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Emergence of a dominant cytotoxic T lymphocyte antitumor effector from tumor-infiltrating cells in the anterior chamber of the eye*

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Summary. Previous studies in mice revealed that resolving intraocular tumors (UV5C25 fibrosarcoma) were infiltrated with mononuclear cells and invoked potent systemic delayed-type hypersensitivity responses without nonspecific tissue destruction. The present study characterized the tumor-infiltrating lymphocyte (TIL) population and established its role as the mediator of specific intraocular tumor rejection. This was accomplished by (a) isolating TIL from resolving intraocular tumors; (b) identifying characteristic surface markers on TIL; and (c) demonstrating in vitro and in vivo antitumor functions. Fluorescence-activated cell sorter analysis of TIL showed 33.4% $Thy1^+$, 19.8% $CD8^+$, 11.1% $CD4^+$, 17.2% $MAC-1^+$, 10.4% $F4/80^+$, and 7.7% $B220^+$. Functional studies indicated that TIL were directly cytolytic for UV5C25 tumor cells. Additionally a tumor-necrosis-factor(TNF)-sensitive cell line (WEHI 164.1) was lysed on cocultivation with TIL, whereas UV5C25 tumor cells were insensitive to lysis by TNF. Precursor CTL analysis demonstrated a high frequency (1/251) of tumor-specific precursors and a low frequency of alloresponsive cells in the TIL population. In vivo analysis by a Winn-type assay demonstrated that only TIL could effect tumor resolution in immunosuppressed hosts. These results demonstrate that although $CD4^+$ T cells and macrophages were present and TNF activity was detected in the TIL population, there was no evidence for nonspecific tissue destruction within the eye. Therefore, this pattern of intraocular tumor rejection is mediated by a lymphocyte population expressesing cell-surface phenotypes and functional characteristics of conventional cytotoxic T lymphocytes. Moreover, the results suggest that a regulatory mechanism within the eye allows for the emergence of one dominant antitumor effector (CTL) while controlling a more destructive mechanism (delayed-type hypersensitivity).

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Introduction

The observation that lymphocytes frequently infiltrate tumors may indicate immune recognition and antitumor activity by the host. However, the prognostic significance of the presence of tumor-infiltrating lymphocytes (TIL) is disputed [21, 50]. Consequently, the identity and function of these cells have been intensely studied. Tumor-infiltrating lymphocytes isolated from human tumors demonstrate various functional properties. Some investigators have shown that freshly isolated TIL demonstrate poor cytolytic activity [20, 32, 49, 52], whereas others have shown that a small percentage of TIL isolates are capable of lysing autologous tumor [53, 57]. Moreover, experiments determining the presence of precursors for cytolytic cells in the TIL population have shown an elevated frequency of antitumor effectors [16, 51, 58]. Taking these points into consideration, it has been suggested that the in situ tumor environment is inhibitory for lymphocyte functioning [1, 14, 28, 30, 33, 52]. Nevertheless, antitumor effectors can be generated from TIL expanded in vitro [31, 34, 60] and animal studies have demonstrated their potential therapeutic efficacy in tumor treatment [43].

Animal models representing the rejection of a syngeneic tumor often utilize highly immunogenic tumors which spontaneously regress following transplantation. Although human tumors are frequently weakly antigenic, these animal models can provide insight into potential host-tumor relationships (reviewed in [26, 56]). In resolving virally induced sarcomas [18, 41] and ultraviolet light-induced fibrosarcomas [23] a predominate mononuclear cell infiltration is observed. Furthermore, the TIL population obtained from immunogenic tumors contains a high frequency of precursor cytolytic T lymphocytes [7]. Even so, other investigators suggest that both macrophages and cytotoxic T lymphocytes (CTL) [15, 19], or T helper lymphocytes and CTL [54] are necessary to bring about tumor resolution. Immune regulatory networks involving T suppressor cells have been well defined in systemic antitumor immune circuits [39, 45] and suggested for intratumor sites [31]. However, it has been difficult to differentiate the functional role of different discrete populations of tumor-infiltrating cells in vivo.

The anatomical integrity of the eye is essential for its functioning. Hence, inflammatory processes, possibly induced by immune mechanisms, could severely interfere with this normal structure and function (reviewed in [47]).

