed by Student's *t*-test and the Kruskal-Wallis nonparametric one-way analysis of variance using SigmaStat, Jandel Scientific, Calif..

Results

TCR phenotype and MHC allele specificity of the cytotoxic effector cells from vaccinated and tumor-bearing mice

In a previous report, we showed that hyperimmunization of naïve mice with killed 2C3 tumor induced highly cytotoxic $CD3^+CD8^+$ $\alpha\beta^+$ and mostly idiotype-specific T cells in spleens [5]. In a subsequent report, we indicated that 2C3-tumor-specific $CD8^+$ CTL were also induced in spleens of tumor-bearing animals [27]. In this study, we investigated TCR and idiotype-specificity of these CTL from tumor-bearing mice. A ^{51}Cr-release cytotoxicity-inhibition assay in the presence of anti-($\alpha\beta^+$ TCR) monoclonal antibody (IgG) revealed (Fig. 1) that anti-tumor $CD8^+$ T cells from the mice bearing early and late

tumors were also $\alpha\beta^+$TCR, like those from vaccinated mice [5]. Interestingly, as we observed with the CTL lines and clones from hyperimmunized mice [5], the cytolytic activity of CTL from the tumor-bearing mice were also partially inhibited by syngeneic mAb anti-idiotypic antibody, 2F7 (Fig. 1). We used the same anti-Id mAb to characterize CTL from 2C3-hyperimmune mice in an earlier study [5]. Inhibition was about 10% when an unrelated mAb, 1BF7, was used as control. Thus, although the CTL from tumor-bearing mice were highly heterogeneous, a significant fraction of them seemed to be like the CTL induced by vaccination, and, however pardoxical this would appear, similar inhibition of T cell activity by antibody has been reported before [5, 7, 32].

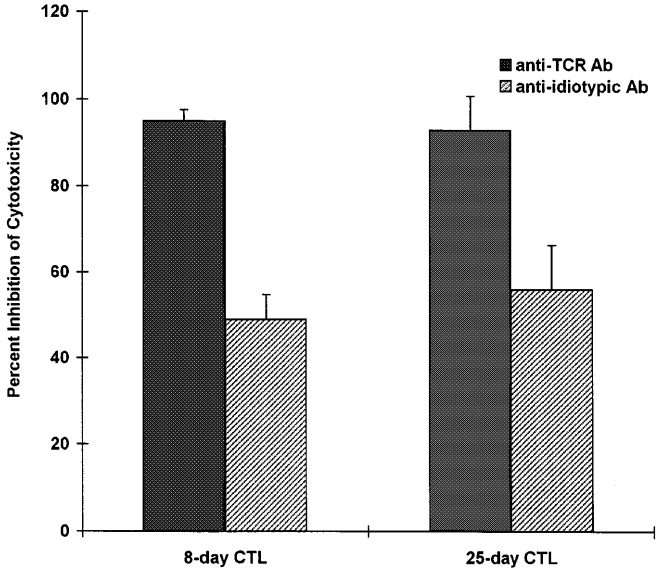

Fig. 1 Phenotype of splenic $CD8^+$ cytotoxic T lymphocytes (CTL) induced during tumor progression in mice. CTL were incubated with 10 μg/ml mAb to the T cell receptor (TCR) αβ or the anti-idiotypic mAb to 2C3 for 1 h at 37 °C and then mixed with ^{51}Cr-labeled 2C3 cells for a 4-h cytotoxicity assay. The percentage inhibition of cytotoxicity was about 10% when an unrelated mAb 1BF7 was used as control at 10 μg/ml. Data are representative of two different experiments involving three individual mice for each category and expressed as means ± SD of triplicate assays

MHC allele specificity of the effector CTL was also determined by the cytotoxicity-inhibition assay using K^d-, D^d- and L^d-specific monoclonal antibodies described in Materials and methods. Unrelated monoclonal antibodies MKD6 (isotype-matched) and TIB 120 (γ2b, κ) served as the control. The percentage inhibition of cytotoxicity by these control antibodies was around 17%–20%. The degree of heterogeneity among the CTL from the three experimental groups was clearly evident from the results in Fig. 2. The data indicate that, while the CTL response in tumor bearers was essentially directed to all three MHC alleles, those generated by prophylactic immunization were primarily K^d-specific and the CTL induced at the late stage were predominantly specific for K^d and L^d alleles.

