Infiltration and Accumulation of Precursor Cytotoxic T-Cells Increase with Time in Progressively Growing Ocular Tumors

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ABSTRACT

Precursors of cytotoxic T-cells (pTc) infiltrate P815 tumors growing progressively within the immunologically privileged anterior chamber (AC) of BALB/c mouse eyes, but directly cytotoxic T-cells cannot be detected in these eyes. To determine if the failure to reject these tumors is due to a relative inability of tumor-specific pTc to gain access to, or be retained by, the tumor-containing eye, we have assayed through time the frequency of pTc in eyes that received P511 tumor cells in the AC or subconjunctival space (SC; a site where the tumors are rejected). P511 tumor cells, a hypoxanthine-amethopterin-thymine medium-sensitive derivative of P815 cells, were selected for these studies because P511 tumor cells can be eliminated from in vitro lymphocyte cultures containing hypoxanthine-amethopterin-thymine medium, permitting us to make accurate estimates of pTc frequencies. To ensure that P511 cells are similar biologically and immunologically to P815 tumor cells, we demonstrated that both P511 and P815 cells form progressively growing tumors when injected into the AC of BALB/c eyes and that recipients of both tumor cell lines develop DBA/2-specific anterior chamber-associated immune deviation. Using cell suspensions harvested from eyes of mice bearing AC or SC P511 tumors, we found that tumor-specific pTc appeared first (day 8) in SC tumor-bearing eyes, compared to their appearance in AC tumor-bearing eyes (day 11). Thereafter, however, the number of pTc detected was significantly greater in eyes bearing progressively growing AC tumors than in SC tumor-injected eyes. The number and frequency of pTc we found in these eyes appeared to correlate directly with the size of the ocular tumor burden. We conclude that failure to reject P511 tumors from the AC can be ascribed neither to a quantitative deficiency in infiltrating tumor-specific pTc nor to an inability to retain pTc at the site. Our findings suggest that immune acceptance of allogeneic ocular tumor grafts may result from failure of infiltrating pTc to differentiate terminally in situ into cytotoxic effector cells.

INTRODUCTION

Minor histoincompatible P815 tumor cells grow progressively following their injection into the immunologically privileged AC of BALB/c mouse eyes (1, 2). These grafts never undergo immune rejection, and Tc cannot be detected within these ocular tumor sites (3). By contrast, P815 tumor cells do not grow progressively when injected into the SC (a nonprivileged site) of BALB/c mice. It is important that fully functional Tc can be detected within SC tumor-bearing eyes, and the temporal appearance of these cells coincides with the onset of tumor graft rejection (4). Taken together, these results suggest that (a) Tc are the relevant effector cells responsible for successful destruction of ocular P815 tumors and (b) the absence of Tc from AC tumors may account for the inability of host mice to reject these tumors. We were surprised, therefore, to learn recently that tumor-specific pTc could be retrieved from P815 tumors growing in both the AC and SC sites (3). The appearance of tumor-specific pTc in the AC site (where the tumor is never rejected) indicates that there is no anatomical barrier within the eye which limits the entry of lymphocytes with the potential of rejecting AC tumors. Therefore, the reasons for immune acceptance of AC tumors may be that (a) a quantitatively lower number of tumor-specific pTc are able to gain access to the eye and/or (b) pTc may fail to differentiate into fully functional effector cells in the eye.

Our attempts to quantify the number of P815-specific pTc in mouse eyes have been frustrated by the fact that the cell suspensions prepared from tumor-containing eyes are heavily contaminated with tumor cells. We and others have documented that if P815 cells are present in cultures, they have the effect of masking/suppressing cytolytic activity of lymphocytes in the same cultures. This problem is especially great in tumor-containing eyes, since there is an ever-increasing number of tumor cells present. We have previously attempted to eliminate tumor cell contamination from ocular cell suspensions by positively selecting with anti-Thy-1 antibodies (thereby enriching for T-cells) (3). Using this approach, we found that the purity of Thy-1 T-cells could be increased from 4 to 71% and that the corresponding pTc frequencies rose from 1/50,000 to 1/6,000. However, significant contamination with P815 cells still persisted, rendering these frequency estimates suspect.