We have previously reported that an immunogenic variant of P815 mastocytoma, P91 [5], undergoes a pattern of tumor resolution in the anterior chamber of the syngeneic mouse eye in which nonspecific destruction of the eye is coincident with tumor resolution. Several key findings suggest that this form of tumor resolution is mediated predominantly by a delayed-type hypersensitivity (DTH) mechanism [22, 36, 37]. As described by others and observed in this intraocular tumor model, characteristic vascular endothelial damage leads to an inflammatory reaction resulting in bulk tumor necrosis and destruction of innocent bystander host tissues [12, 17, 61]. By contrast, in other intraocular tumor models, rejection can be mediated by a nondestructive immune mechanism, in which both CTL and very potent DTH responses are present systemically yet rejection leaves the eye anatomically intact [22]. Histologically, this latter form of tumor rejection is characterized by an intense intratumor mononuclear cellular infiltration, chiefly by Thy1$^+$, CD8$^+$ lymphocytes.

In this report we present data that support the hypothesis that the nondestructive pattern of intraocular tumor resolution is mediated by tumor-infiltrating cytotoxic T lymphocytes. Although tumor-specific DTH mechanisms are available to the host and tumor-necrosis factor (TNF) secretion by TIL is apparent in vitro, these forms of nonspecific tumor immunity are excluded from eyes undergoing tumor rejection. This tumor model, therefore, is useful for analyzing regional immune regulatory mechanisms that influence the expression of antitumor effector elements within an organ.

Materials and methods

Mice. Adult female BALB/c (H-2^d) and C57BL/6 (H-2^b) were purchased from The Jackson Laboratory (Bar Harbor, Me) and used as experimental subjects when they were between 6 and 8 weeks of age.

Tumor lines. UV5C25 fibrosarcoma, an ultraviolet-light-induced tumor of BALB/c (H-2^d) origin, was originally obtained from Dr. Margaret L. Kripke (M. D. Anderson Hospital and Tumor Institute, Houston, Tex) and UV1591/28$^+$ fibrosarcoma (C3H, H-2^k) from Dr. James Forman (UT Southwestern Medical School, Dallas, Tex). Cells were grown in monolayer cultures in Dulbecco's modified Eagle's minimal essential medium (MEM; Gibco, Grand island, NY) supplemented with 10% heat-inactivated fetal bovine serum 1% L-glutamine, 1% vitamin solution, 1% sodium pyruvate and 1% streptomycin/penicillin/fungizone (complete MEM). P815 (H-2^d) was maintained in suspension cultures in complete MEM. Yac-1 (H-2^a), EL-4 (H-2^b) and J744.1 (H-2^d) tumor cells were maintained in suspension culture in RPMI 1640 supplemented with 10% fetal bovine serum (Gibco), 1% nonessential amino acids, 1% sodium pyruvate, 1% L-glutamine, 0.01 M HEPES, and streptomycin/penicillin/fungizone (complete RPMI).

Antibodies. Supernatants from the following hybridomas were precipitated with ammonium sulfate using standard procedures: IgG2b secreting anti-Thy1.2 rat hybridoma 53-6.72, and IgG2a secreting anti-CD4 rat hybridoma GK1.5. Monoclonal antibodies reactive to Mac-1 (macrophages and granulocytes), B220 (B cells), and F4/80 (macrophages) were kind gifts from Dr. Pam Witte (UT Southwestern Medical School, Dallas, Tex). Fluorescein-conjugated affinity-purified F(ab')$_2$ fragment of mouse anti-(rat IgG) (heavy and light chains) was purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, Pa).

Anterior chamber inoculations. A modified quantitative technique for depositing a definite number of tumor cells into the anterior chamber of the mouse eye has been described [38]. Adherent UV5C25 tumor cells were collected from culture plates and washed once with Hanks' balanced salt solution, (HBSS) and 1×10^6 cells were injected into the anterior chambers of deeply anesthetized BALB/c mice.

