INDUCTION OF ANTERIOR CHAMBER-ASSOCIATED IMMUNE DEVIATION REQUIRES AN INTACT, FUNCTIONAL SPLEEN*

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When allogeneic P815 mastocytoma cells (derived from DBA/2 mice) are injected into the anterior chamber of the eyes of BALB/c mice, they take advantage of the immunologic privilege within this site (1). The tumor cells proliferate relentlessly until the injected eye is destroyed and/or the tumor invades the cranial vault by direct extension and kills the host. We have reported previously that the intracameral (IC)[1] success of P815 is accompanied by a profound alteration in the capacity of tumor-bearing BALB/c mice to respond to minor histocompatibility antigens of DBA/2 tissues. BALB/c mice with progressively growing intraocular P815 tumors are unable to reject orthotopically grafted DBA/2 skin, although the animals retain the capacity to reject third party C57BL/6 skin grafts (2). The alteration in immune responsiveness in these animals resembled F_1 lymphocyte-induced immune deviation described in rats by Kaplan and Streilein (3). To identify the unique systemic immune responses of animals bearing allogeneic tissues in their anterior chambers we propose the generic term, anterior chamber-associated immune deviation (ACAID).

We have described three separate manifestations of ACAID in BALB/c mice inoculated IC with P815 cells: (*a*) progressive growth of the tumor locally; (*b*) transient, significant growth of tumors at subcutaneous (SC) sites where P815 cells have also been injected; and (*c*) prolonged (and usually indefinite) acceptance of DBA/2 skin grafts placed on their thoracic cages. Our previous studies of the ACAID phenomenon in rats produced evidence that alloantigens presented via the anterior chamber evoke both destructive and protective host immune responses (4). The data further suggested that regulation of the relative participation of protective/destructive host forces was focused within the intact spleen (5). In this paper, we describe the results of experiments designed to examine a putative splenic role in ACAID as it is elicited in BALB/c mice by the IC inoculation of P815 mastocytoma cells.

Materials and Methods

Experimental Animals. Adult female BALB/c (H-2^d) and DBA/2 (H-2^d) mice were purchased from The Jackson Laboratory, Bar Harbor, Maine, and used as experimental subjects between 3 and 5 mo of age.

Tumor Cells. P815 mastocytoma (DBA/2; H-2^d) cells were cultivated in suspension cultures in Falcon 75-cm^2 tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson & Co.,

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[1] *Abbreviations used in this paper:* ACAID, anterior chamber-associated immune deviation; HBSS, Hanks' balanced salt solution; IC, intracameral; SC, subcutaneous.

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Oxnard, Calif.) using Dulbecco's modified Eagle's minimal essential medium supplemented with 10% heat-inactivated fetal calf serum and gentamycin (0.05 mg/ml; Schering Corp., Kenilworth, N. J.). EL-4 lymphoma (C57BL/6; H-2^b) was maintained by serial passage as ascites in C57BL/6 mice. Monocellular suspensions of P815 cells and EL-4 cells were washed in Hanks' balanced salt solution (HBSS) and resuspended in HBSS for SC and IC injections.

Anterior Chamber Injections. A modified quantitative technique for depositing a definite number of tumor cells into the anterior chamber of the mouse eye has been described in detail elsewhere (1). Briefly, a microglass pipette (~80 μm in diameter) was fitted into a sterile infant-feeding tube (5 French; Cutter Laboratories, Inc., Berkeley, Calif.) that was mounted onto a sterile 0.1-ml Hamilton syringe (Hamilton Co., Inc., Whittier, Calif.). A Hamilton automatic dispensing apparatus was fitted onto the loaded syringe and was used to dispense 5-μl quantities of P815 cell suspensions into the anterior chamber of mouse eyes.

SC Injections. 1×10^5 tumor (P815 or EL-4) cells suspended in 0.1 ml of HBSS were injected SC into the right rear flanks at various times relative to anterior chamber injections, as described in Results.

Skin Grafting. Full thickness skin grafts were prepared as described elsewhere (6). Grafts were applied orthotopically and held in place with plaster of Paris bandages. Casts were removed 8 d later and the grafts were inspected daily for evidence of rejection. Destruction was judged complete when all remnants of surface epidermis were gone. Median survival times were calculated where appropriate.