To describe the pTc frequency within tumor-bearing eyes more accurately, we have obtained P511 tumor cells, a HAT-sensitive derivative of the P815 tumor cell line. Since P511 cells are selectively eliminated in vitro if cultured in the presence of HAT, this approach gave us the opportunity to produce tumor cell-free lymphocyte suspensions for pTc analysis. In this communication, we report that P511 cells strongly resemble the parent P815 cell line in that they form progressively growing tumors in the AC of eyes of BALB/c mice and induce ACAID. Using P511 cells, we have discovered that during progressive tumor growth in the AC, the frequency of tumor-specific pTc increases through time and eventually reaches levels significantly greater than those achieved at SC sites. These results permit us to conclude that the reason for immune acceptance of AC P815 tumors is not that fewer pTc gain access to the tumor-containing eye, leading us to suspect that a block in terminal differentiation of pTc that infiltrate AC tumors prevents the host from rejecting these histoincompatible intraocular tumors.

MATERIALS AND METHODS

Animals. Adult female BALB/c-H-2" mice and DBA/2-H-2" mice were raised in our own animal colony and used in experiments when they were between 2 and 5 months of age. Animals were treated according to the Association for Research in Vision and Ophthalmology resolution on the use of animals in research.

Tumor Cell Inoculations. P511 mastocytoma cells (DBA/2 origin) are a HAT-sensitive derivative of P815 cells and were generously provided by Dr. T. Boon (Ludwig Institute for Cancer Research, Brussels, Belgium). Tumor cells were grown in suspension cultures in 75-
cm² tissue culture flasks (Costar) with RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (HyClone), 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Gibco), 2.0 mM glutamine (Gibco), and 20 mg/ml gentamicin (Gibco Laboratories). Tumor cells were isolated from cell cultures, washed in Hank’s balanced salt solution, and resuspended in the salt solution for inoculations. A quantitative technique for depositing a definite number of tumor cells into the AC or SC of the mouse eye has been described (1). Mice were deeply anesthetized with 0.66 mg i.m. of ketamine hydrochloride (Vetalar; Parke, Davis and Co., Detroit, MI). P511 cells, at a concentration of 2 x 10^8 cells/3 μl, were injected into the anterior chamber or subconjunctiva (3 μl/eye). Recovery of Cells from Tumor-containing Eyes. Tumor-containing eyes were removed from anesthetized mice on the designated day after inoculation. Single-cell suspensions containing tumor cells and mononuclear cells were prepared by a collagenase treatment described previously (4). The ocular tissue was minced in a Petri dish containing 7 ml of collagenase (Sigma) at 150 units/ml in RPMI 1640 and incubated for 90 min at 37°C. The debris was separated from the released cells which were then washed three times and resuspended in RPMI 1640 complete culture medium. The mean number of lymphocytes recovered per eye 14 days after AC or SC inoculation of P815 cells was 0.85 x 10^6 and 0.5 x 10^6, respectively. The mean number of lymphocytes recovered from normal un inoculated eyes was 2.0 x 10^6 cells.

Limiting Dilution Assay for pTc. Twenty-four replicate cultures containing varying numbers of responder cells and 2 x 10^8 X-irradiated (2000 rads) DBA/2 stimulator cells in 200 μl culture medium (supplemented with 15% rat spleen concanavalin A culture supernatant as described previously (5)) were set up in 96-well round-bottomed microtiter plates. The cultures were incubated for 7 days at 37°C after which the medium was discarded, and 200 μl of culture medium containing 2 x 10^6 ^51Cr-labeled P511 targets were added to the cultures. The plates were centrifuged (150 x g for 3 min), incubated for 6 h at 37°C in 5% CO₂, and centrifuged again, and the ^51Cr release was measured as described previously. Individual cultures were considered positive if the ^51Cr release was 3 SD above background cultures containing stimulators and targets alone.

Dr. Peter Krammer (Heidelberg, Germany) kindly provided a computer program for the analysis of the data from limiting dilution assays. The program calculated both the frequency of pTc and the 95% confidence limits using the maximum likelihood and the minimum χ² methods. The χ² value was determined along with the corresponding probability for the goodness of fit for the frequency to the Poisson model. All limiting dilution data reported herein conformed to the single-hit kinetics of the Poisson distribution.