Isolation of tumor-infiltrating cells. Tumor-containing eyes were removed between days 18 and 24 after tumor inoculation and placed in complete RPMI media. The anterior segment of the eye was removed and placed in a separate petri dish where the tumor was carefully dissected from the anterior chamber using a dissecting microscope. Pooled tumors were minced with scissors, placed in 10 ml HBSS containing 10 mg collagenase (Sigma grade IV, Sigma Chemical Corp., St. Louis), 25 units hyaluronidase (Sigma Chemical Corp, St. Louis, Mo), and 1.0 mg DNase (Sigma), and incubated for 1.5 h on a rocker at 37° C. The suspension was centrifuged, the tissue pellet was pressed through a wire mesh screen, washed twice with HBSS, and centrifuged. The cell pellet was resuspended in complete RPMI and passed through nylon mesh (Tetko Inc., Elmsford, NY).

FACS analysis. Tumor-infiltrating cells were incubated with primary antibodies at the appropriate dilution for 30 min on ice, washed with complete RPMI, and then incubated with fluoresceinated secondary antibody at a 1:40 dilution for 30 min on ice. The cells were washed with medium and resuspended in 0.7 ml media for analysis with a Facstar flow cytometer (Becton Dickson, Mountain View, Calif).

In vitro boosting. A mixed lymphocyte/tumor cell culture for the generation of anti-UV5C25-tumor specific cytotoxic T cells was performed as previously described [22]. Briefly, 2.5×10^7 responder spleen cells and 2.5×10^5 stimulator tumor cells were cultured in complete RPMI supplemented with 50 µM 2-mercaptoethanol. Allo-responsive spleen cell effectors, boosted in vitro, were obtained from mice sensitized with mitomycin-C-treated spleen cells (anti-H-2^d and anti-H-2^b) or LTK cells (anti-H-2^k). Boosting flasks contained 3×10^7 spleen cells mixed with 6×10^5 stimulator cells and incubated for 4 days.

CTL assay. CTL activities were assayed by using a ^{51}Cr-release assay as described [22]. Labelled target cells (1×10^3–3×10^3) were added to serially diluted effector cells in 96-well round-bottomed microtiter plates. Effector-to-target ratios ranged from 100:1 to 12.5:1 and were performed in groups of three to four wells. After a 6-h incubation plates were centrifuged and 100 µl supernatant was collected and counted in a gamma counter. The cytotoxicity was calculated as follows:

$$\text{Specific release (\%)} = \frac{\text{experimental release} - \text{spontaneous release}}{\text{total release} - \text{spontaneous release}} \times 100$$

Table 1. Fluorescence-activated cell sorter analysis of tumor-infiltrating lymphocyte (TIL) population[a]

Experiment	Percentage (% total) positive for phenotypes:					
	Thy1	CD8	CD4	Mac-1	F4/80	B220
1	39.4	25.9	10.8	–	–	–
2	36.1	28.4	13.2	–	–	–
3	34.7	12.8	8.4	–	–	–
4	20.3	15.7	7.0	27.8	18.6	6.7
5	39.4	19.5	12.2	9.9	4.2	7.8
6	30.8	16.3	14.8	13.9	8.5	8.5
Mean	33.4	19.8	11.1	17.2	10.4	7.7
(SEM)	(2.9)	(2.5)	(1.2)	(5.4)	(4.3)	(0.5)

[a] For each experiment 15–20 tumor-containing eyes were enzymatically digested and the TIL cell suspension was stained with monoclonal antibodies reactive to the above phenotypic markers. A total of 5000 events were recorded for each monoclonal antibody and the total percentage was determined by analysis using TIL stained with secondary antibody alone as background

where ^{51}Cr release is measured as cpm. Maximum release was determined by treatment of target cells with 100 μl 1.0 M HCl and spontaneous release by incubating target cells with medium. Spontaneous release was $<39\%$ maximum release for all tumor targets.

TNF assay. A standard method to assess TNF activity was used [40]. Various dilutions of effector cells were incubated with the TNF-sensitive target WEHI 164.1 for 18 h and the plates processed as above for determination of cytolytic activity. TNF-containing culture supernatant from J774.1 tumor cells (B. Beutler, personal communication) was sterile-filtered and frozen. Aliquots were thawed and used at a final dilution of 1:2.