Effects of mitogenic stimulus on proliferation and cytotoxicity of CTL

To characterize the $CD8^+$ T cells from the vaccinated as well as from the 2C3 tumor bearers, 5×10^5 splenic $CD8^+$ T cells, isolated by panning, were stimulated

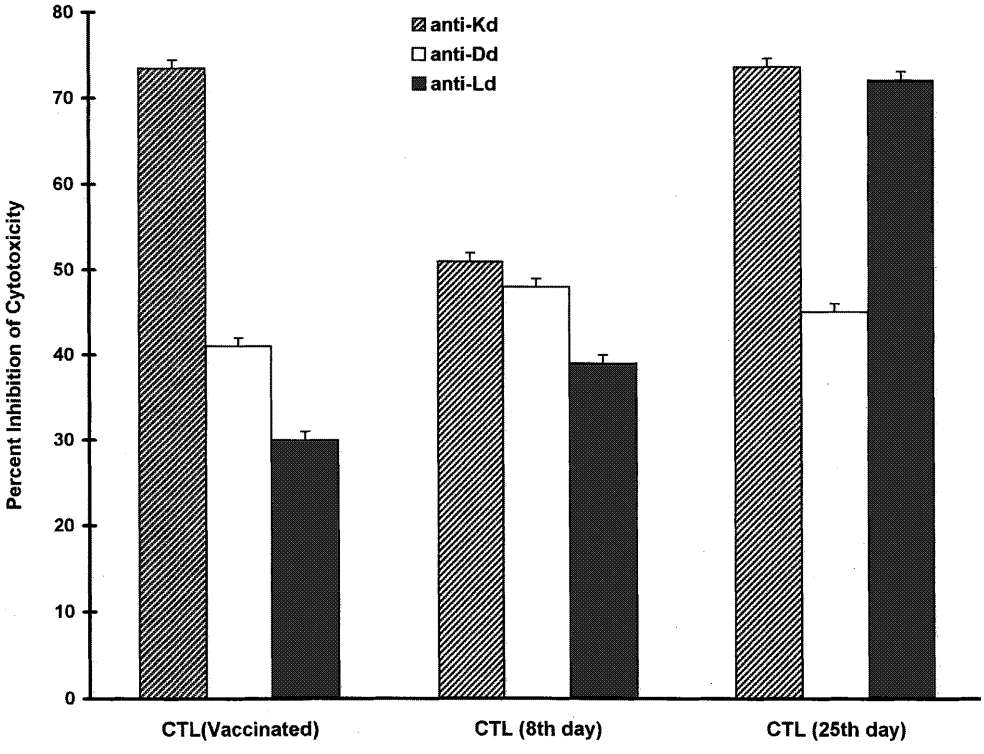

Fig. 2 Allele-specific MHC restriction of $CD8^+$ CTL from vaccinated and tumor-bearing mice. The percentage inhibition of cytotoxicity was based on cytotoxicity assays carried out using ^{51}Cr-labeled 2C3 cells and CTL from different experimental groups in the presence or absence of monoclonal antibodies specific for K^d, D^d and L^d alleles (10 μg/ml). Data are representative of two different experiments involving three individual mice for each category and expressed as means ± SD of triplicate assays

