CYCLOPHOSPHAMIDE-FACILITATED ADOPTIVE IMMUNOTHERAPY OF AN ESTABLISHED TUMOR DEPENDS ON ELIMINATION OF TUMOR-INDUCED SUPPRESSOR T CELLS*

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Although the passive transfer of T cell-mediated immunity to growth of a tumor implant is relatively easy to demonstrate, it has proven extremely difficult to demonstrate that passively transferred, tumor-sensitized T cells have any therapeutic effect against an established growing tumor (1). The most likely reason for the refractoriness of established immunogenic tumors to adoptive immunotherapy with tumor-sensitized T cells was supplied by previous publications from this laboratory (2, 3), which show that progressive growth of an immunogenic tumor evokes the generation in its host of a T cell-mediated mechanism of immunosuppression. The existence of this mechanism of immunosuppression was revealed in two ways: first, by showing that although passively transferred, tumor-sensitized T cells failed to cause the regression of tumors growing in normal mice, they caused the complete and permanent regression of the same-sized tumors growing in mice that had been made T cell deficient by thymectomy and irradiation; and second, by demonstrating that failure of passively transferred, sensitized T cells to cause the regression of tumors in normal mice was associated with the presence in these mice of splenic T cells capable of inhibiting adoptive T cell-mediated regression of tumors growing in T cell-deficient mice. On the basis of this and other evidence, it was hypothesized (2, 3) that the progressive growth of an immunogenic tumor evokes the generation of a state of T cell-mediated concomitant immunity that undergoes T cell-mediated negative regulation before enough effector T cells are produced to destroy the tumor. It was further hypothesized (4) that any attempt to cause tumor regression by the passive transfer of tumor-sensitized T cells represents an attempt to superimpose an adoptive immune response on an already ongoing concomitant immune response that may be undergoing negative regulation, depending on the size of the tumor. If this line of reasoning is correct, it should follow that any treatment that prevents the generation of concomitant immunity and of the suppressor T cells that negatively regulate it, should facilitate the antitumor function of passively transferred, tumor-sensitized T cells.

The purpose of this paper is to show that a cyclophosphamide-treated, tumor-bearing recipient can substitute for a T cell-deficient, tumor-bearing recipient for demonstrating that an established tumor can be caused to completely regress by the

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passive transfer of tumor-sensitized T cells. It will show that, whereas passive transfer of tumor-sensitized T cells has no effect on the growth of an immunogenic fibrosarcoma, and whereas cyclophosphamide treatment causes only a temporary halt in tumor progression, combination therapy with cyclophosphamide plus sensitized T cells causes the tumor to completely and permanently regress. It will show, in addition, that tumor regression caused by combination therapy with cyclophosphamide and immune cells can be completely inhibited by intravenous infusion of cyclophosphamide-sensitive suppressor T cells from the spleens of tumor-bearing donors.

Materials and Methods

Mice. Specific-pathogen-free BALB/c and CB6F1 (BALB/c × C57BL/6) mice were supplied by the Trudeau Institute Animal Breeding Facility. They were free of known viral pathogens, as evidenced by the results of routine serological screening performed by the Animal Diagnostic Testing Service of Microbiological Associates, Bethesda, MD.

Tumor. The methylcholanthrene-induced, Meth A fibrosarcoma, syngeneic in BALB/c mice, was originally obtained from Dr. Lloyd J. Old of the Memorial Sloan-Kettering Cancer Center, New York. It was grown to large number as an ascites in the peritoneal cavities of BALB/c mice, harvested and washed in phosphate-buffered saline (PBS), resuspended in Fisher’s medium (Grand Island Biological Co., Grand Island, NY) containing 20% fetal calf serum and 10% dimethyl sulfoxide, and cryopreserved in small vials over liquid nitrogen. For each experiment, a vial was thawed, the tumor cells washed in PBS, and 2 × 10⁶ implanted in the peritoneal cavities of CB6F1 mice. Tumor cells were harvested 6 d later in PBS, washed in PBS, and resuspended appropriately for implantation. Tumors were initiated in the right hind footpad by intrafootpad injection of 10⁶ or 2 × 10⁶ tumor cells in a volume of 0.5 ml PBS. Tumor growth was monitored against time by measuring changes in the thickness of the footpad with dial calipers.