CTL precursor frequency assay. A standard procedure for determining the CTL precursor frequency for an antitumor response was optimized for the UV5C25 tumor [6]. Briefly, 1×10^6 irradiated syngeneic spleen cells (accessory cells) and mitomycin-C-treated UV5C25 or EL-4 stimulator cells (2×10^3 or 5×10^3/well, respectively) were added to round-bottom plates. Effector cells included normal spleen and lymph node cells, day 18–24 tumor-bearing spleen and lymph node cells, and TIL. Spleen and lymph node populations were passaged once through nylon wool. Limiting dilutions of these populations were added to plates (24 wells/dilution) and incubated at 37° C in a humidified atmosphere containing 5% CO_2. On day 7, the medium was flicked off and 100 μl complete medium and 100 μl $Na_2{}^{51}CrO_4$-labelled tumor cells (2×10^3/well) were added. After a 6-h incubation, the plates were centrifuged and 100 μl supernatant was removed and counted in a gamma counter. Positive wells were those that had radioactivities greater than three times the standard deviation of the radioactivity (cpm) of labelled tumor cells incubated in media alone. Regression analysis was performed as described by Taswell [48] and statistical significance determined by X^2 methods.

Winn assay. In vivo antitumor activity was investigated by using a Winn-type assay, in which putative antitumor effectors or control cell populations were mixed with viable tumor and injected intracamerally into syngeneic hosts. BALB/c hosts were exposed to 500 rad gamma radiation in a Gammacell (Atomic Energy of Canada Ltd., Ottawa, Canada) containing a ^{137}Cs source 1 day prior to intracameral injection. The inoculum consisted of effector cells (5×10^5 normal spleen cells or 5×10^4 TIL) mixed with 5×10^5 UV5C25 tumor cells. Tumor growth in the anterior chamber was evaluated by slit-lamp biomicroscopy.

Results

We have previously reported that syngeneic intraocular tumors can undergo one of two basic forms of immune rejection [22]. One pattern of rejection involves a violent intraocular inflammatory response, which culminates in complete atrophy of the tumor-containing eye. The evidence strongly suggests that this form of tumor rejection is mediated by a DTH effector mechanism [22, 36, 37]. By contrast, a second form of intraocular tumor rejection is characterized by a homogeneous infiltration of lymphocytes, piecemeal necrosis of individual tumor cells without damage to juxtaposed normal ocular tissues, and preservation of the physiological and anatomical integrity of the eye [22]. In the present study we wished to identify the intratumor cellular effectors in the nondestructive pattern of tumor rejection.

Isolation of tumor-infiltrating cells

The first step in establishing the mechanism for the nondestructive pattern of intraocular tumor rejection is to isolate the infiltrating effector cell from the resolving tumor lesion and determine the surface phenotype of the immune cell. Our ability to separate lymphocytes efficiently from other host cells and tumor cells was hampered by the very small mass of tumor collected even when pooling tumors from 20–25 eyes. Moreover, since purification techniques can result in the loss of certain subpopulations of cells [42, 59], we felt that a more accurate functional analysis would be obtained by using the total cell suspension. Although the tumor-infiltrating cell suspension contained tumor cells, as well as infiltrating lymphocytes, we have chosen to use the term tumor-infiltrating lymphocytes (TIL) to emphasize the functional attributes of the cells as they relate to tumor rejection. In a typical isolation, the total recoverable population from 20 mouse eyes averaged 1.3×10^7 cells.

Fluorescence-activated cell sorter (FACS) analysis of TIL

In preliminary experiments, in vitro cultured UV5C25 fibrosarcoma cells were stained with the monoclonal antibodies shown in Table 1 and analyzed by FACS analysis. Background staining of tumor cells alone was negative for

Table 2. Cell-mediated cytotoxicity of control and TIL cell populations against various tumor targets[a]

Effector population	Specific lysis (%) of targets:		
	UV5C25	EL-4	UV1591/28+
Normal spleen[b]	12.8 (1.0)	4.6 (3.2)	2.0 (0.8)
Immune spleen[b]	57.7 (1.8)	3.6 (5.1)	6.9 (0.8)
Normal spleen	0.0 (2.5)	0.0 (2.6)	5.8 (1.2)
Immune spleen	6.9 (2.3)	5.8 (4.0)	13.2 (1.7)
Immune TIL	33.4 (2.8)	2.8 (1.2)	12.2 (0.2)
C57BL/6 anti-BALB/c[b]	87.4 (2.9)	ND	ND
BALB/c anti-C57BL/6[b]	ND	56.0 (3.8)	ND
BALB/c anti-H-2[k] (E:T = 50:1)	ND	ND	93.8 (4.8)

