

T CELL-MEDIATED IMMUNOSUPPRESSION AS AN
OBSTACLE TO
ADOPTIVE IMMUNOTHERAPY OF THE P815
MASTOCYTOMA AND ITS METASTASES*

BY EARL S. DYE AND ROBERT J. NORTH

From the Trudeau Institute, Inc., Saranac Lake, New York 12983

The numerous reports (1) showing that suppressor T cells are generated in response to growth of immunogenic tumors serve to provide an explanation for the paradoxical growth of these tumors in their immunocompetent syngeneic hosts. A recent publication (2) from this laboratory revealed, for example, that progressive growth of a transplantable murine tumor, the Meth A fibrosarcoma, results in the generation in the host of a T cell-mediated state of immunosuppression that prevents the regression of this tumor by passively transferred, tumor-sensitized T cells from immune donors. The presence of suppressor T cells in the tumor-bearing host was revealed in two ways: first, by showing that in order to demonstrate adoptive T cell-mediated regression of established tumors, it was necessary to use tumor-bearing recipients that had been made T cell deficient by thymectomy and gamma irradiation; second, by demonstrating that adoptive T cell-mediated regression of tumors in these T cell-deficient recipients could be inhibited by prior infusion of splenic T cells from T cell-intact, tumor-bearing donors. It was concluded that the failure of passively transferred, sensitized T cells to cause tumor regression in T cell-intact mice was caused by the presence in these mice of a tumor-induced state of T cell-mediated immunosuppression.

The main purpose of this paper is to show that a strikingly similar mechanism of immunosuppression is generated in response to growth of the P815 mastocytoma. It will show, in addition, that passive transfer of sensitized T cells not only causes complete regression of the primary tumor but also causes the destruction of tumor metastases.

Materials and Methods

Mice. B6D2 (C57BL/6 × DBA/2), F₁, CB6 (BALB/c × C57BL/6)F₁, DBA/2, and BALB/c mice of either sex were used when they were between 8 and 12 wk of age. They were supplied by the Trudeau Institute Animal Breeding Facility.

Tumors. The P815 mastocytoma syngeneic in DBA/2 mice was originally obtained from Dr. Virginia Evans of the National Cancer Institute, Tissue Culture Section, Bethesda, Md. The Meth A fibrosarcoma syngeneic in BALB/c mice was obtained from Dr. Lloyd J. Old of the Memorial Sloan-Kettering Cancer Center, New York. The tumors were subcultured for

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several weeks in Fisher's medium (Grand Island Biological Co., Grand Island, N. Y.) containing 15% fetal calf serum (FCS)¹ before being passaged several times as ascites in the peritoneal cavities of syngeneic mice. The tumor cells thus grown were washed and resuspended in Fisher's medium containing 20% FCS and 10% dimethyl sulfoxide, dispensed into a large number of small vials, and cryopreserved over liquid nitrogen. Before each experiment, a vial was thawed and the cells washed in phosphate-buffered saline (PBS) and grown as an ascites in semisyngeneic mice. The ascites tumor cells were harvested 6–8 d after implantation in PBS containing 10 U heparin/ml. After two washes in PBS, they were resuspended to the desired concentration in PBS for implantation in experimental, semisyngeneic recipients. The standard procedure for experimental animals was to inject 10⁶ tumor cells in the right hind footpad, and to follow tumor growth by measuring changes against time in the thickness of the footpad with dial calipers.

Tumor Excision. Tumors to be excised were initiated by implanting 10⁶ P815 tumor cells intradermally in the belly region. At progressive stages of tumor growth, mice were selected for tumor excision and anesthetized with Nembutol. Intradermal tumors were carefully excised along with a generous portion of surrounding normal tissue, and the incision closed with stainless steel surgical clips. After tumor excision, mice were examined periodically for evidence of tumor metastases and regrowth of their primary tumor. Mice showing evidence of tumor regrowth at the excision site were excluded from the study so that survival curves only reflected death from metastatic disease.

