GENERATION AND DECAY OF THE IMMUNE RESPONSE TO A PROGRESSIVE FIBROSARCOMA

II. Failure to Demonstrate Postexcision Immunity After the Onset of T Cell-mediated Suppression of Immunity

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It was shown in the preceding companion paper (1) that early progressive growth of an immunogenic fibrosarcoma results in the acquisition by its syngeneic host of concomitant immunity to growth of a challenge implant, and of tumor-sensitized Ly-1^-2^+ T cells that are capable of adoptively immunizing against an established tumor in γ-irradiated recipients. It was shown, in addition, that after the tumor reaches a certain size, concomitant immunity and tumor-sensitized T cells are progressively lost, and that this is associated with the generation of Ly-1^+2^- suppressor T cells capable, on passive transfer, of inhibiting the expression of adoptive immunity against an established tumor in T cell-deficient (TXB)[1] recipients. The evidence was interpreted as showing that progressive tumor growth evokes a mechanism of T cell-mediated concomitant immunity that is down-regulated by suppressor T cells before it develops sufficiently in magnitude to destroy the tumor. This knowledge that progressive tumor growth evokes a concomitant immune response that subsequently decays under the influence of suppressor T cells must surely be taken into account in assessing the results of the most commonly used test for tumor immunogenicity (2–4): determining whether surgical removal of a tumor results in immunity to growth of a subsequent implant of cells of that tumor. It might be expected, in this regard, that the immunological consequences of excising an immunogenic tumor would be determined by whether excision is performed during the generation of concomitant immunity or after it has decayed under the influence of suppressor T cells. It is apparent from the literature (5) that little is known about the relationship between the ability to demonstrate postexcision antitumor immunity and the state of concomitant immunity of the host at the time of excision.

This paper will show that excising the meth A fibrosarcoma during the time that concomitant immunity is being generated results in the preservation of this immunity for a long period of time. In contrast, excising the tumor after T cell-mediated suppression of concomitant immunity does not result in the reemergence of immunity. Instead, the host remains unresponsive to the meth A tumor

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[1] *Abbreviations used in this paper:* PBS, phosphate-buffered saline; TXB, T cell-deficient.

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and retains a population of suppressor T cells capable of passively transferring suppression for at least 31 d.

Materials and Methods

The materials and methods used in this study are described in the companion paper (1), except regarding tumor excision and the SA-1 sarcoma. Tumor excision was performed on tumors that were initiated by implanting 10^6 meth A cells intradermally in the midline of the abdomen. At specified days, the tumor-bearing mice were anesthetized with Nembutol (Abbot Laboratories, North Chicago, IL) and the tumors were removed together with 3 mm of the surrounding skin and abdominal wall with scissors. The incision was closed with surgical clips and the mice observed for the reemergence of a tumor at the site of excision or the development of lymph node metastases. Of mice observed over a period of 60 d, only 5% developed lymph node enlargement, and this occurred within 4 wk. This is in keeping with the fact that the meth A fibrosarcoma is poorly metastatic. SA-1 sarcoma cells, syngeneic in A/J mice, were stored over liquid nitrogen. For each experiment a vial was thawed, cells washed in phosphate-buffered saline (PBS), and 10^6 of them used to initiate ascites tumors in syngeneic mice. The tumor cells were harvested 4 d later in heparinized PBS, washed, and resuspended appropriately in PBS. Footpad tumors were initiated by injection of 10^6 SA-1 cells in the right hind footpad.