in vitro with ConA (5 μg/ml) in quadruplicate in 96-well plates [31]. Parallel groups were also stimulated with the plate-immobilized anti-CD3 monoclonal antibody as described [30]. After incubation for 48 h at 37 °C in a 5% CO_2 incubator, each well of the plate was treated with 1 μCi [^{3}H]dT and incubated for 16 h. It is evident from the data in Table 1 that the $CD8^+$ T cells from both the vaccinated group and the tumor bearers proliferated well in response to ConA as well as anti-CD3 antibody. However, the degree of [^{3}H]dT incorporation was significantly higher for the $CD8^+$ T cells from the tumor bearers. The overall uptake of [^{3}H]dT by the CTL line from the vaccinated group was quite low, possibly because this line, unlike the CTL that were freshly prepared from tumor-bearing mice, represents an enriched antigen-specific long-term population [5]. Interestingly, similar [^{3}H]dT uptake by antigen-specfic CTL has also been observed by others [16]. However, in terms of cytotoxic activity, only the CTL from the vaccinated group registered a precipitous decline after mitogenic stimulation, but not those from the late tumor bearers (Table 2).

Table 1 Effect of mitogenic stimulation on the proliferation of cytotoxic T lymphocytes (CTL) from vaccinated and tumor-bearing mice. $CD8^+$ T cells from the vaccinated (the CTL line) and tumor-bearing mice were stimulated in quadruplicate in a 96-well plate in a total volume of 200 μl containing 5×10^5 cells and 5×10^4 killed 2C3 cells for 3 days. To examine the effect of mitogens, one experimental group was treated with 5 μg/ml concanavalin A (*ConA*) and the other with anti-CD3 monoclonal antibody in plates coated with the antibody. The controls were run without ConA or anti-CD3. After 48 h, 1 μCi [^{3}H]dT was added in each well and incubated for another 18 h. The cells were harvested on glass filters using a Skatron cell harvestor, and thymidine incorporation was measured in a liquid scintillation counter. Results represent averages of three separate experiments each involving three individual mice in each category. For the CTL line, the results represent the average of three separate experimental results

Treatment	[^{3}H]dT incorporation (cpm)		
	CTL line (from vaccination)	CTL (8-day tumors)	CTL (25-day tumors)
Control	125.8 ± 38	6065 ± 1535	3896 ± 1869
ConA	415.6 ± 44.5	13 706 ± 2537	14 020 ± 7990
Anti-CD3	1008 ± 242	14 106 ± 3944	16 108 ± 9352

Effects of mitogenic stimulus on cytokine secretion

To assess whether these CTL differed on the basis of the cytokines they elaborated, their cytokine secretion profile was assessed before and after mitogenic stimulation. The results in Table 3 indicate that the CTL from all three experimental groups secreted primarily IFNγ, but they also secreted IL-4 at a lower concentration. Exposure to the mitogens, however, significantly affected this secretion profile with the vaccinated group registering a twofold increase in IFNγ secretion. In contrast, there was no significant change in IFNγ secretion by the CTL from the mice bearing either early or late tumors early after treatment with the mitogens. Furthermore, all three groups differed significantly in IL-4 secretion with the vaccinated group registering nearly a twofold increase after treatment with anti-CD3 antibody, and the tumor bearers showing about a four- or fivefold increase. However, the effects were quite different from those of the ConA-stimulated CTL from the late-tumor-bearing mice. Only this group registered any significant increase in IL-4 secretion after treatment with ConA.

Table 2 Effect of mitogenic stimulation on the cytolytic ability of CTL from vaccinated and tumor-bearing animals. CTL were stimulated in a total volume of 1 ml containing 5×10^6 cells and 5 μg/ml ConA in RPMI-1640 medium. For anti-CD3 stimulation the same number of cells were incubated in a 24-well plate coated with anti-CD3 monoclonal antibody. Controls consisted of CTL not subjected to Mitogens. After 48 h, the cells were harvested and their cytotoxicity was determined in a 4-h cytotoxicity assay against radiolabeled target 2C3 cells. Results represent the average of three separate experiments involving three individual tumor-bearing mice in each category per experiment. For the CTL line, the results represent the average of two separate experimental results