Tumor-immune Donor Mice. Mice were immunized by injecting them intradermally in the belly region, or in a hind footpad with 2×10^6 tumor cells admixed with 100 μ g of Formalin-killed *Corynebacterium parvum* (Burroughs Wellcome Co., Research Triangle Park, N. C.) in a volume of 0.05 ml of PBS. This results in an initial 9-d period of tumor growth followed by complete tumor regression within 2 wk. During this time, strong specific immunity to growth of a tumor implant develops (E. S. Dye and R. J. North, unpublished observations). Spleen cells were harvested from these mice soon after complete tumor regression, diced into small pieces, and gently pushed through a 200-mesh stainless steel screen into PBS containing 1% FCS. The cells were triturated with a Pasteur pipette to break up clumps, and passed through six layers of sterile surgical gauze. They were then washed in PBS and resuspended to an appropriate concentration in PBS for intravenous injection via a lateral tail vein.

Donors of Suppressor Cells. Suppressor cell donors were mice carrying 15-d progressively growing tumors initiated by a hind footpad injection of 2×10^6 tumor cells. The spleen cells of these donors were prepared in the same way as immune spleen cells.

T Cell-deficient Test Recipients. Mice were made T cell-deficient (TXB) at 4 wk of age by thymectomy followed 7 d later by 900 rad of whole body gamma irradiation delivered by a ¹³⁷Ce irradiator at a dose rate of 29.5 rad/min. The mice were infused intravenously with 10⁷ syngeneic bone marrow cells within 2 h of irradiation and employed in experiments after an additional 6 wk.

Treatment with Anti-Thy-1.2 Antibody. Lyophilized anti-Thy-1.2 F7D5 monoclonal IgM antibody (Sera Lab, Accurate Chemical & Scientific Corp., Westbury, N. Y.) was dissolved in a volume of distilled water equal to the original volume of ascites. It was divided and stored at –70°C and thawed as needed. When tested against mouse thymocytes, the apportioned antibody solution had a cytotoxic titer of >1 in 50,000. Spleen cells were treated at 5×10^7 /ml in a 1:1,000 dilution of the antibody for 45 min on ice. The cells were then centrifuged and incubated for 30 min at 37°C in the same volume of a 1:15 dilution of rabbit serum (Low-Tox-M rabbit complement; Accurate Chemical & Scientific Corp.) as a source of complement. After washing twice in PBS-FCS, the cells were resuspended to the desired concentration in PBS for intravenous infusion.

Results

Demonstration of Adoptive T Cell-mediated Tumor Regression Requires T Cell-deficient Recipients. In the first series of experiments, the P815 mastocytoma and the Meth A

¹Abbreviations used in this paper: FCS, fetal calf serum; PBS, phosphate-buffered saline; TXB, thymectomized T cell-deficient mice.

fibrosarcoma growing in normal mice or in T cell-deficient mice, were compared in terms of their susceptibility to intravenously infused spleen cells from appropriately immunized donors. The results in Fig. 1 show that the two tumors behaved in the same way. In both cases, intravenous infusion of immune spleen cells resulted after about a 6-d delay in complete tumor regression, but only if the tumors were growing

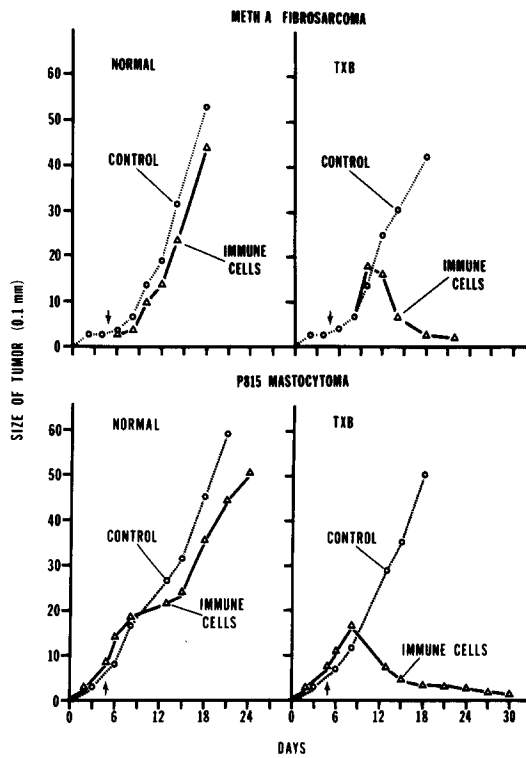


FIG. 1. Evidence that intravenous infusion of 1.5×10^8 spleen cells from immune donor mice on day 4 of tumor growth results, after a delay, in complete tumor regression in T cell-deficient recipients (TXB), but not in normal recipients. Means of five mice per time point.