Results

Postexcision Immunity Depends on Possession of Concomitant Immunity at Time of Excision. By showing that the removal of chemically induced, transplantable tumors can result in specific immunity of the host to growth of a subsequent implant of tumor cells, Foley demonstrated in 1953 (6) that syngeneic tumors can possess transplantable rejection antigens. It seems to have been assumed from the time of this demonstration that it is the surgical removal of the tumor that causes the emergence of immunity. However, in view of the knowledge that the growth of immunogenic tumors can result in the generation of concomitant immunity to growth of a tumor cell implant (1, 7), it is possible that there is no need to surgically remove these tumors to demonstrate their immunogenicity. It is possible, instead, that by preventing death of the host, removal of the tumor simply serves to allow an already acquired mechanism of immunity to persist. In keeping with this possibility, one might expect that immunity to the growth of a challenge implant would not result whether excision is performed before concomitant immunity is generated or after it decays under the influence of suppressor T cells. To investigate these possibilities, mice whose 3, 6, 9, or 16 d meth A tumors had been excised were tested up to 4 wk after excision for their ability to express immunity to growth of a 10^6 challenge implant given in a hind footpad. It can be seen in Fig. 1 that only the excision of a 6 or 9 d tumor resulted in a significant level of long-lived immunity to the growth of the standard challenge implant, and that this immunity existed at the time the tumors were excised. In contrast, excision of a 3-d tumor resulted in a low level of immunity that was very short-lived, and excision of a 16-d tumor resulted in practically no immunity to a challenge implant given up to 28 d later. Thus, on the basis of the results in the preceding companion paper (1), we suggest, on the one hand, that excising a 3-d tumor failed to result in postexcision immunity because the 3-d tumor was not large enough to have caused the generation of a significant level of concomitant immunity. On the other hand, the 16-d tumor was more

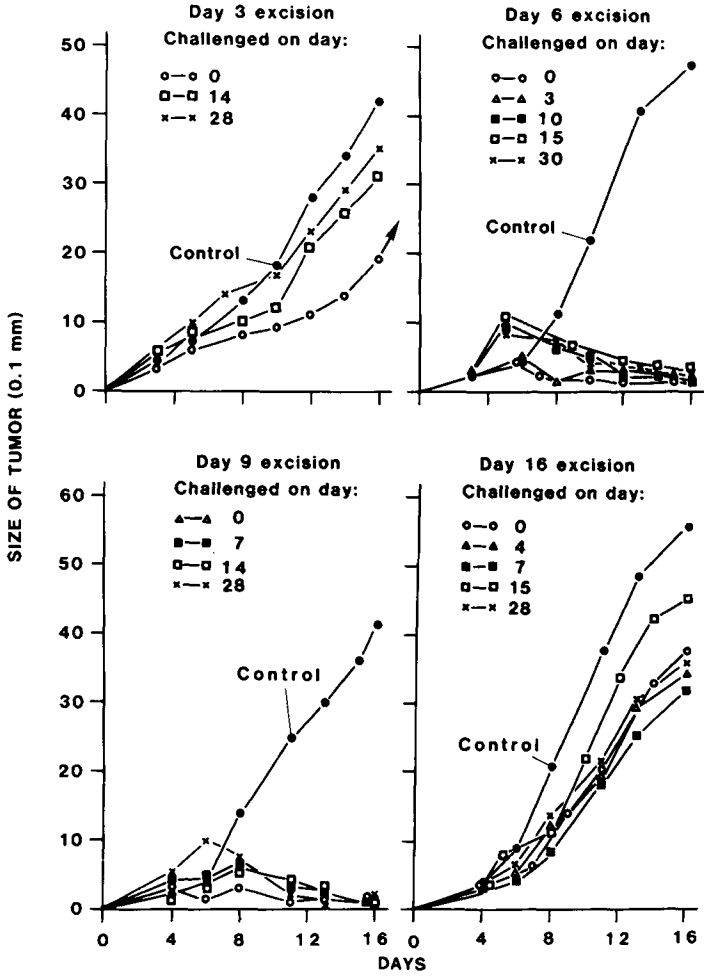

FIGURE 1. The immunological consequence of surgical removal of a 3, 6, 9, or 16 d meth A tumor. Removal of a 6 or 9 d tumor left the host with immunity to growth of an intra-footpad implant of 10^6 tumor cells. In contrast, excision of a 3-d tumor left the host with partial and short-lived immunity to growth of the same sized implant, and excision of a 16-d tumor left the host with practically no immunity at all. Means of five mice per group.

than large enough at the time of excision to have caused the generation of T cell-mediated suppression of concomitant immunity.