Treatment	Specific cytotoxicity (%)		
	CTL line (from vaccination)	CTL (8-day tumors)	CTL (25-day tumors)
Control	41	72 ± 5.9	23.5 ± 10.3
ConA	11.5	64.1 ± 8.1	22.18 ± 11.6
Anti-CD3	10.5	55 ± 18.5	12 ± 4.3

Table 3 Effect of mitogenic stimulation on interleukin-4 (IL-4) and interferon γ (IFNγ) secretion by the CTL from vaccinated and tumor-bearing animals. CTL were stimulated in a total volume of 1 ml containing 5×10^6 cells and 5 μg/ml ConA in RPMI-1640 medium. For anti-CD3 stimulation, the same number of cells were incubated in a 24-well plate coated with anti-CD3 monoclonal antibody. Controls consisted of CTL not subjected to mitogens. After 48 h, the supernatants were harvested and IL-4 and IFNγ secretions were measured by cytokine ELISA kits from Endogen Inc., Mass.

Treatment	CTL line[a] from vaccination		CTL[b] from 8-day mice with 8-day tumors		CTL[b] from mice with 25-day tumor	
	IL-4 (pg/ml)	IFNγ (ng/ml)	IL-4 (pg/ml)	IFNγ (ng/ml)	IL-4 (pg/ml)	IFNγ (ng/ml)
Control	177.05	13.64	567 ± 150	29.3 ± 2.01	188.1 ± 32.9	29.365 ± 2.01
ConA	222.3	28.35	500.5 ± 33.47	34 ± 1.72	986 ± 77.5	34.207 ± 1.7
Anti-CD 3	309.7	28.41	2225 ± 122.9	31.7 ± 3.47	719 ± 80	31.912 ± 3.47

[a] Average of two separate experimental results
[b] Results of three separate experiments involving three tumor-bearing mice in each category per experiment

TCR Vβ gene expression in the anti-2C3 CTL

To determine TCR Vβ usage in CTL induced against killed as well as actively growing 2C3 tumors, TCR Vβ repertoire expression was analyzed by RT-PCR and primers specific for the Vβ gene subfamily, using RNA isolated from $CD8^+$ splenic T cells from vaccinated or tumor-bearing mice.

Extracted RNA was reverse-transcribed to cDNA, which was amplified using the radiolabeled constant-region primer and each of the 23 primers specific for the Vβ subfamily. The PCR products were analyzed in denaturing polyacrylamide/urea gel followed by autoradiography. Various TCR Vβ subfamily cDNA were seen in the form of bands as described [1]. It is also evident from the autoradiographs (Fig. 3) that the CTL population from the vaccinated animals displayed strong bands corresponding to subfamilies Vβ 9 and 10 and very weak bands corresponding to Vβ 1 and 11. The TCR Vβ profile of CTL from early-tumor bearers corresponded with the strong expression of Vβ 1, 9, 10, 11, and 12, with relatively weak expression of Vβ 6. A similar study with the CTL from the late-tumor bearers indicated amplifications of Vβ 1, 6, 9, 10, 11, 12, 14, 15, and 16, with Vβ 1 showing weakest expression. Normal BALB/c splenocytes usually expressed all but the unexpressed pseudogenes, namely Vβ 5.3, 17, 19 and the superantigen-deleted subfamily of Vβ 3 and 20 [1]. Thus, it appears that the CTL from the vaccinated animals had the most restricted expression of TCR Vβ, and those from the animals bearing late tumors the least. It is noteworthy that Vβ 1, 9, 10 and 11 expression was common in all cases, possibly because they represented the shared components in Vβ gene usage for both regular and active processes of immunization.