[a] Values indicate percentage specific cytotoxicity (±SEM) calculated from three or four wells in a 6-h ^{51}Cr-release assay at an effector: target (E:T) ratio of 100:1, except as noted. A representative experiment is shown. Similar direct cytolytic activity of TIL for UV5C25 tumor targets has been found in 2 additional experiments (mean specific lysis: 54.4±11.4)

[b] Effector cells were boosted in vitro in bulk cultures for 4 days or 6 days with the appropriate mitomycin-c-treated tumor or spleen cells. ND, not determined

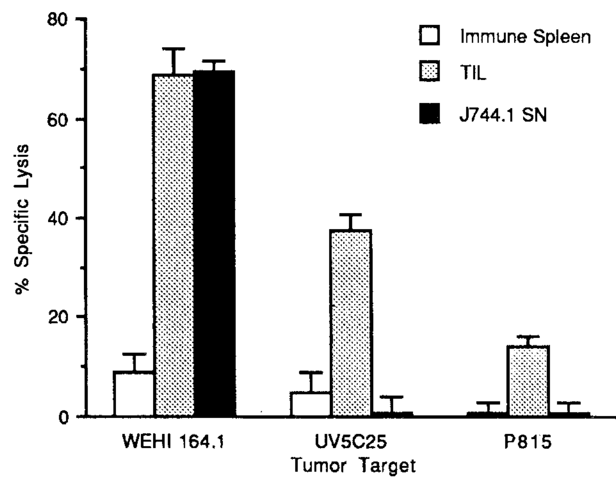

Fig. 1. Natural cytotoxic activity of TIL population (*dotted bars*); immune spleen cells (*open bars*); or tumor-necrosis-factor-containing J774.1 supernatant (*solid bars*). *Bars* represent percentage specific lysis of indicated targets at an E:T of 100:1 following an 18-h incubation. Spontaneous release: < 32%. A representative experiment from three separate experiments is shown

all mAb except B220 (<5%; data not shown). Table 1 shows the result of FACS analysis of the TIL population. An average of 33.4% of the cells were Thy1$^+$, 19.8% CD8$^+$, and 11.1% were CD4$^+$. These results indicate that the majority of the infiltrating lymphocytes were T cells and expressed the characteristic phenotype of a cytotoxic/suppressor T cell. Only a small portion (7.7%) of the cells were B cells, as demonstrated by staining with the B220 marker. Within the TIL population macrophages/granulocytes comprised 17.2%, as identified by the Mac-1 antigen, and the majority (10.4% F4/80$^+$) of these cells were macrophages [2]; however, the percentage of macrophages was substantially less than that of T cells (Thy1$^+$) and was further substantiated by in situ immunoperoxidase studies (data not shown). Natural killer (NK) cells were present in exceedingly low numbers (0.8% NK 2.1$^+$; data not shown). Thus, the results indicate that the predominant infiltrating lymphoid cell expressed the surface determinants characteristic of cytotoxic T cells.

Cytotoxic activity of TIL

The next step in confirming the role of cytotoxic T lymphocytes in this pattern of intraocular tumor rejection was to demonstrate their functional activities in a controlled setting. Accordingly, TIL were examined for their cytolytic activity against UV5C25 cells in vitro. A direct 6-h ^{51}Cr-release assay was used to measure tumor-specific cytolysis by TIL. We have previously shown that tumor-bearing hosts generated in vitro boosted CTL activity and that this activity was specific for the UV5C25 tumor [22]. These results were confirmed in Table 2. But, when freshly isolated unboosted spleen cells or TIL effectors from tumor-bearing mice were used in a direct cytotoxicity assay, only the TIL population was cytolytic for UV5C25 fibrosarcoma. Moreover, the TIL were unable to lyse third-party EL-4 or UV1591/28$^+$ target cells. Since a portion of the cells in the TIL cell suspension are tumor cells and could function as cold-target inhibitors in this assay, the cytolytic activity demonstrated by the chromium-release assay represents the minimum amount of cytolytic activity. Therefore, this result is consistent with the notion that the infiltrating lymphocytes are actively lysing tumor cells in situ.