Failure of Immunity to Emerge After Excising a 16-d Tumor Is Associated with an Inability to Regenerate Concomitant Immunity to a Second Tumor. The foregoing results show that excising a tumor that is large enough to have induced a state of T cell-mediated immunosuppression fails to result in the emergence of postexcision immunity to an implant of tumor cells. This could mean that excision of the tumor either allowed the host to return to a normal state of immunological responsiveness to the tumor or caused the state of T cell-mediated immunosuppression that existed at the time of tumor excision to persist. If the latter possibility is correct, then it would follow that a host that has had its 16-d tumor

removed should be unable to generate concomitant immunity to growth of a second tumor. That this proved to be the case is shown in Fig. 2 where it can be seen that whereas mice bearing a meth A tumor for the first time generated concomitant immunity to a tumor implant by day 9 of tumor growth, mice bearing a second 9-d meth A tumor that was initiated 1 wk after the excision of a 16-d primary tumor, failed to express concomitant immunity to the same sized

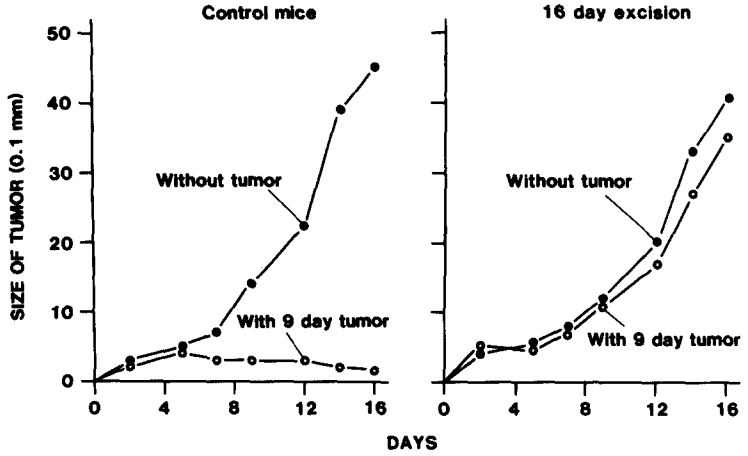

FIGURE 2. Evidence that a host whose 16-d meth A tumor is surgically removed does not regenerate to a normal state of immunological responsiveness to this tumor, as indicated by the absence of a capacity to regenerate concomitant immunity to a second tumor. (*Left*) Growth of an intra-footpad challenge of 10^6 tumor cells in control mice (without a tumor) and mice bearing a 9-d primary tumor. (*Right*) Behavior of the same-sized intra-footpad challenge implant in mice whose 16-day tumors were removed 16 d earlier (without tumor) and mice whose 16-d tumors were removed 16 d earlier and were bearing a 9-d tumor initiated intradermally in the belly region 7 d after excision of the first tumor. Concomitant immunity was not generated against the second tumor. Means of five mice per group.

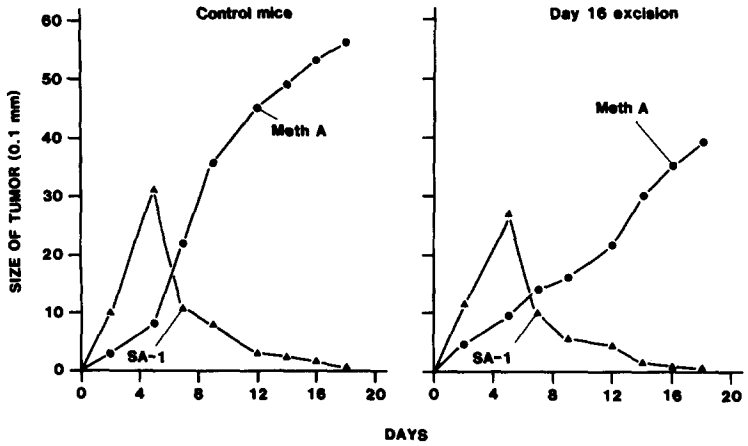

FIGURE 3. Evidence that a host whose 16-d meth A tumor is surgically removed 2 wk earlier retains the ability to reject an allogeneic tumor. (*Left*) Growth of an intra-footpad challenge of 10^6 allogeneic SA-1 or 10^6 meth A cells in normal control mice. (*Right*) Growth of the same-sized implants of SA-1 cells or meth A cells in postexcision mice.

implant. This result indicates, therefore, that the host remained immunosuppressed after the removal of its 16-d tumor. However, this immunosuppression does not represent a state of generalized nonspecific immunosuppression, as evidenced by the retained ability of the animals to reject an implant of an allogeneic tumor, the SA-1 sarcoma. It can be seen in Fig. 3 that the SA-1 grew to the same size in control and postexcision mice, before being rapidly rejected with the same efficiency by both.