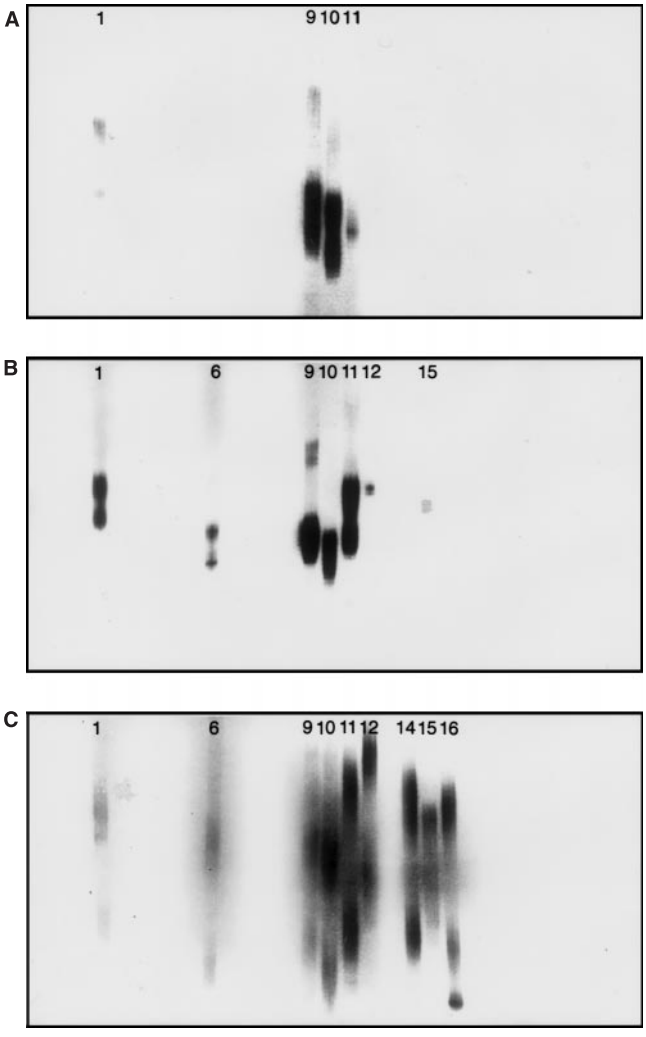

Fig. 3A–C TCR Vβ gene expression in anti-2C3 $CD8^+$ CTL from vaccinated and tumor-bearing mice. Total cellular RNA was reverse-transcribed into cDNA, and then amplified for 35 cycles, using each of 23 different Vβ-specific primers and a radiolabeled Cβ primer. **A** Autoradiograph of CTL cDNA from the vaccinated mice indicating amplification of TCR Vβ genes 1, 9, 10 and 11. **B** Autoradiograph of CTL cDNA from the early-tumor bearer indicating amplification of TCR Vβ genes 1, 6, 9, 10, 11, 12 and 15. **C** Autoradiograph of CTL cDNA from the late-tumor bearer indicating amplification of TCR Vβ genes 1, 6, 9, 10, 11, 12, 14, 15 and 16

Discussion

In this study, three potential splenic sources of therapeutic CTL, namely the vaccinated or hyperimmunized group, and the early- and late-tumor-bearing mice, have been evaluated. The issue we have addressed relates to defining their phenotypic and functional characteristics in terms of mitogen sensitivity, MHC allele specificities and TCR Vβ usage. It appears that the antitumor $CD8^+$ CTL elicited in hyperimmunized mice, and those evoked during active growth of a B lymphoma 2C3, represent overlapping as well as distinct populations of the effector T cells, and that the CTL from the tumor-bearing mice undergo significant phenotypic and functional changes during tumor progression. Obviously the assessment of such differences is important in determining their therapeutic efficacy and usefulness.

We previously observed that, while the vaccination-induced CTL promote effective protection against tumor growth, those induced in the tumor bearers are effective only at the initial stages of tumor growth. The $CD8^+$ effector T cells from the late stages of tumor growth are, in contrast, critically dependent on IL-4 for their cytotoxic activity and, even in combination with IL-4, they prolong, but do not provide comparable long-term survival of tumor-bearing mice, as the vaccination-induced effector T cells do [27].