Depletion of Lymphocyte Subpopulations by Complement-mediated Lysis. Lymphocytes recovered from the draining lymph nodes of AC and SC tumor-bearing mice were treated with various monoclonal antibodies plus complement in a negative selection procedure to determine which cell surface molecules the precursor cytotoxic T-cells express. Effector cells were treated with a 1:1000 dilution of Thy-1.2 antibody (New England Nuclear), a 1:500 dilution of CD8 antibody (Cedarlane, San Diego, CA), or a 1:1000 dilution of CD4 antibody (Becton Dickinson, Mountain View, CA) for 30 min on ice at a concentration of 5 x 10⁶ cells/ml of antibody in RPMI 1640. The cells were washed twice, resuspended in RPMI 1640 containing a 1:20 dilution of rabbit complement (Pel-Freez Biologicals, Rogers, AR), incubated for 30 min at 37°C, and washed three times with medium. Antibody plus complement-treated cells were resuspended at a concentration of 5 x 10⁶ cells/ml. Cell counts were adjusted to reflect the viability of cells receiving complement only.

Delayed Hypersensitivity Response to P511 Tumor Cells. DH was measured as described previously (6). Briefly, mice received inoculations of 2 x 10⁸ P511 tumor cells into the AC or SC of the eye. On the designated day after inoculation, 0.5 x 10⁶ X-irradiated (10,000 rads) P511 cells suspended in 10 μl of Hanks’ balanced salt solution were injected s.c. into the right ear pinnae. Both ears of each mouse were measured in mm using an engineer’s micrometer immediately before ear challenge and 24 and 48 h later. The left ear served as a negative control. Additional controls included normal mice challenged with X-irradiated P511 cells. Results were expressed as Specific ear swelling = (24-h measurement – 0-h measurement) experimental ear – (24-h measurement – 0-h measurement) negative control ear x 10⁻³ mm

Data were analyzed by a two-tailed Student’s t test in which significant differences were accepted at P < 0.05.

Adaptive Transfer of Suppression. Groups of five BALB/c mice received AC inoculations of 2 x 10⁵ P511 tumor cells on day 0, the tumor-containing eyes were enucleated on day 7, and single cell suspensions of spleen cells were obtained on day 10. As a positive control, spleen cells were similarly prepared from normal mice. Groups of five normal recipient mice each received i.v. inoculations (5 x 10⁶ cells/mouse) of the experimental or positive control spleen cell preparations. Within 2 h all mice received a priming dose of 2 x 10⁶ P511 tumor cells per mouse (total volume, 3 μl) administered into the SC of the eye. As a negative control, a third group of five mice received neither i.v. inoculations nor the priming SC dose of P511 tumor cells. The DH response of all mice was assayed 10 days later as described above.

RESULTS

The validity of our experiments depends on P511 tumor cells being equivalent to P815 tumor cells in their ability to grow progressively within the AC of minor histoincompatible BALB/c mice and to induce ACAID. The following experiments examined the intraocular tumor growth of P511 tumor cells within privileged (AC) and nonprivileged (SC) sites. Groups of BALB/c mice received AC or SC inoculations of 2 x 10⁵ P511 tumor cells, and the subsequent tumor growth was observed by visual examination. P511 tumor cells grew progressively within the AC; by day 10 postinoculation the tumor completely filled the AC, and by day 14 the eyes were twice their normal size. By contrast, the same number of tumor cells was rapidly rejected from the SC of the eye (data not shown). Previous experiments have shown that progressive P815 tumor growth within the AC correlates with an altered systemic immune response which has been termed ACAID and is characterized by a suppressed tumor-specific DH responsiveness (reviewed in Refs. 7 and 8). To determine if progressive P511 AC tumor growth is also associated with the induction ACAID, the following experiments were performed.