Postexcision Immunosuppression Is Associated with the Protracted Possession of Ly-1$^+$2$^-$ T Cells Capable of Passively Transferring Suppression to TXB Recipients. If, as the preceding results suggest, excising a tumor that is large enough to have induced immunosuppression does not result in the return of normal immunological reactivity, then it is likely that the host retains the suppressor T cells that were generated before excision was performed. This was investigated by excising 16-d tumors from a number of mice and dividing them into groups according to whether they were used as donors of suppressor T cells just before excision or 1, 7, 21, or 31 d later. Suppression was measured in terms of the capacity of one organ equivalent (1.5×10^8) of spleen cells to inhibit, on passive transfer, the capacity of one organ equivalent of immune spleen cells infused 3 h earlier to cause the regression of an established tumor in a TXB test recipient (8, 9).

It can be seen in Fig. 4 that the ability of spleen cells from donors with a 16-d tumor to inhibit the expression of adoptive immunity did not disappear after excision of the tumor. Instead, appreciable suppression of the expression of adoptive immunity could be demonstrated with spleen cells harvested up to 31 d after tumor excision. It is apparent, however, that suppression slowly and progressively decayed over this 31 d period.

The cells with suppressor function present in the spleen 2 wk after the excision

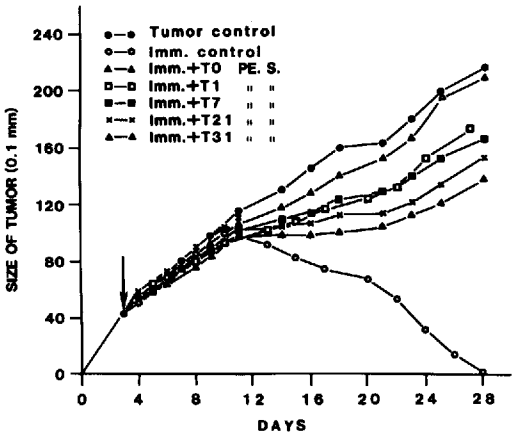

FIGURE 4. Mice that had their 16-d tumors removed continued to possess splenic T cells capable, on passive transfer, of suppressing the expression of adoptive immunity. When TXB recipients bearing 3-d tumors were infused with 1.5×10^8 spleen cells from donors preimmunized 3 wk earlier with 10^6 meth A cells admixed with 100 μg of *C. parvum*, the tumors in all of these recipients underwent complete regression (Imm. control). Tumor regression failed to occur, however, if the recipients were also infused 3 h later with one organ equivalent ($\sim 1.5 \times 10^8$) of spleen cells from donors whose 16-d tumors were removed from 1 d earlier (Imm. + T1 PE.S) to 31 d earlier (Imm. + T_{31} PE.S). Means of five mice per group.