Splenectomy. Mice were anesthetized with 0.6 mg of ketamine hydrochloride (Vetalar; Parke, Davis & Co., Detroit, Mich.) given intramuscularly. Through a left lateral body wall incision the spleen was exteriorized and the splenic pedicle was severed. Trivial local hemorrhage required no ligation of the splenic blood vessels. Control animals were similarly treated but the spleen and the splenic pedicle were left intact (sham splenectomy).

Splenic Reconstitution with Spleen Fragments. Mice were splenectomized and immediately reconstituted with fragments of autochthonous spleens. Briefly, after splenectomy, each whole spleen was aseptically sliced into either 4 (large) or 16 (tiny) pieces. These fragments were immediately re-implanted into the peritoneal cavity of the original donor. Peritoneal and skin incisions were closed with 5-0 chromic catgut and metal wound clips.

Splenic Reconstitution with Spleen Cell Suspensions. Mice were splenectomized and reconstituted with autochthonous, monocellular spleen suspensions. After splenectomy, spleen cell suspensions were prepared by pressing whole spleens through sterile 60-mesh stainless steel screens. The entire cellular contents from each spleen were washed (330 *g* for 10 min) once in HBSS, resuspended in 0.5 ml of HBSS, and injected intraperitoneally into the original donor.

Observation and Recording of IC Tumor Growth. Mice injected IC with P815 cells were observed daily with a dissecting microscope (×8). Ocular tumor growth was scored according to the percent of the anterior chamber occupied by mastocytoma (1). Eyes were observed for pathological changes, including glaucomatous enlargement, corneal opacity and abrasion, perforation of the capsule of the eye and invasion of the orbit by the tumor, neoangiogenesis, and corneal inflammation.

Observation and Recording of SC Tumor Growth. We have previously demonstrated that simultaneous injection of P815 cells into the anterior chamber and into the SC flank of BALB/c mice resulted in the development of palpable SC tumors (1). We employed this phenomenon as an additional measure of ACAID. Panels of splenectomized and sham-operated mice were injected SC and IC with 10^5 P815 cells at different temporal intervals. Controls consisted of splenectomized and sham-operated mice injected IC with 10^5 P815 cells and SC with 10^5 EL-4 cells. The injected flanks of these mice were palpated daily and the diameter of detectable SC tumors was measured with metric calipers.

Results

Effect of Splenectomy on ACAID

ORTHOTOPIC SKIN GRAFT SURVIVAL. P815 cells, at a concentration of 10^5 cells/5 μl, were injected into the anterior chambers of eyes of panels of BALB/c mice that had

been splenectomized or sham operated 7 d earlier. 14 d after IC injection of P815 cells, recipients were given orthotopic DBA/2 skin grafts. Plaster dressings were removed 8 d later and the grafts were observed daily for evidence of rejection. The results are summarized in Table I. DBA/2 skin grafts placed on sham-splenectomized BALB/c mice bearing ocular P815 cells healed well, and in most of these animals remained in healthy condition 30 d after grafting. In contrast, DBA/2 skin grafts placed on BALB/c mice whose spleens had been removed 7 d before IC inoculation of P815 cells were rejected acutely. No grafts survived beyond 11 d. By comparison, the tempo of rejection of DBA/2 skin grafts placed on sham-operated or splenectomized BALB/c mice that received no IC inoculation of P815 cells resembled that of untreated control BALB/c mice; median graft survival times of ~13 d were observed. These data indicate that an intact spleen is essential for ACAID as expressed as prolonged acceptance of DBA/2 skin allografts.

GROWTH OF SC P815 TUMORS. P815 cells (10^5) fail to grow into palpable tumors when injected into the flanks of normal BALB/c mice. We have previously demonstrated that simultaneous injection of P815 cells IC and SC into the flank of BALB/c mice results in the transient growth of SC tumors, another expression of ACAID. To examine the possible participation of an intact spleen in this phenomenon, P815 cells (10^5) were inoculated simultaneously IC and SC into BALB/c mice of the following panels consisting of 8–10 animals each: (*a*) splenectomized, or (*b*) sham operated 7 d before injections. Similar mice that received P815 cells IC and EL-4 cells SC (EL-4 is a lymphoma derived from C57BL/6 mice) were used as controls. SC tumors became palpable in the flanks of sham-operated mice that received P815 cells SC. However, no tumors developed in any of the other panels. The lack of tumor development in the mice of the panel that received P815 SC after splenectomy is consistent with the results of the skin grafting experiments described above, and indicates that an intact spleen is essential for ACAID expressed as SC P815 tumor growth. The data from the recipients of EL-4 cells underscore the fact that ACAID is an immunologically specific state.