Induction of ACAID by P511 Tumor Cells. Groups of BALB/c mice received AC or SC inoculations of 2 x 10⁵ P511 tumor cells into one eye, while control mice received no inoculations. Ten days later the tumor-containing eyes were enucleated, and 4 days thereafter (day 14 p.i.) all mice were challenged for delayed hypersensitivity in the ear pinnae with X-irradiated (10,000 rads) P511 tumor cells. The specific ear swelling was measured at 24 and 48 h as a measure of the DH response. The results of a representative experiment are displayed in Fig. 1. SC P511-inoculated mice generated vigorous DH responses to the tumor cells. By contrast, mice receiving AC P511 inoculations had significantly reduced DH responses (P < 0.05) compared with positive controls. To confirm that ACAID and a suppressed DH response were present, the next series of experiments was performed.

Putative splenic suppressor cells from AC P511-inoculated mice were adoptively transferred into normal recipient mice, which were subsequently given a priming dose of tumor cells to determine if the induction of DH was suppressed. Groups of BALB/c mice received AC inoculations of 2 x 10⁵ P511 tumor cells on day 0, the tumor-containing eyes were enucleated on day 7, and single cell suspensions of spleen cells were prepared on day 14. As a positive control, spleen cells from normal mice

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were inoculated with a priming dose of P511 tumor cells in the SC of the eye (2 x 10⁵ tumor cells/mouse). As a negative control, a third group of mice received neither i.v. inoculation of spleen cells nor a priming SC dose of tumor cells. Ten days later, all three groups of mice were ear challenged with X-irradiated P511 tumor cells, and the specific ear swelling was measured. The results of a representative experiment are shown in Fig. 2 and indicate that mice receiving spleen cells from AC-inoculated mice had significantly reduced DH responses to the P511 tumor cells as compared with the positive control (P < 0.05). These results indicate that P511 cells inoculated into the AC generate splenic DH suppressor cells. We conclude that the progressive tumor growth following the AC inoculation of P511 tumor cells into BALB/c mice coincides with the induction of ACAID, and in this regard P511 and P815 tumor cells are virtually identical.

Mice with P511-induced ACAID Are Primed for Cytotoxic T-Cells. One of the interesting aspects of ACAID is that, even though these mice have suppressed DH responses, they are primed for tumor-specific cytotoxic T-cell responses (5, 9). The following experiments were performed to ensure that AC inoculation of P511 tumor cells primes mice for a tumor-specific cytotoxic T-cell response. BALB/c mice received AC or SC inoculations of P511 cells as in previous experiments; 15 days later, the draining cervical lymph node cells were recovered and assayed for the frequency of specific pTc in a limiting dilution analysis. Normal cervical lymph node cells were used as a negative control in these assays. Prior to the pTc assay, the cells were divided into four equal groups and treated with (a) complement only, (b) anti-CD4 mAb plus complement, (c) anti-CD8 mAb plus complement, or (d) both anti-CD4 and anti-CD8 mAb plus complement. The results are displayed in Table 1. Following both AC and SC inoculation of P511 tumor cells, mice contained an expanded pool of pTc among the cells recovered from the tumor-bearing eyes of mice harboring both AC and SC P815 tumors (3). The following experiments were performed to determine if tumor-specific pTc also infiltrated into the eyes of mice harboring AC and SC P511 tumors. Groups of BALB/c mice received 2 x 10⁵ AC or SC inoculations of P511 tumor cells. Fourteen days later, the tumor-containing eyes were enucleated, and single cell suspensions were prepared by collagenase treatment of the ocular tissue. The recovered cells were then used in a limiting dilution assay to determine the pTc frequency. These cultures contained HAT to selectively eliminate the P511 tumor cells; exogenous interleukin 2 was also added to expand the lymphocytes. Two types of negative control were used: (a) cells from uninoculated contralateral eyes of AC and SC tumor-bearing mice; and (b) cells from normal eyes of BALB/c mice that had not received any tumor cell inoculations. The results of a representative experiment are displayed in Fig. 3 and reveal that pTc are present within AC and SC tumors at a frequency of 2760 and 191/10⁶ lymphocytes, respectively. Neither uninoculated contralateral eyes nor normal eyes contained detectable pTc frequencies. We conclude that tumor-specific pTc can infiltrate into P511 tumor-containing eyes and that these cells do not migrate indiscriminately into eyes which do not harbor tumor cells.