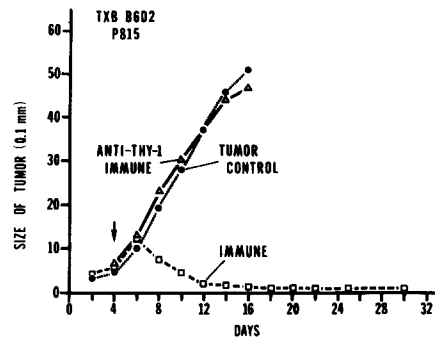


FIG. 2. Evidence that donor spleen cells that mediate regression of the P815 mastocytoma in T cell-deficient recipients are T cells. Treatment of spleen cells with monoclonal anti-Thy-1.2 plus complement ablated their capacity to cause tumor regression. Means of five mice per time point.

in recipients that had been made T cell deficient by thymectomy and gamma irradiation.

Evidence that regression of the P815 mastocytoma was mediated by T cells was obtained by determining whether immune spleen cells from mastocytoma-immunized donors failed to cause tumor regression if they were first incubated with anti-Thy-1.2 antibodies and complement. The results in Fig. 2 show that anti-Thy-1.2 treatment completely ablated the capacity of these spleen cells to regress tumors growing in T cell-deficient recipients. Therefore, there can be little doubt so far as this model is concerned that the cells that mediate regression of the P815 mastocytoma, like those that mediate regression of the Meth A fibrosarcoma (2), are Thy-1-positive T cells.

Passively Transferred, Sensitized T Cells Destroy Tumor Metastases. Unlike the Meth A fibrosarcoma, which does not metastasize, the P815 mastocytoma metastasizes to the draining lymph nodes, spleen, and eventually the lungs. As a result, the P815 tumor kills its host much sooner than the Meth A tumor. In addition, it was observed that T cell-deficient mice injected intradermally, or in a hind footpad with 10^6 P815 cells developed metastases and died 2–3 wk earlier than normal mice implanted with a similar number of tumor cells. Based on this observation, it seemed likely that the passive transfer of immune cells into tumor-bearing, T cell-deficient mice described in the previous section, not only caused regression of the primary tumor, but also eliminated already seeded metastases. Evidence to support this interpretation is illustrated in Fig. 3, which shows the results of an experiment designed to measure the survival time of T cell-deficient mice and normal mice that had their primary intradermal tumors excised on days 3 or 6 of tumor growth. It can be seen from the

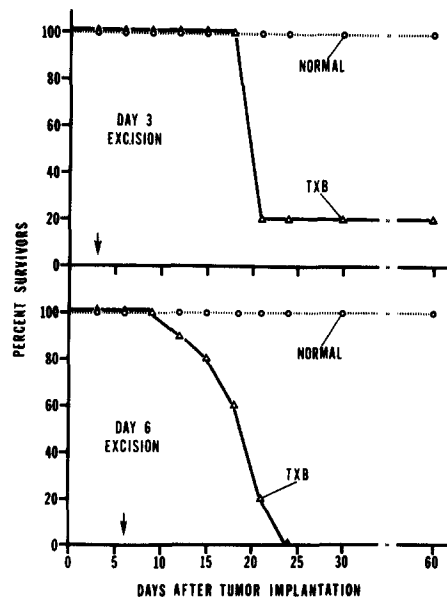


FIG. 3. Evidence that intradermal growth of the P815 tumor causes lethal tumor metastases to be seeded much sooner in T cell-deficient (TXB) mice than in normal mice. Tumors growing in normal and TXB mice were excised either 3 or 6 d after implantation, and the mice were examined periodically for tumor metastases and survival. The survival curves show that 80% of the TXB mice were seeded with metastases by day 3 of tumor growth and 100% by day 6. Mice with metastases had a median survival time of 19 d. In contrast, T cell-competent mice remained free of tumor metastases for the 60-d period of observation. Percent survivors is based on 10 mice per group.