GROWTH OF P815 TUMORS IN ANTERIOR CHAMBERS. The fact that P815 cells grow unabatedly in the anterior chamber of BALB/c eyes, but not at other body sites, suggests that progressive IC tumor growth is yet another expression of ACAID. To test the role of the spleen in ACAID expressed IC, panels of BALB/c mice were sham

TABLE I

Effect of Splenectomy on ACAID Induced in BALB/c Mice by IC Inoculation of P815 Cells

Experimental panels		Pattern of DBA/2 skin graft survival, day of inspection										Graft survival*
		8	9	10	11	12	13	14	15	20	30	
SPLX	*P815 IC*											%
No	No	5‡	5	5	5	5	5	2	1	0	0	0
No	Yes	15	15	15	15	15	15	14	14	13	12	80
Yes	No	9	9	9	9	7	7	2	1	0	0	0
Yes	Yes	7	1	1	1	0	0	0	0	0	0	0

Mice were splenectomized on day −7, injected IC with P815 cells on day 0, and grafted with DBA/2 skin on day 14.

* Percent of grafts that survived beyond 30 d.

‡ Data presented as number of grafts surviving on each day of inspection.

operated or splenectomized 7 d before IC inoculation of P815 cells. Eyes of these mice were examined daily with a dissecting microscope (×8) and tumor growth scored according to the proportion (%) of the anterior chamber occupied by grossly visible tumor tissue. The results, shown in Fig. 1, reveal that both spleen-intact and splenectomized mice developed ocular tumors. In spleen-intact mice, the tumors grew rapidly, filling the anterior chamber within 7 d after injection. Progressive growth subsequently perforated the globe by the 14th d and invaded the orbit. In contrast, the pace of tumor growth in the eyes of splenectomized mice was considerably slower, the anterior chamber not being filled with tumor until 17 d after inoculation. Moreover, perforation of the globe and invasion of the ocular orbit was never observed. Thereafter, gradual resolution of the tumor took place without evidence of an acute inflammatory response. Whereas tumor cells in the eyes of spleen-intact animals invaded the cranial vault, no such invasion was observed in splenectomized animals. Instead, all of these animals survived, although resolution of the ocular tumor resulted in destruction of the eye. Thus, ACAID, which permits aggressive

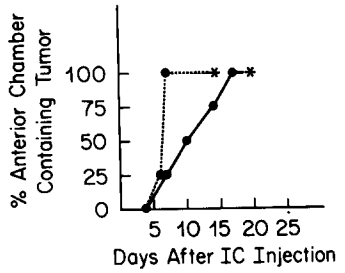

FIG. 1. Growth patterns of anterior chamber tumors in splenectomized (——) and sham-splenectomized (- - -) BALB/c mice following IC inoculation of 10^5 P815 mastocytoma cells. Ischemic necrosis, (*). Each point represents the mean accumulation of tumor within the anterior chamber as assessed by visual inspection with a dissecting microscope, e.g., 25 = 25% of anterior chamber occupied by tumor mass. There were 10 mice per group.

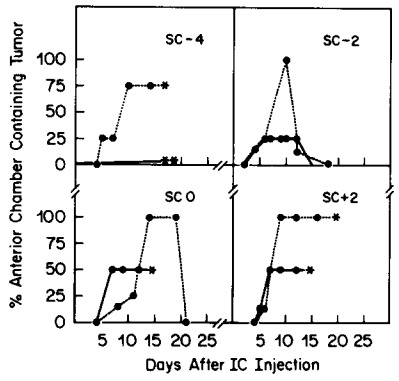

FIG. 2. Influence of P815 mastocytoma cells inoculated SC on growth patterns of IC inoculated P815 cells. BALB/c mice were either splenectomized (——) or sham splenectomized (- - -) 7 d before IC inoculation of 10^5 P815 cells. Label in upper right corner of each panel represents time interval between SC and IC inoculations of P815 cells. Minus sign indicates that SC inoculation preceded IC inoculation by (N) days. Responses that reach the baseline signify that tumor resolution has taken place. There were 10 animals per group. Necrosis, (*). No ocular tumors developed, (**).

invasion of the eye and periocular tissues by P815 tumor, is dependent upon an intact spleen.