On the basis of our previous experiments, we suspected that contaminating tumor cells among the cells recovered from tumor-containing eyes artificially lowered the pTc frequency values obtained by the limiting dilution assay. The following experiments utilized the HAT-sensitive P511 tumor cells to test directly the effect of tumor cell contamination on the

![Fig. 1. DH responses after inoculation of 2 x 10⁵ P511 tumor cells into the AC or SC. Fourteen days after inoculation the mice were challenged in the ear pinna with 0.5 x 10⁶ X-irradiated (10,000 rads) P511 cells, and the degree of ear swelling was assessed 48 h later. Negative controls were normal mice challenged with X-irradiated tumor cells. Columns, mean of five animals; bars, SEM.](image)

![Fig. 2. Adoptive transfer of impaired DH response following AC inoculation of P511 tumor cells. Spleen cells (50 x 10⁶), from BALB/c mice that received AC inoculations of P511 tumor cells on day 0, were enucleated on day 7, and were sacrificed on day 10, were infused i.v. into normal recipient mice. Within 2 h, recipients received a priming dose of P511 tumor cells in the SC. Ten days later, mice were ear challenged with X-irradiated P511 tumor cells, and the specific ear swelling was measured. Positive controls were recipient mice which received i.v. inoculations of normal spleen cells, and negative controls were normal mice which received only an ear challenge.](image)

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<th>Table 1 Frequency of tumor-specific precursors of cytotoxic T-cells following anterior chamber inoculation of P511 tumor cells</th>
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<td>Mean pTc frequency/10⁶ lymph node cells</td>
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† BALB/c mice received inoculations of P511 cells into the AC or SC of a single eye. Fourteen days later the draining cervical lymph node cells were recovered, and the tumor-specific pTc frequency was determined by a limiting dilution assay. Cervical lymph node cells from uninoculated mice served as a negative control.

The lymph node cells were treated with various mAb plus complement treatments in a negative selection experiment.
The results of these experiments are displayed in Fig. 4. In the presence of HAT, the pTc frequency among lymphocytes recovered from AC and SC tumor-containing eyes was 2207 and 565/10^6 lymphocytes. By contrast, in the absence of HAT, the pTc frequencies were significantly less, at 216 and 117/10^6 lymphocytes, respectively. It is interesting to note that the difference in pTc frequencies between cultures with and without HAT was greater among the cells recovered from AC tumor-bearing eyes compared with SC tumor-containing eyes. This corresponds to the much larger tumor burden that is present within AC tumor-containing eyes at 14 days after inoculation. These results confirm that contaminating tumor cells contribute to an artificially low estimate of the pTc frequency among lymphocytes within these tumor sites.

**Infiltration and Accumulation of pTc within Tumor-containing Eyes through Time.** In experiments reported previously, we observed that directly cytotoxic Tc were present within SC tumor-containing eyes but absent from progressively growing AC tumor-containing eyes (3). To determine if the absence of Tc from AC tumors is the result of a decreased infiltration and/or accumulation of pTc within AC tumor-containing eyes, the following series of experiments was performed. AC and SC inoculations of P511 tumor cells were administered to groups of BALB/c mice. On days 5, 8, 11, 14, 18, and 27 postinoculation the eyes were enucleated, and the pTc frequency was determined as in the previous experiments. All culture media contained HAT. The results of these experiments are displayed in Fig. 5. Following the inoculation of tumor cells into the SC of the eye, pTc were first detected between days 5 and 8 p.i. We have previously shown that tumor cells are completely eliminated from the SC tumor-containing eyes between days 8 and 18 p.i. During this interval, the pTc frequency rose from 45 to a maximum of 3086/10^6 lymphocytes on day 18. After elimination of the tumor, the number of pTc diminished between days 18 and 27 to 141/10^6 lymphocytes. By contrast, following AC inoculation of tumor cells, pTc were not detected until day 11 p.i. Thereafter, during progressive tumor growth, the frequency rose from 1653 on day 11 p.i. to a maximum of 9326/10^6 lymphocytes on day 18 p.i. and remained at that approximate frequency through day 28. These results reveal that while pTc first appear in SC tumor-bearing eyes, thereafter the frequency of pTc within AC tumor-containing eyes is at least as great as and generally greater than that found in SC tumor-containing eyes.