Natural cytotoxic activity of TIL population

Tumor lysis by natural cytotoxic mechanisms is mediated mainly by tumor necrosis factor [40]. Furthermore, macrophages are primarily responsible for TNF production (reviewed in [4]). In the present experiments, the observation that approximately 10% of the host-infiltrating cells were macrophages (F4/80$^+$) would suggest that lysis mediated by natural cytotoxic mechanisms may contribute to UV5C25 tumor rejection. The results in Fig. 1 show natural cytotoxic activity mediated by immune spleen cells, the TIL population or TNF-containing J744.1 supernatant against various tumor targets. Although the TIL population demonstrates significant killing of UV5C25 and WEHI 164.1 targets following an 18-h incubation, UV5C25 tumor targets are not susceptible to lysis by TNF-containing supernatant. These results suggest that the TIL population is able to produce TNF adequately, yet this mechanism may not be involved in the direct lysis of the fibrosarcoma in situ.

Frequency of cytolytic precursors

The cornerstone of immunology is the principle that antigen-specific lymphocytes undergo clonal selection and expansion following exposure to their relevant antigen. Furthermore, a lymphocyte population actively engaged as effector cells in the destruction of a tumor should contain a high frequency of tumor-specific cytotoxic precursor cells. Accordingly, the frequency of precursors to cytolytic cells in the TIL population was determined by limiting dilution analysis. The precursor cytotoxic T lymphocyte assay was optimized for low numbers of responsive cells in a series of prior experiments. Although tumor cells are present in the TIL suspension, the precursor CTL assay used very low numbers of responding cells (250–2000/well);

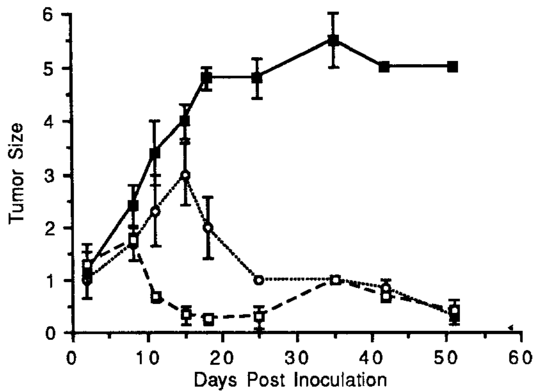

Fig. 2. Winn assay in the anterior chamber. BALB/c mice were injected intracamerally with 5×10^5 UV5C25 tumor cells alone (...), or exposed to 500 rad gamma irradiation 1 day prior to receiving 5×10^5 tumor cells mixed with 5×10^5 normal spleen cells (———), or mixed with 5×10^4 TIL (-----). The anterior chamber was evaluated by slit-lamp biomicroscopy and graded from 1 to 6 on the basis of the percentage of tumor occupying the anterior chamber (1 = 10%; 2 = 25%; 3 = 50%; 4 = 75%; 5 = 100%; 6 = progressive tumor growth). Values represent the mean of five or six mice per group (experimental) or three mice per group (nonirradiated). *Bars* represent SEM

Table 3. Frequency of precursor cytotoxic T lymphocytes

Population	Anti-UV5C25		Anti-H-2^b (EL-4)	
	$CD8^+$	P^a	$CD8^+$	*P*
Control lymph node	1/202 000	<0.05	1/735	<0.001
Immune lymph node	1/580 000	<0.05	1/1325	<0.005
Immune spleen	1/221 000	<0.05	–	–
TIL	1/251	<0.005	1/19 904	<0.05

[a] *P* value based on χ^2 analysis for a single-hit limiting-dilution model

therefore, the small percentage of contaminating tumor cells would have been killed in those wells containing differentiating, proliferating, and functional CTL. The results in Table 3 show that there is a dramatic increase in the frequency of $CD8^+$ precursor CTL in the TIL population (1/251) compared to both normal (1/202000) and immune spleen cell populations (1/221 000).