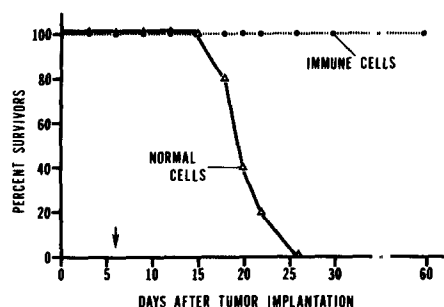


FIG. 4. Evidence that immune cells can inhibit the growth of already seeded tumor metastases in a T cell-deficient host. Intradermal P815 tumors, growing in T cell-deficient mice, were excised on day 6 of tumor growth, and 4 h later the mice were infused with either 1.5×10^8 immune spleen cells or 1.5×10^8 normal spleen cells. The survival curves show that whereas immune spleen cells protected mice from tumor metastases for the 60-d period of observation, recipients of normal spleen cells all died of tumor metastases with a median survival time of 19 d. Percent survivors is based on 10 mice per group.

survival curves that 80% of the T cell-deficient mice were seeded with metastases by day 3 of tumor growth and 100% by day 6. In contrast, control mice all survived the 60-d period of observation and showed no signs of developing metastases.

Because the T cell-deficient mice develop metastatic disease before the 6th d of primary tumor growth (Fig. 3), and because the passive transfer of tumor-sensitized T cells on day 4 of tumor growth does not cause the regression of tumors in T cell-deficient recipients for a further 6 d (Fig. 1), it follows that the passively transferred T cells must have caused the destruction of already established metastases. Direct evidence to support this interpretation can be seen in Fig. 4, which shows the results of an experiment that tested the capacity of 1.5×10^8 tumor-immune spleen cells, or 1.5×10^8 normal spleen cells, to protect T cell-deficient recipients that had their primary tumors excised on day 6 of tumor growth. It can be seen that an infusion of immune spleen cells 4 h after tumor excision protected these mice from tumor metastases for the 60-d period of observation, whereas recipients of normal spleen cells died rapidly with a median survival time of 19 d. There is no doubt, therefore, that the survival of adoptively immunized, tumor-bearing, T cell-deficient recipients depended on the capacity of intravenously infused, sensitized T cells to seek out and destroy metastases as well as the primary tumor.

Inhibition of Adoptive T Cell-mediated Tumor Regression by Spleen Cells from Tumor-bearing Donors. It was anticipated on the basis of the foregoing results, and those obtained previously with the Meth A fibrosarcoma (2), that adoptive T cell-mediated regression of the P815 mastocytoma in T cell-deficient recipients would be inhibited by an appropriately timed infusion of spleen cells from T cell-intact, tumor-bearing donors. That this was the case can be seen in Fig. 5, which shows the results of an experiment that measured the effect of an intravenous infusion of 1.5×10^8 spleen cells from 15-d tumor-bearing donors on regression caused by the same number of spleen cells from immune donors. It can be seen that an infusion of spleen cells from immune donors completely inhibited the ability of the immune spleen cells to cause tumor regression in T cell-deficient test recipients. Normal spleen cells, in contrast, had no inhibitory effect.

Evidence that the spleen cells from tumor-bearing donors that inhibit the expression

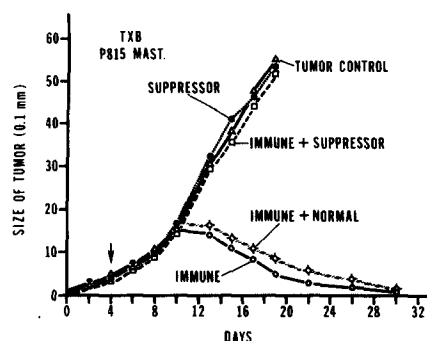


FIG. 5. Evidence that adoptive T cell-mediated regression of the P815 mastocytoma in T cell-deficient recipients can be inhibited by intravenous infusion of spleen cells from tumor-bearing donors. On day 4 of tumor growth, the recipients received 1.5×10^6 spleen cells from 15-d tumor-bearing donors, or normal donors 1 h before receiving 1.5×10^8 spleen cells from immune donors. Means of five mice per time point.