ROLE OF SPECIFIC SENSITIZATION. The next series of experiments was designed to determine whether immunization of BALB/c mice with DBA/2 alloantigens could influence the growth of P815 cells injected IC into splenectomized recipients. The experimental protocol was: panels of BALB/c mice were sham operated or splenectomized 7 d before IC inoculation of P815 cells. Mastocytoma cells (10^5) were injected SC into these mice 2 or 4 d before, simultaneous with, or 2 or 4 d after the IC injection. Controls were panels of mice that were sham operated or splenectomized and inoculated IC with P815, but received no SC injection of tumor cells. Growth patterns of intraocular tumors, evaluated and scored as previously described, are shown in Fig. 2. In general, SC injection of BALB/c mice with P815 cells is capable of immunizing the hosts, thereby thwarting subsequent growth of the intraocular neoplasms. In spleen-intact mice, the SC injection must precede the IC injection by at least 7 d to achieve this effect. This requisite interval was foreshortened in splenectomized mice. SC injection of P815 cells as late as 4 d before IC challenge, prevented the growth of ocular tumors in these animals. As the interval between SC and IC injections was shortened, and especially when the SC injection followed the IC injection, the effectiveness of the host anti-tumor response became progressively more feeble. Not only did larger tumor masses develop IC, but intense inflammatory reactions were observed. Although the host response effectively destroyed the tumor cells and the hosts survived, the eyes were destroyed in the process. Thus, splenectomy can conspire with a regimen of specific sensitization to interfere with the development of ACAID. However, careful timing of the two components appears to be required if preservation of a functional eye is desired.

Reconstitution of ACAID in Splenectomized Mice. We next attempted to identify, if possible, the splenic mechanism(s) that is important in the development of ACAID. We first prepared single cell suspensions from normal BALB/c spleens and inoculated one-donor equivalents intraperitoneally into recipient BALB/c mice whose spleens had been removed surgically. 4 d later, P815 cells were inoculated IC in these recipients; 2 wk later, they were grafted orthotopically with DBA/2 skin. The survival of these test grafts and the growth pattern of the intraocular tumors were examined and scored daily. In companion experiments, normal BALB/c spleens were sliced into (*a*) large fragments, 4 per spleen; or (*b*) tiny framgents, 16 per spleen; one donor-equivalent was placed intraperitoneally into freshly splenectomized BALB/c recipi-

TABLE II

Restoration of ACAID in Splenectomized BALB/c Mice Inoculated IC with P815 Cells

Reconstitution with	Pattern of DBA/2 skin graft survival, day of inspection										Fate of ocular tumors*
	8	9	10	11	12	13	14	15	20	30	
None	7	1	1	1	0	0	0	0	0	0	7/0
Monodisperse spleen cells	9	7	5	5	5	5	5	5	4	4	7/2
Large spleen fragments	8	5	4	4	4	4	4	4	4	3	0/8
Small spleen fragments	6	4	2	1	0	0	0	0	0	0	6/0
No splenectomy	8	8	8	8	8	8	7	7	7	6	0/8

* Ocular tumors were either destroyed or grew progressively into enormous orbital tumors: destroyed/progressive growth.

TABLE III

Effect of Timed Splenectomy on Development of ACAID in BALB/c Mice Inoculated IC with P815 Cells

Time of splenectomy in relation to IC P815 inoculation	Pattern of DBA/2 skin graft survival, day of inspection										Graft survival*
	8	9	10	11	12	13	14	15	20	30	
											%
7 d before	7	1	1	1	0	0	0	0	0	0	0
4 d after	7	7	6	2	0	0	0	0	0	0	0
6 d after	8	6	3	2	1	0	0	0	0	0	0
8 d after	9	8‡	8	8	8	8	8	8	5	5	55
10 d after	6	6	6	5	5	5	5	5	5	4	67
14 d after	10	10	10	10	10	10	10	10	10	9	90
None	8	8	8	8	8	8	7	7	7	6	75

* Percent of grafts that survived beyond 30 d.