Here we further delineate the differences among the three types of antitumor effector T cell, which are all 2C3-specific and $\alpha\beta^+$ $CD3^+$ $CD8^+$ T cells [5, 27]. The extent of their heterogeneity is clearly evident from the analyses of MHC allele specificity, responsiveness to mitogen, and TCR Vβ usage. Clearly the CTL from the vaccinated group show relatively restricted diversity as their response is primarily K^d-specific. On the other

hand, the CTL from the tumor-bearing animals are more heterogeneous and do not represent any dominant population recognizing any specific MHC allele. Clearly this suggests that there is a shift in the antigenic profile of the tumor during active growth and that this is reflected in the involvement of more than a few major CTL epitopes defined by different MHC alleles. The observations of Chang et al., who reported changes in antigenic specificity among the effector T cells in tumor-bearing mice but not in the immunized group, lend support to this contention [6].

Inhibition of cytotoxicity of the CTL from the tumor-bearing mice with syngeneic anti-idiotype mAb, though paradoxical, parallels our previous finding with the CTL lines and clones from 2C3-vaccinated mice [5]. This is, however, not without precedent, as others have reported similar inhibition as well [7, 32, 34]. As a possible explanation, it has been suggested that there may be overlap between B and T cell epitopes [7]. Furthermore, although the optimal binding of MHC class 1 usually occurs with peptides of 8 or 9 amino acids, those spanning 15–20 residues may also bind, albeit with a lower affinity [22]. As the longer peptides bind, they tend to bulge out in the middle [12]. Using a 2C3 idiotype-transfected P815 cell line (H-2^d mastocytoma), we are currently investigating whether a similar bulging of the idiopeptide would account for the inhibition of CTL activity mediated by the anti-Id mAb.

The CTL from the vaccinated and tumor-bearing animals are also distinguished on the basis of how they respond to stimulation with ConA and anti-CD3 antibody. We observe that the CTL from the vaccinated group take up the least amount of tritiated thymidine, and their cytolytic activity also declines by about 70%. Similar findings have also been reported by others [31]. In contrast, the cytolytic activity in the early-tumor bearers remains relatively unchanged and, at the late stages, the CTL activity declines significantly in response to anti-CD3 antibody. It is known that ConA exerts inhibitory effects on antigen-specific lysis by uncloned CTL [30, 31], and this appears to be the case with the CTL from the vaccinated animals. Sitkovsky et al., suggested that ConA blocks CTL activity by interfering with CTL surface structures that are critically involved in the recognition or lysis of target cells [2]. The inhibitory effects of anti-CD3 monoclonal antibody on antigen-specific lysis are possibly due to competition for the TCR receptors that are involved in polyclonal activation and also in CTL–target interaction. However, this does not explain why there is relatively little change in cytolysis by the CTL of tumor bearers after exposure to these mitogens. One likely explanation is that a tumor growing actively may compromize the activities of antigen-presenting cells which, as contaminants with the enriched $CD8^+$ T lymphocytes, may limit the access and action of mitogens on T cells [2, 13, 29].

Differential effects of exposure to mitogens are also evident in the cytokine secretion profile of these T cells. While all three groups respond similarly to the activation stimuli by secreting IL-2 [27], the CTL from the vaccinated group are primarily IFNγ secretors, secreting twice as much as the unstimulated control. Production of IL-4 by these cells is significantly low and few changes are seen upon stimulation. By contrast, the CTL from both groups of tumor bearers register few changes in secretion of IFNγ, but a significant increase in that of IL-4. For the early-tumor-bearing animals, stimulation with anti-CD3 is clearly more effective and similar to what we previously observed following antigen-specific stimulation [27]. Furthermore, compared to the CTL from 25-day-tumor-bearing animals, the 8-day CTL secrete six- and tenfold more of IFNγ and IL-4 respectively after antigen-specific stimulation for 48 h [27]. Thus, although these CTL are all $CD8^+$, $\alpha\beta TCR^+$, and are directed against 2C3 tumor, they represent phenotypically and functionally distinct and heterogeneous groups, and this explains the differences in their therapeutic potential.