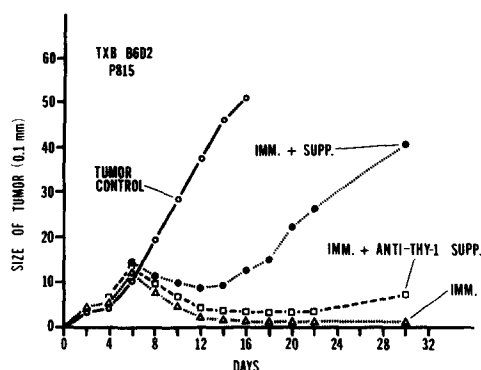


FIG. 6. Treatment of spleen cells from 15-d tumor-bearing donors with anti-Thy-1.2 plus complement essentially eliminated the capacity of these cells to inhibit immune spleen cells from regressing the P815 mastocytoma in T cell-deficient recipients. Means of five mice per time point.

of adoptive immunotherapy are T cells is supplied in Fig. 6, where it can be seen that their suppressor activity was almost totally eliminated by incubating them with anti-Thy-1.2 antibodies and complement.

It will be noted that suppression was not as strong in this experiment as in the experiment shown in Fig. 5. That this was probably a result of the presence of fewer suppressor cells in the spleens of these suppressor donors is shown in Fig. 8 below.

Effect of Timing of Suppressor Cell Infusion on Adoptive Immunity. A characteristic feature of adoptive T cell-mediated regression of the P815 mastocytoma, and the Meth A fibrosarcoma (2), is that there is a relatively long delay (6–7 d) between the time that immune cells are infused intravenously and the time the tumors begin to regress. One possible reason for this delay might be that the infused immune T cell population needs to replicate in order to reach a large enough number to cause regression. According to this interpretation, the stimulus for T cell replication would be supplied by antigens of the recipient's growing tumor, and the function of suppressor T cells would be to prevent this replication. If so, then one would expect suppressor T cells to be more effective if they were infused before immune T cells

have had time to expand in number. This possibility was investigated by measuring the degree to which adoptive T cell-mediated tumor regression was inhibited when 1.5×10^8 suppressor spleen cells were infused 2 d before, at the time of, or 4 d after the infusion of 1.5×10^8 immune cells. It was found (Fig. 7) that when suppressor cells were infused either at the time of, or 2 d before immune cells, tumor regression was completely suppressed. In contrast, when suppressor cells were infused 4 d after immune cells, the onset of suppression was greatly delayed in that tumor growth was halted for 8 d before suppressor function emerged. It can be predicted on the basis of these results that suppressor T cells will exert little or no influence if they are infused after tumor regression has already commenced. This possibility is currently being tested.

Effect of Varying the Ratio of Immune T Cells to Suppressor T Cells on Tumor Regression.

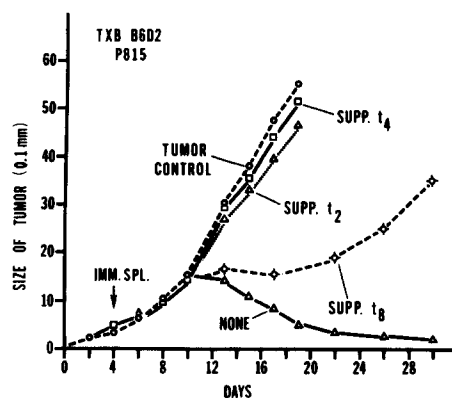


FIG. 7. Effect on tumor regression in T cell-deficient recipients of varying the time of infusing suppressor spleen cells relative to the time of infusing immune spleen cells. Immune spleen cells were infused on day 4 of tumor growth, and suppressor cells were infused 2 d before, at the time of, or 4 d later. Delaying the infusion of suppressor cells by 4 d resulted in a greatly reduced suppressor effect. Means of five mice per time point.