If there is a localized inflammatory response, it is possible that lymphocytes infiltrating into the tumor arrive at the tumor site nonspecifically. If this were the case, then the precursor CTL frequencies for different specificities would be the same. To test this, the precursor CTL frequency was determined for a specific allogeneic response. In the TIL population, the frequency of anti-H-2^b responsive $CD8^+$ cells was 1/19900 (Table 3). This indicates that there is a 80-fold lower likelihood of finding an allogeneic-directed precursor CTL than of finding a UV5C25-specific precursor CTL in the TIL population. Moreover, in the normal lymph node population, the reverse situation existed. There was a 275-fold greater chance of finding an alloresponsive precursor CTL (1/735) than finding a UV5C25-specific precursor CTL (1/202000). Collectively, these results strongly indicate that tumor-specific precursor CTL are highly enriched in the TIL population.

In vivo activity of TIL

The hypothesis we have proposed is that the tumor-infiltrating lymphocytes are responsible for tumor rejection. Therefore, it should be possible to isolate these cells, demonstrate their functional activities in vitro, and finally show that such cells will produce similar in vivo effects when transferred to a new host. The next series of experiments considered the final step in this analysis by determining whether TIL were capable of mediating tumor rejection when transferred to immunosuppressed recipients. Viable tumor cells were mixed with normal spleen cells (E:T = 1:1) or TIL (E:T = 0.1:1) prior to injection into the anterior chamber of irradiated syngeneic hosts. Tumor growth was evaluated by biomicroscopy and the extent of tumor growth was graded from one to six. As shown in Fig. 2, the tumor grew vigorously in hosts given normal spleen cells, but was rejected quickly with minimal pathological sequelae in hosts receiving tumor and TIL. It is particularly noteworthy that the clinical characteristics of tumor rejection in the hosts treated with TIL were identical to those that occurred in normal immunocompetent hosts.

Discussion

We have previously hypothesized that tumor rejection within the eye can proceed through two mechanisms. One is DTH-mediated, occurs without a lymphocytic infiltration, involves nonspecific tissue destruction, and results in complete atrophy of the eye. The other pattern (CTL-mediated) characteristically demonstrates infiltrating $Thy1^+$, $CD8^+$ cells, which appear to be attached directly to tumor cells, and produces minimal damage to host tissues [22]. This study characterized these tumor-infiltrating antitumor effectors by in vivo and in vitro techniques.

Although several different cell types were identified in the TIL population, the weight of evidence suggests that the major antitumor effector within the intraocular tumor was a CTL. FACS analysis showed that 1/3 of the cells were T cells, as identified by the Thyl phenotype, and the majority (64%) were $CD8^+$. Although B cells were found in the TIL population (7.7%), we have not been able to demonstrate UV5C25-specific serum antibody by immunofluorescence [22]. Therefore, it is unlikely that antitumor antibody contributes to tumor rejection; however, the function of these B cells remains unclear.

Cells isolated from peripheral lymphoid organs are frequently noncytolytic unless they are boosted in vitro in the presence of a source of interleukin-2 and autologous tumor stimulator cells [8, 13]. Similarly, in this tumor model, spleen or lymph node cells did not demonstrate direct cytolytic activity; by contrast, the TIL population was directly cytolytic to fibrosarcoma cells.

Results from precursor cell analysis showed a high frequency of tumor-specific precursor CTL present in the TIL population. This observation may be interpreted in two ways: (a) precursor CTL migrate to the intraocular tumor site or (b) mature CTL enter the tumor site and differentiate, giving rise to memory CTL that are detected in the assay. The results in Table 3 show that the number of UV5C25-specific precursor CTL in tumor-bearing lymph node cells (1/580000) is 2.5 times less than that found in a normal lymph node population (1/202000). This suggests that tumor-specific precursors apparently leave peripheral

lymphoid tissue and migrate to the tumor-containing eye. The precursor CTL frequency for an allogeneic response in the TIL population was much lower than that for the specific tumor. Since the number of cells responding to a single alloantigen is approximately 2% of the UV5C25-specific responding cells and is less than the percentage of clones found in a clonally expanding antitumor cytotoxic response demonstrating cross-reactivity to allogeneic targets [7], it is unlikely that nonspecific emigration of precursor cells into the tumor had occurred. Therefore, the data support our conclusion that tumor-specific pCTL migrate to the tumor-containing eye.