The analysis of Vβ expression in these effector cells permits further delineation of the nature of specific CTL induced in the vaccinated as well as tumor-bearing mice [4, 17, 19, 26]. The issue is whether tumor progression or regression in a syngeneic system is accompanied by the recurrent presence of any specific CTL population characterized on the basis a specific TCR Vβ chain rearrangement. Using a murine mastcytoma P815, Levraud et al., observed two recurrent T cell clones amidst an array of diverse T cells infiltrating the tumor [17]. Casanova and Maryanski observed restricted usage of TCR in the case of MHC-presented self-antigens [4]. There are other reports demonstrating conserved intra-individual TCR-variable-region segments in melanoma patients [19]. Since we are not aware of similar studies on Id-specific splenic CTL, we ask whether there is a predominance of one or more CTL clones, characterized in terms of TCR Vβ expression, in the vaccinated as well as in tumor-bearing mice. Certainly the former exhibit very restricted heterogeneity, displaying primarily the usage of Vβ 9 and 10 subfamilies and considerably weaker bands corresponding to Vβb 1 and 11. But the TCR Vβ profiles of the CTL from tumor-bearing animals also include the subfamilies mentioned, and many others. Indeed, Vβ 9 and 10 represent the most dominant "public" TCR Vβ gene usage in both vaccinated and tumor-bearing mice. Interestingly, the CTL from the early-tumor-bearing mice that are highly effective in adoptive immunotherapy closely resemble those of the vaccinated group in Vβ gene usage, although they include CTL of the TCR Vβ 6 and 12 subfamilies. In contrast, those of the late stages are very diverse, coinciding with the likely changes in antigenic profiles that accompany successful and actively growing tumors. It is, however, important to note that, even at the late stage of tumor growth, 2C3-specific public CTL responses are still represented by the TCR cDNA band clusters present in the Vβ 1, 9, 10 and 11 subfamilies. Although the $CD8^+$ T cells represent a heterogeneous population, including some that may not be directed against the tumor, the relatively restricted response is

clearly an indication that a specific T cell population was induced by the tumor, killed or active.

The various cDNA for the TCR Vβ gene subfamily are obtained in the form of clusters or broad bands by the RT-PCR technique, as has been reported by others focusing mostly on the overall T lymphocyte response rather than on tumor-specific $CD8^+$ CTL [1, 26]. Dealing with the latter, the present study reveals significant differences even within the same Vβ subfamilies in all three experimental groups described here. The CTL from the vaccinated group exhibit more than one band for the Vβ 10 gene, suggesting the involvement of different D or J gene segments. Similar subfamily heterogeneity is also observed in the Vβ 11 gene of CTL from the early-tumor bearers, and in Vβ 11, 12, 14 and 16 genes of CTL from late-tumor bearers. Investigation is underway to determine if this heterogeneity occurs because of size differences of the CDR3 region resulting from various V-D-J or V-J combinations, as has been suggested [1, 26].

The mechanisms responsible for the long-lasting tumor-specific protection observed after vaccination with the killed or attenuated tumor cells, but not when the live tumor grows in vivo, are not understood well [3, 10, 33]. It is likely that, with the killed tumor as vaccine, the host encounters a relatively limited pool of antigens but with considerable involvement of innate immunity. The latter not only processes the dominant antigens present in the killed tumor, but also provides a cytokine milieu that is conducive to induction of oligoclonal CTL responses. This possibly happens in the early stages but not the late stages of the tumor growth. However, the CTL induced at the late stages could become therapeutically more effective in the presence of cytokines, such as IL-4 in the case of 2C3 tumor [27]. Whether this prophylactic application of IL-4 and other cytokines produces a change in the profile of tumor antigens, or in their processing and presentation by the innate immune components is not understood. It also remains to be addressed whether, in the tumor-bearing hosts, cytokines such as IL-4 promote a relatively restricted CTL response dominated by a few dominant TCR Vβ subfamilies, similar to those observed following vaccination with killed 2C3 tumor.

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