‡ All of these grafts underwent severe inflammatory crises, although most recovered.

ents. These animals also were inoculated IC with P815 cells and subsequently grafted with DBA/2 skin. The results of these experiments are presented in Table II. From the standpoint of test skin allografts, ACAID was restored in almost 50% of the splenectomized animals that had received monodispersed spleen cells or large splenic fragments: these animals accepted DBA/2 skin grafts for more than 30 d. However, recipients of spleen cell suspensions displayed patterns of ocular tumor growth more consistent with splenectomized recipients; their IC tumors grew modestly, then resolved, leaving behind a phthisic, nonfunctional eye. In contrast, ocular tumors grew to enormous size in splenectomized animals reconstituted with large splenic fragments; this pattern of growth resembled closely the growth of P815 cells in anterior chambers of spleen-intact BALB/c mice. Necropsy of these animals 30 d after grafting with DBA/2 skin revealed large nodules of easily identifiable and viable splenic tissue attached to peritoneal surfaces. As a consequence, these fragments assumed the intact spleen's role of promoting ACAID. In contrast, splenectomized recipients of tiny fragments of spleen failed to express ACAID; instead, they displayed ocular tumor growth and graft rejection patterns consistent with the asplenic state. This unexpected finding suggests that the spleen's contribution to the development of ACAID is complicated and may comprise several components. One component resides among lymphoid cells that are dissociable from normal spleens rendered into single cell suspensions. However, there must be another component(s) that resides among nonlymphoid stromal cells and/or a unique architectural quality of the spleen; it is this latter component(s) that allows large, but not small, splenic fragments to promote immune deviation in splenectomized mice who develop progressively growing intraocular P815 tumors.

Time Course of Splenic Dependence of ACAID. The experiments in which P815 cells were injected at varying intervals into IC and SC sites suggested that development of ACAID is a dynamic process. The results of the splenectomy experiments described thus far merely confirm that removal of the spleen before IC injection of P815 cells prevents the development of ACAID. The next experiments were designed to examine the temporal sequence during which the spleen exerts its putative role. Accordingly, panels of BALB/c mice, inoculated IC on day 0 with P815 cells, were subjected to

splenectomy at 4, 6, 8, 10, and 14 d thereafter. All mice were test-grafted orthotopically with DBA/2 skin on day 14 and graft survival was assessed as a measure of the development of ACAID. The results of these experiments, summarized in Table III, are particularly illuminating. Whatever the role of the spleen in the development and expression of ACAID, it has not had sufficient time to exert its action within 4 d of IC injection of P815 cells. Mice splenectomized at 4 d rejected DBA/2 skin grafts acutely. By 14 d, however, the splenic role in ACAID appeared to be complete: 9 of 10 mice splenectomized at this time retained their test skin grafts indefinitely (beyond 30 d). In fact, the spleen had accomplished its task in some animals as early as 8 d; the effect was virtually complete in mice injected IC 10 d earlier with P815 cells. These results indicate that a specific interval (between 6 and 10 d after IC inoculation) is required for an intact spleen to promote the development of ACAID. Of equal interest is the realization that an intact spleen is not required thereafter for the continued expression of ACAID. In mice whose spleens were removed 14 d after IC injection of P815 cells, progressive tumor growth was observed within the orbit. The tumor relentlessly invaded the cranial vault as though the host's spleen were intact.

Discussion

BALB/c mice inoculated either SC or IC with allogeneic P815 mastocytoma cells suffer profoundly different consequences. The SC challenge elicits a vigorous host immune response that prevents the tumor cells from establishing a viable graft; from clinical appearances, these mice are perfectly healthy. In contrast, the IC challenge represents a major threat to the health of the host. At the very least, the tumor invades the eye locally, causing blindness. In most IC-injected animals, however, a systemic perturbation of the host's immune response occurs, a phenomenon we have called "anterior chamber-associated immune deviation". ACAID, which expresses itself as progressive tumor growth within the eye, transient growth of SC injected P815 cells, and prolonged acceptance of DBA/2 skin grafts, is immunologically specific.

The studies reported here focus on the role of the spleen in the development of ACAID. BALB/c mice, otherwise destined to die from the effects of tumor cells growing within their eyes, survive this challenge if their spleen has been removed before IC inoculation. Moreover, in the absence of a spleen, a specific immunization protocol with SC injected P815 cells can be devised that results in an immune response so effective it destroys the tumor before the eye's functional integrity has been disrupted.