As mentioned previously, eyes bearing AC-injected tumors increase in size dramatically through time. Thus, beyond day 14, eyes bearing AC tumors are considerably larger than are their SC-injected counterparts. It was important, therefore, to estimate the number of tumor-specific pTc present per eye. To calculate this, the frequency of pTc per 10^6 lymphocytes was multiplied by the mean number of lymphocytes recovered per eye.
eye. In addition, in order to adjust for the difference in the size of the intraocular tumor, the number of viable tumor cells recovered per eye was also determined. The results of these calculations are displayed in Fig. 6. Following SC P511 inoculation, the number of tumor cells per eye peaked on day 8 p.i. at $5.0 \times 10^6$ and then steadily decreased until no tumor cells were detected on day 18 p.i. In these eyes, the number of pTc per eye peaked on day 14 p.i. (789 pTc/eye) and steadily decreased through day 27 p.i. By contrast, following AC P511 inoculation, the tumor burden on day 8 p.i. was much larger ($1.83 \times 10^7$ tumor cells/eye). Thereafter, the number of viable tumor cells decreased, probably due to cellular necrosis in areas of the eye where the blood supply is limiting. Importantly, a significantly greater number of pTc were present within the AC tumor-containing eyes, as compared with SC tumor-containing eyes, on every day assayed. The maximum number of pTc per eye peaked on day 14 p.i. (3580 pTc/eye) and diminished thereafter. Thus, within AC tumors, the number of pTc per eye is greater than within SC tumors. In addition, the frequency of pTc mirrors the size of the tumor burden, although the time course of pTc infiltration is delayed with respect to changes in tumor burden, within these animals. We conclude from these results that the inability of pTc to differentiate into Tc and to reject AC tumors cannot be ascribed to a lower number of pTc reaching the tumor-containing eye or to their inability to be retained at the site.

**DISCUSSION**

**BALB/c** mice are readily capable of rejecting minor histoincompatible P815 tumor cells injected subconjunctivally, but they are unable to contain and destroy P815 cells injected into the anterior chamber of the eye. Although the DBA/2-specific systemic immune response of mice bearing AC P815 tumors is deviant (these mice display ACAID) (1, 2, 9, 10), their lymphoid organs are primed for DBA/2-specific pTc, just as are the lymphoid tissues of conventionally immune mice that have rejected P815 cells from the subconjunctival space (5, 9). Moreover, tumor-specific precursor Tc can be detected at both SC and AC tumor-containing sites (3). However, fully functional and directly cytotoxic T-cells can be harvested only from eyes in which SC tumors are being rejected (4). One possible explanation for the failure of BALB/c mice to reject AC P815 tumors is that insufficient numbers of pTc gain access to, or are retained by, the tumor-containing site. In the past, our efforts to describe quantitatively the frequency of DBA/2-specific pTc in tumor-bearing eyes has been frustrated by the fact that cell suspensions prepared from these eyes were heavily contaminated with P815 cells. It is well known that P815 cells can interfere with in vitro detection of specific pTc activity. To circumvent this difficulty, we have used a HAT-sensitive variant of P815, termed P511, which has enabled us to assess with more precision the numbers of eye-infiltrating, tumor-specific pTc. Our results indicate that DBA/2-specific pTc can be detected in SC tumor-bearing eyes earlier than in AC tumor sites; however, the delay amounts to only 3 days. Thereafter, the frequency of pTc rises dramatically at both tumor sites. Eventually, a significantly higher frequency of pTc is found in the AC tumor site. We conclude that the failure to reject AC tumors cannot be attributed to either insufficient access to or retention of pTc within this site.

In the solid tissue transplantation literature, considerable evidence exists to suggest that infiltration of pTc into the allograft site is merely one step in the multistage process that eventually culminates in graft rejection (11-13). Terminal differentiation of pTc into fully functional Tc takes place, apparently in situ. Moreover, this step in Tc differentiation is mediated by factors provided by T helper cells (14, 15). By implication, T-cells able to deliver "help" must also infiltrate grafts prior to rejection. We presume that a similar process unfolds at ocular tumor allograft rejection sites. Thus, when BALB/c mice successfully reject P511 or P815 cells injected into the SC, we believe that both pTc and "helper" T-cells infiltrate the site. In fact, preliminary evidence from our laboratory confirms this prediction, since lymphokine-secreting T-cells enter these sites by 8 days postinoculation. It is pertinent to this discussion to point out that mice rejecting SC P815 tumors also display systemic, DBA/2-specific delayed hypersensitivity (6). We suspect that the T-cells responsible for providing help to SC tumor-infiltrating pTc are the same T-cells that mediate delayed hypersensitivity. As we have already reported, fully functional Tc can be detected in these eyes coincident with regression of the SC tumor.