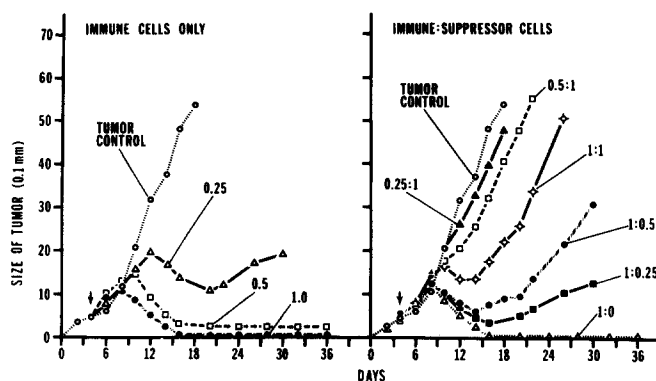


FIG. 8. Effect of varying the ratio of immune spleen cells to suppressor spleen cells infused on tumor regression in T cell-deficient recipients. A ratio of 1:1 equals 1.5×10^8 immune spleen cells to 1.5×10^8 suppressor spleen cells given 1 h apart on day 4 of tumor growth. It is obvious from the graphs on the right that the larger the number of immune cells infused, the sooner the onset of regression. It can be seen in the graphs on the left that the larger the proportion of suppressor cells, the greater was the level of suppression expressed.

The experiments up to this point were based on the use of one organ equivalent ($\sim 1.5 \times 10^8$) of donor spleen cells to passively transfer immunity and suppression. It was important for the design of future experiments, however, to determine the effect of varying the ratio of immune T cells to suppressor T cells infused. The results of an experiment that measured the effect on tumor regression of infusing different ratios of immune to suppressor cells into T cell-deficient recipients bearing 4-d tumors is shown in Fig. 8. It can be seen in the right panel that decreasing the number of suppressor cells relative to the number of immune cells resulted in increasing periods of delay before suppressor function was expressed. In the case of the smallest proportion of suppressor cells infused, enough time elapsed to enable effector T cells to cause almost complete tumor regression. Therefore, in all cases, suppressor function eventually emerged and the tumors regrew at a rapid rate. The left panel of Fig. 8 serves to show, in the absence of suppressors, that the larger the number of immune spleen cells infused, the earlier the onset of tumor regression.

Discussion

This paper shows that growth of the P815 mastocytoma in semisyngeneic mice evokes the generation of a state of T cell-mediated immunosuppression. This conclusion is based on two major findings: first, that it is possible to regress the P815 mastocytoma by passive transfer of tumor-sensitized T cells, but only if the tumors are growing in recipient mice that have been made T cell-deficient by thymectomy and gamma irradiation; second, that adoptive T cell-mediated regression of this tumor in T cell-deficient recipients can be inhibited by an infusion of splenic T cells from T cell-intact tumor-bearing donors. It follows, therefore, that failure of passively transferred, sensitized T cells to regress tumors in T cell-intact mice is caused by the presence in these mice of a tumor-induced mechanism of T cell-mediated immunosuppression. The results as a whole are strikingly similar, therefore, to those obtained previously (2) with the Meth A fibrosarcoma, and make it highly likely that the same mechanism of immunosuppression will be found to be generated in response to the growth of many other immunogenic tumors.

The results with the P815 mastocytoma, however, are immunotherapeutically more interesting, because this tumor metastasizes to cause systemic disease. Moreover, it does so much more rapidly in the T cell-deficient test recipients used in this study. It was shown, by excising primary tumors at progressive time intervals, that metastases are seeded before day 6 of primary tumor growth in T cell-deficient mice, but not in normal mice. Considering the knowledge that intravenous infusion of immune cells on day 4 of tumor growth does not cause tumor regression in T cell-deficient recipients until 6 d later, it follows that survival of these recipients not only depended on the capacity of the infused T cells to destroy the primary tumor, but also on the capacity of these cells to seek out and destroy established metastases. It was demonstrated, in support of this interpretation, that all T cell-deficient mice that received immune spleen cells 4 h after removal of their day 6 primary tumors survived a 60-d period of observation and remained free of palpable tumor metastases. In contrast, the recipients of normal spleen cells all died of tumor metastases with a median survival time of 19 d. So far as the P815 mastocytoma is concerned, it seems obvious that tumor cells that give rise to metastases are not selected on the basis of their capacity to avoid destruction by T cell-mediated immunity.