Claims for unique immunologic properties of the spleen have been made many times (7–9). Recently, with the advent of the notion of immunoregulation and a prominent role for suppressor cells, the spleen has come to be regarded as the major, though not the only, site in which suppressor cells develop, mature, and/or are amplified. Two independent laboratories have produced data consistent with the hypothesis that the spleen harbors populations of suppressor cells that can down-regulate alloimmune responses to minor histocompatibility antigens (10, 11). However, other investigators failed to find comparable splenic properties in similar alloimmune responses (12, 13). Controversy also surrounds the spleen's putative involvement in contact hypersensitivity (14) and tumor immunity (15, 16). The controversy stems at least in part from heterogeneity among the immune phenomena being studied. The dramatic and highly reproducible role identified for the spleen in

ACAID offers an ideal opportunity to investigate the generation of suppression mechanisms within this organ.

Development of the splenic-dependent phase of ACAID begins about 4 d after P815 cells are inoculated IC into BALB/c mice. During the next 6 d, this phase is virtually completed, and ACAID becomes established as a systemic pattern of immune reactivity, freed of its splenic dependence. It is presumed that during this 6-d "window", alloantigenic signals from the anterior chamber of the eye are delivered intravenously to the spleen, wherein they are transduced into a predominately protective rather than destructive immune response. The fact that the spleen is no longer required for ACAID after 10 d implies that the protective response has disseminated systemically and is self-sustaining. We have no direct information about the cellular basis of the process taking place within the spleen. Reconstitution experiments with monodisperse spleen cells and with splenic fragments transplanted intraperitoneally into splenectomized mice only partially resolved the dilemma. The results suggest that although splenic cells participate in the induction of ACAID, the whole of the spleen's role must be greater than the sum of its parts. Perhaps this realization addresses unique architectural features of this organ, and/or its distinctive stroma and microenvironment. Numerous strategies have been employed in other murine systems to identify subpopulations of functionally distinct lymphocytes, and we intend to bring those to bear on these phenomena. However, considerable ingenuity will be required to analyze that component of the spleen's role in ACAID that is not restored solely by monodisperse spleen cells, but requires the use of large (but not small) splenic fragments.

In this model, the precision with which the host immune response destroys intraocular tumors varies considerably. In the experiments reported here, splenectomy before IC ioculation of P815 cells equips the host with a vigorous immune response that destroys the ocular tumor; but the anatomic integrity of the eye is destroyed in the process. A much more precise immune response is evoked in splenectomized BALB/c mice who are immunized with P815 cells SC several days before intraocular challenge with P815 cells; tumor destruction within the eyes of these animals is accomplished without significant disruption of the functional integrity of the eye. This is the second instance in which an elegantly specific, yet highly effective alloimmune response has been generated in animals with ACAID. We have recently found that, if the immunogenetic disparity between P815 cells (DBA/2) and their potential IC recipients involves both *K* and *D* regions of the *H-2* complex, the tumor cells are destroyed, whereas the eye is spared (17). Alloantigenic differences restricted to only one or the other of these two loci evoke an immune response that causes innoncent bystander ocular damage while destroying its specific tumor targets. Thus, we have been able to identify several important factors that govern the precision of the alloimmune response as it expresses itself within the eye. If these experiments address the clinical realm of ocular neoplasms, then perhaps successful therapeutic strategies can be developed that take these factors into account.

Summary

Anterior chamber-associated immune deviation (ACAID) expresses itself in BALB/c mice inoculated intracamerally with P815 cells in three ways: progressive growth of the tumor within the eye, transient growth of P815 cells injected subcutaneously, and

prolonged acceptance of DBA/2 skin allografts. The spleen was found to play a crucial role in the development of ACAID. Splenectomized animals bearing intracameral P815 tumors reject DBA/2 skin grafts in an accelerated manner. A functioning spleen was required during the first 10 d after intracameral inoculation of P815 cells, but not thereafter. Reconstitution experiments revealed that the spleen's ability to support the induction of ACAID depends partly upon its constituent lymphoid cells, but also upon either a stromal component or a unique architectural arrangement that can only be restored with splenic fragments. The data hold promise that therapeutic protocols using appropriately timed splenectomy and specific immunization can be devised to induce hosts bearing intraocular tumors to mount an immune response sufficiently vigorous to destroy the tumor within the eye, and sufficiently precise to preserve the functional and anatomic integrity of the eye.

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