Two possible explanations can be advanced to explain the inability of BALB/c mice to reject AC P815 and P511 tumors. On the one hand, these mice may be unable to provide a source of help for the pTc, which we now know infiltrate these tumors in large numbers. As mentioned above, BALB/c mice that have received AC injections of P815 cells develop a deviant systemic immune response characterized primarily by the failure to display DBA/2-specific delayed hypersensitivity (1). Thus, the fact that P815 cells injected into the AC induce ACAID in BALB/c mice may contribute to their failure to reject the AC tumor since circulating T-cells that mediate delayed hypersensitivity are not generated (16). On the other hand, delivery of helper signals within the eye itself may be impaired. Over the
past several years, our laboratory, as well as others, has demonstrated that the microenvironment of the anterior chamber of the eye is extremely inhospitable to antigen-driven lymphocyte activation (17). In part, the immunosuppressive properties of the ocular environment are dictated by unique cells of the tissues that surround the anterior chamber. Few, if any, conventional antigen-presenting cells can be identified phenotypically or functionally in the cornea, iris, or ciliary body (18). Moreover, cells harvested from the iris and ciliary body have the capacity to suppress directly alloantigen-driven T-cell activation (19). In addition, the aqueous humor is itself suppressive of T-cell activation, a property that can be ascribed in part to the presence of the inhibitory cytokine, transforming growth factor β (20, 21). If, as we believe, the local conversion of infiltrating pTc to Tc is critical to tumor rejection and if this conversion depends upon local delivery of T-cell help, then we propose that the reasons why BALB/c mice are unable to reject AC P815 tumors include their failure to generate systemic T-cells that mediate delayed hypersensitivity and the immunosuppressive properties of the anterior chamber which mitigate against successful delivery of help in situ.

We were surprised to find that, through time, P511 tumors within the AC accumulated far more DBA/2-specific pTc than did their SC tumor counterparts. It is doubtful that this greater pTc frequency at the AC site stems from differences in the total number of pTc generated in the two types of tumor recipients. We have shown in previous experiments that the clonal expansion of pTc within the central lymphatic compartments (lymph nodes, spleen) is quantitatively equivalent following AC and SC injection of tumor cells (5). Rather, we suspect that the greater number of pTc in AC tumor-bearing eyes reflects the greater magnitude of the tumor itself at this site. Using a limit dilution approach to assess intraocular tumor mass, we have previously reported that the number of viable tumor cells in AC tumor-bearing eyes at 8 days p.i. is far greater than the number of tumor cells that can be harvested at this time from eyes that received SC tumor cells (6). Since pTc can readily bind to and form stable conjugates with antigen-bearing tumor cells, we suspect that the ever-expanding number of pTc reflects retention of pTc at the site, bound to the progressively increasing number of tumor cells. The fact that such large numbers of pTc are present in these ocular tumors implies that therapeutic strategies aimed at activating these lymphocytes might be able to initiate a rejection process.

Our experiments also reveal a delay in appearance of tumor-specific pTc in AC tumors, compared to SC tumors. Since only the latter tumors are rejected, we cannot dismiss the possibility that this temporal difference in infiltration of pTc is important to the eventual outcome. However, at this time, we cannot offer a suitable explanation of why early infiltration of a tumor allograft site with pTc should lead to a favorable outcome. Perhaps the early SC tumor-infiltrating lymphocytes, which we identify in our in vitro assays as pTc, have other properties which we have not yet examined. We already know that DBA/2-specific CD8+ lymphocytes that infiltrate the SC tumor site are capable of secreting interleukin 2. It may be that lymphocytes secreted by lymphocytes that infiltrate SC but not AC tumor sites will turn out to be the critical determinants of whether rejection occurs.

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