Macrophages bearing the F4/80 antigen were also detected within the TIL population. These cells could contribute to tumor cytolysis by direct tumor lysis or by secreting TNF. In fact significant natural cytotoxic activity, as detected by the lysis of a TNF-sensitive target, was demonstrated in cultured TIL; by contrast, UV5C25 fibrosarcoma was insensitive to lysis by TNF-containing supernatant (Fig. 1) or recombinant TNF (500 units) (unpublished results). However, recent evidence suggests that TNF can synergize with gamma-IFN in mediating tumor cytolysis by a slow-acting mechanism [9]. Therefore, although TNF-mediated cytolysis of UV5C25 tumor cells was not detected in the present assay system, it may, nonetheless, contribute to tumor destruction by other mechanisms. Macrophages within the TIL population apparently do not contribute to direct tumor cytolysis since macrophage-sensitive P815 tumor targets [44] were weakly lysed in this assay. Hence, it is possible that TNF is secreted locally, yet this activity within the eye does not appear to mediate direct tumor cytolysis, hemorrhagic necrosis, or destructive tumor resolution, but favors instead specific CTL activity. There are two mechanisms by which TNF secretion may favor CTL activity. First, it has been shown that TNF-α increases the expression of high-affinity interleukin-2 receptors on activated T cells and consequently, synergizes with interleukin-2 to stimulate and enhance T cell proliferation [46]. This synergistic activity could, therefore, stimulate intraocular antitumor CTL to proliferate. Secondly, TNF-α can interact with vascular endothelial [35] or smooth muscle cells [55] to induce the synthesis/release of interleukin-1, which attracts and activates lymphocytes (reviewed in [11]). Since the iris (located at the posterior aspect of the anterior chamber) contains a rich source of vascular tissue and smooth muscle, it is conceivable that TNF secretion by tumor-infiltrating macrophages stimulates interleukin-1 release, thus permitting the rapid localization and proliferation of antitumor CTL.

We could not account for the lack of nonspecific DTH-like tissue destruction since (a) $CD4^+$ T lymphocytes were present in the TIL population, as determined by FACS analysis, and (b) intraocular tumor-bearing hosts demonstrated remarkable systemic DTH responses to UV5C25 antigens, as measured by footpad swelling responses [22]. Since very few specifically sensitized cells are necessary to transfer DTH responses [25] or are found within DTH lesions [27], we were puzzled that these $CD4^+$ cells were not initiating a DTH-like destructive response within the eye. Several recent findings, however, may explain this apparent dichotomy. First, it has ben shown that T helpers ($CD4^+$) demonstrate two functional subsets based on lymphokine secretion (reviewed in [29]). It is possible that the $CD4^+$ cells within the intraocular tumor represent T helper (Th-2) cells that have limited capacity to initiate a DTH reaction. Moreover, $CD4^+$ cells can induce the development of CTL at a regional site. This has been demonstrated by Mann et al. [24], who showed that kidney-tubular-antigen-specific $CD4^+$ cultured T cells were capable of locally inducing $CD8^+$ nephritogenic effector T lymphocytes typically found within the renal lesions, but were unable to transfer the disease. It is, therefore, possible that $CD8^+$ T cells are the primary effectors within the eye, yet $CD4^+$ cells play an important inductive/regulatory role in the ocular lesion.

The expression of class II molecules is important for the induction of DTH reactions (reviewed in [10]). It is possible that the difference in the two general patterns of intraocular tumor resolution is determined by variable tumor expression of class II antigens. In preliminary experiments we have not detected class II antigens on UV5C25 or P91 tumors even under the conditions of interferon γ treatment (unpublished data), which is known to increase class II expression on various cells [3]. Other studies are under way to correlate the type of tumor rejection observed in the eye and tumor major histocompatibility (MHC) antigen expression.

The data reported here emphasize that within a given organ, a single immunological effector mechanism may predominate. In the case of UV5C25 tumor rejection in the anterior chamber of the eye, CTL effectors predominate while DTH mechanisms appear to be actively down-regulated locally. Understanding how immunological effector mechanisms are regulated in the eye could influence the management of various eye diseases as well as provide insight into the control of neoplasms at other regional sites.

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