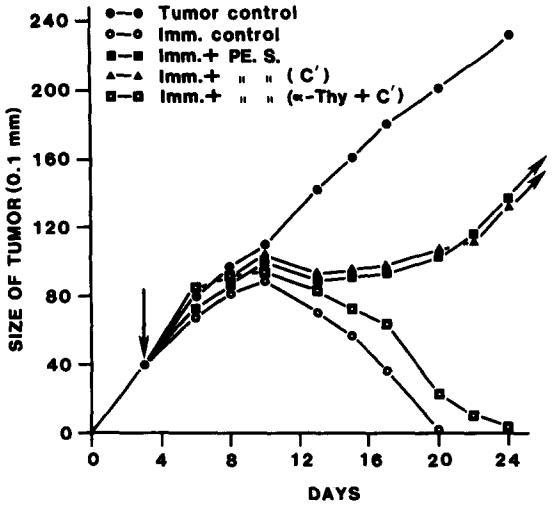

FIGURE 5. The suppressor cells in the spleen of mice whose 16-d tumors were removed 2 wk earlier were T cells. Infusion of immune cells alone (Imm. control) into TXB recipients bearing a 3-d tumor (arrow) resulted in complete tumor regression. However, infusion 3 h after immune cells of 1.5×10^8 spleen cells from donors whose 16-d tumors were removed 14 d earlier (Imm. + PE.S [postexcision suppressor cells]) prevented tumor regression from occuring. This suppressor activity was eliminated by treating the spleen cells with anti-Thy-1.2 antibody and C′ but not with C′ alone. Means of five mice per group.

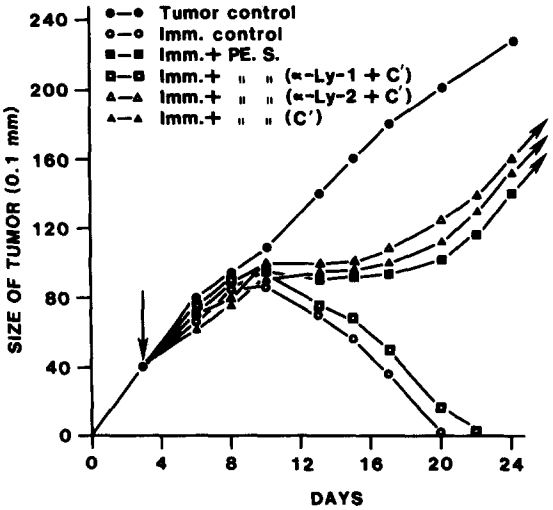

FIGURE 6. Evidence that postexcision suppressor T cells are Ly-1^+2^-. An established tumor in TXB recipients underwent complete regression after the infusion of immune cells alone (Imm. control) but not after infusion of immune T cells plus suppressor T cells (Imm. + PE.S) from suppressor donors whose 16-d tumors were removed 2 wk earlier. The suppressor function of the spleen cells was eliminated by treatment with anti-Ly-1 antibody and C′, but not by treatment with anti-Ly-2 antibody and C′. Means of five mice per group.

of a 16-d tumor were T cells, as evidenced by the finding (Fig. 5) that they were functionally eliminated by treatment with anti-Thy-1.2 antibody and complement. Moreover, like the T cells responsible for active suppression of concomitant immunity in the preceding paper (1), the suppressor T cells that were retained after the excision of a 16-d tumor were of the Ly-1^+2^- phenotype, in that they were eliminated by treatment with anti-Ly-1 antibody and complement but not by anti-Ly-2 antibody and complement (Fig. 6).

Discussion

This study shows that predicting the immunological consequences of the surgical removal of an immunogenic tumor requires a knowledge of the kinetics of the host's concomitant immune response to the tumor. According to the results in the preceding companion paper (1), growth of the nonmetastasizing meth A fibrosarcoma in its syngeneic host results, between days 6 and 9, in the generation of concomitant immunity to the growth of a tumor implant and in the parallel generation of Ly-1^-2^+ T cells capable of passively transferring immunity against an established tumor to γ-irradiated recipients. In addition, the companion paper showed that both the decay of concomitant immunity and the loss of Ly-1^-2^+-sensitized T cells that occurs after day 9 of tumor growth is associated with the progressive generation of Ly-1^+2^- suppressor T cells able to suppress the expression of adoptive immunity against an established tumor in TXB recipients. It perhaps is not surprising, therefore, that the present paper shows that in order for tumor excision to result in immunity to growth of a subsequent implant of tumor cells, excision must be performed while concomitant immunity is being generated and expressed. Therefore, the excision of a tumor does not cause immunity to be generated, but serves to preserve a state of immunity that already exists and which otherwise would undergo rapid T cell-mediated suppression if the tumor were allowed to increase in size. The results suggest that earlier categorization of tumors as immunogenic, on the basis of postexcision immunity to growth of a challenge implant (2–4, 10–13), may not have required excision of the tumors at all. This study also shows that it is possible, by excising a tumor when it is too small to evoke the generation of concomitant immunity, or when it is large enough to induce T cell-mediated suppression of immunity, to falsely conclude that a tumor is nonimmunogenic. This might be the reason why a given tumor can be immunogenic in the hands of some investigators but nonimmunogenic in the hands of others.