It was argued in a previous publication (2) that the generation of suppressor T cells would seem the most plausible explanation for why immunogenic tumors grow progressively in their immunocompetent hosts, in spite of the generation of an initial state of concomitant anti-tumor immunity. There is *in vitro* evidence (3) in this connection, that cytolytic T cells are generated in syngeneic mice in response to growth of the P815 mastocytoma, but that the response is of low magnitude and short duration. Indeed, it was shown in this same study (3) that the loss of cytolytic T cells is associated with the acquisition by the host of T cells that can suppress a secondary cytolytic response to the P815 mastocytoma *in vitro*. Similar evidence has been obtained in this laboratory (C. D. Mills, R. J. North, and E. S. Dye, unpublished observations). There seems little doubt, therefore, that tumor-induced T cell-mediated immunosuppression is generated in response to growth of the P815 mastocytoma, and that this will prove to be the major obstacle to attempts to regress this tumor by immunotherapy. The specificity of suppression is currently under study using the Meth A fibrosarcoma and the P815 mastocytoma in semisyngeneic D2CF₁ mice.

So far as adoptive immunotherapy is concerned, it is apparent from a review of the literature (4), that although there is relatively little difficulty in adoptively immunizing against the growth of tumor implants, great difficulty is experienced in demonstrating adoptive T cell-mediated immunity against established tumors. In fact, to cause even partial regression of established tumors requires the infusion of very large numbers of sensitized cells. However, an exception to this general finding recently has been described for a syngeneic, virus-induced rat tumor, where successful immunotherapy of large tumors was achieved by intravenous infusion of sensitized T cells generated *in vitro* (5). We have repeatedly failed to regress the P815 mastocytoma and Meth A fibrosarcoma in T cell-intact mice, even by repeatedly infusing very large numbers of immune spleen cells. This indicates that, in the case of these tumors, the state of T cell-mediated immunosuppression is relatively strong.

A significant finding revealed by this study is that although immune T cells can be functionally dominant in the presence of relatively small numbers of suppressor T cells, suppression eventually dominates. It was shown, for example, that whereas one spleen equivalent of suppressor cells can completely prevent one spleen equivalent of immune cells from regressing tumors in T cell-deficient test recipients, reducing the number of suppressor cells infused to a quarter spleen equivalent allows infused immune cells to cause almost complete tumor regression. However, suppressor function eventually emerged, and the tumors regrew at a rapid rate. A similar result was obtained by delaying the time that suppressor cells were infused. Taken together, these results indicate the possibility that the function of suppressor cells in this model is to inhibit the replication of passively transferred immune T cells, and thereby to prevent these cells from reaching a large enough number to cause tumor regression. Thus, delaying the time of infusion of suppressor cells or infusing a smaller number allows enough time for the infused immune cells to expand in number in response to antigens supplied by the recipient's growing tumor. The reason suppressor function eventually emerges and becomes dominant might be that suppressor T cells also are stimulated to replicate in response to antigens supplied by the growing tumor. This hypothesis is currently being tested.

Even so, it remains to be explained why the spleens of immune donors themselves and of normal donors do not contain suppressor precursors that can be stimulated to

replicate and differentiate in response to tumor antigens in the T cell-deficient test recipient. One possibility might be that even though suppressor precursors are present in immune and normal spleens, they are present in such small numbers that the tumors have enough time to be completely regressed by infused immune T cells before suppressor T cells replicate to dominant numbers. Alternatively, the precursors of suppressor T cells may be relatively short-lived and may be newly formed cells released by the thymus, as appears to be the case for the Lyt-123 subpopulation of suppressor T cells that cooperate with other T cells in other models of T cell-mediated suppression (6, 7). These precursors would not be available in the thymectomized T cell-deficient test recipients used in this study.

Summary

Progressive growth of the P815 mastocytoma in semisyngeneic mice evokes the generation of a T cell-mediated mechanism of immunosuppression that inhibits the capacity of passively transferred, tumor-sensitized T cells from regressing this tumor in recipient mice. This conclusion is based on two findings: (a) that it is possible to demonstrate adoptive T cell-mediated regression of established tumors, but only if the tumors are growing in T cell-deficient recipients, and (b) that adoptive T cell-mediated regression of tumors in these recipients can be inhibited by the infusion of splenic T cells from T cell-intact, tumor-bearing donors. The results of additional experiments designed to measure the effect of decreasing the number of suppressor cells and the time that they are infused, relative to immune cells, indicate that the function of suppressor cells in this model is to inhibit the replication of passively transferred immune T cells. The results obtained with the P815 mastocytoma are similar to those obtained previously with a chemically induced fibrosarcoma. They show, in addition, that passively transferred immune cells are capable of destroying already seeded metastases in T cell-deficient recipients.

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