However, this study does not provide information about the mechanism of postexcision immunity. It remains possible, therefore, that it is functionally different from the concomitant immunity that exists at the time of excision. It is likely, for example, that if concomitant immunity represents a state of active immunity mediated by cytolytic effector T cells, as suggested in the companion paper (1), then excising a 9-d tumor might result in the rapid decay of this active immunity and in the subsequent emergence of a long-lived state of immunological memory. Indeed, recent studies in this laboratory (Bursuker and North, manuscript in preparation) have revealed that long-lived, postexcision immunity is based on the possession of a T cell population physiologically different from the effector T cells that mediate the concomitant immunity that exists at the time

excision is performed. Needless to say, a state of immunological memory never develops if a tumor is allowed to grow progressively, because the host dies. But even if the host were to survive for a long period of time, immunological memory still would fail to develop, because an increasing load of tumor antigen would result in the generation of a state of T cell-mediated immunosuppression. Moreover, according to the results of this study, the state of immunosuppression persists even after the tumor that caused its generation is excised. Consequently, a host that has had a 16-d meth A tumor removed remains unable to regenerate concomitant immunity to a second tumor and continues to possess, for at least a 31-d period, Ly-1^+2^- splenic T cells capable, on passive transfer, of suppressing the expression of adoptive immunity against an established tumor in TXB test recipients. Therefore, the host continues to possess suppressor T cells with the same Ly phenotype as the suppressor T cells that actively suppressed concomitant immunity (1). The level of suppression, however, progressively decays over this 31-d period. Moreover, a more recent study (Bursuker and North, manuscript in preparation) has revealed that T cells able to suppress the expression of adoptive immunity in tumor-bearing TXB recipients disappeared by day 60 postexcision, but that the mice surprisingly continued to exhibit diminished capacity to generate concomitant immunity. This study has shown, in addition, that the diminished capacity for generating concomitant immunity is associated with an ability to regenerate suppressor T cells in an accelerated manner, indicating the possession of "memory suppression" as recently shown by results from another laboratory (14). The persistence of this long-lived state of T cell-mediated immunosuppression in the apparent absence of antigen, likens tumor-induced immunosuppression to immunological tolerance of the type that can be generated against histocompatibility antigens (15, 16). For it is now well established that tolerance of histocompatibility antigens is actively sustained by suppressor T cells that enable it to be passively transferred to appropriate recipients (17).

It should be pointed out finally that because surgical removal of an immunogenic tumor does not result in the abridgement of tumor-induced immunosuppression, it is unlikely that immunotherapy of systemic disease will be more successful if performed after the removal of the primary tumor burden. For immunotherapy to be successful under these conditions, agents would need to be administered that would preferentially eliminate suppressor T cells.

Summary

This study shows that surgical removal of the meth A fibrosarcoma from its semisyngeneic host fails to result in postexcision immunity to growth of a tumor implant unless the host already has acquired a mechanism of concomitant immunity to growth of an implant. Therefore, tumor excision does not cause immunity to be generated but preserves a mechanism of concomitant immunity that already exists and which otherwise would eventually undergo down-regulation under the influence of suppressor T cells. Removal of the tumor after it has grown large enough to cause the T cell-mediated suppression of concomitant immunity does not result in the reemergence of immunity. Instead, the host remains unable to generate concomitant immunity to a second tumor for a long

period of time and retains, for at least 31 d, suppressor T cells able to passively transfer suppression to appropriate recipients. Like the suppressor T cells responsible for active suppression of concomitant immunity, the suppressor T cells responsible for "memory" suppression are of the Ly-1^+2^- phenotype. The results indicate that progressive tumor growth results in a state of immunological tolerance of tumor-specific, transplantation antigens that can persist in the apparent absence of tumor antigens.

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