Adoptive Immunization. Donor mice were immunized against the Meth A tumor by injecting them intradermally with 2 × 10⁷ Meth A cells admixed with 100 μg of formalin-killed Corynebacterium parvum (Burroughs Wellcome, Greenville, NC). This is known (5) to result in a 9-d period of tumor growth followed by complete tumor regression. The onset of tumor regression is associated with immunity to growth of a tumor implant and with the presence in the spleen of T cells capable of passively transferring immunity to a tumor implant (5). The immunized mice were used as donors 3-4 wk after tumor regression. Their spleens were diced into small pieces and gently pushed and washed through a 60-mesh stainless screen with PBS. The resulting cell suspension was triturated with a pasteur pipette to break up clumps, passed through six layers of sterile surgical gauze, and washed and resuspended in PBS for intravenous infusion. Recipient mice received 1.5 × 10⁸ spleen cells in a volume of 0.5 ml.

Anti-Thy-1.2 Treatment. Spleen cells were incubated at 10°C for 30 min at 5 × 10⁷/ml in a 1:1,000 dilution of monoclonal IgM anti-Thy-1.2 antibody supplied as ascites fluid by Accurate Chemical & Scientific Corp., Westbury, NY. The cells were washed in PBS and incubated at the same concentration in a 1:15 dilution of rabbit serum from the same supplier for 30 min at 37°C. After a further wash in PBS, the cells were resuspended for intravenous infusion.

Cyclophosphamide. Cyclophosphamide (Cytoxan) was purchased from Mead Johnson & Co., Evansville, IN. It was dissoluted in sterile water and injected intravenously in a dose of 100 mg/kg, unless otherwise stated.

Results

Failure of Cyclophosphamide or Immune Cells Alone to Cause Tumor Regression. That cyclophosphamide has a limited therapeutic effect against a Meth A footpad tumor is shown in Fig. 1, where it can be seen that a dose of 160 mg/kg given on day 5 or 9 of tumor growth caused only a small degree of tumor regression over several days, after which the tumor resumed growing at its original rate. Fig. 1 shows, in addition,
that the antitumor effect of cyclophosphamide was the same in mice that had been given 650 rad of whole-body gamma-irradiation 1 h before tumor implantation. This result allows the conclusion that the antitumor effect was caused by the direct action of the drug on the tumor, and was not caused by a capacity of the drug to enhance an antitumor immune response. The same results were obtained with the 100 mg/kg dose of cyclophosphamide routinely employed in the experiments that follow. However, the result of giving this smaller dose of the drug was to cause tumor growth to plateau for several days, rather than to cause tumor regression.

In keeping with previous findings (2, 3), an intravenous infusion of $1.5 \times 10^8$ spleen cells from the tumor immune donor mice had no effect on the growth of a 4-d tumor (Fig. 2). In fact, this number of immune spleen cells had only a slight inhibitory effect on tumor growth when infused within 1 h of implanting tumor cells. In other words, the standard number of immune spleen cells used in this study was incapable even of preventing the growth of a tumor implant.
Complete Tumor Regression after Combination Therapy with Cyclophosphamide and Immune Cells. The proceeding results showed that treatment with cyclophosphamide caused only temporary regression of an established Meth A fibrosarcoma, whereas intravenous infusion of immune cells has no effect at all. The purpose of the experiments in this section was to determine whether combination therapy with cyclophosphamide plus immune cells would result in complete tumor regression. The design of the experiments was based on the knowledge (2, 3) that passive transfer of immune T cells can cause the regression of the Meth A tumor growing in mice made T cell deficient by thymectomy and irradiation, but not in normal mice. It was reasoned, therefore, that any treatment that causes either a temporary or permanent deficiency of T cells should allow passively transferred immune T cells to express their antitumor function. That cyclophosphamide treatment facilitates the expression of adoptive immunotherapy against an established tumor is shown in Fig. 3, where it can be seen that an injection of 100 mg/kg of cyclophosphamide on day 4 of tumor growth followed 1 h later by intravenous infusion of $1.5 \times 10^8$ immune spleen cells resulted in complete tumor regression in all mice. No regrowth of tumors was observed during a 60-d period of observation. In contrast, and in agreement with the findings above, cyclophosphamide alone had only a marginal effect on tumor growth, and immune cells alone had no effect at all. A previous publication (2) showed that spleen cells which on passive transfer cause regression of established Meth A tumors are T cells, as evidenced by their destruction by treatment with anti-Thy-1.2 antibody and complement.

It was found, in addition, that combination therapy with cyclophosphate and immune cells is capable of causing the regression of relatively large Meth A tumors. It can be seen in Fig. 4 that giving 100 mg/kg of cyclophosphamide 1 h before infusing immune spleen cells caused the complete regression of 3-, 6-, and even 9-d tumors. It should be pointed out that a 9-d footpad tumor is three times the size of a normal footpad, and that cyclophosphamide alone contributed only marginally to the regression of tumors of this size. Therefore, the success of combination therapy does not depend on the ability of cyclophosphamide to reduce the tumor below a certain critical size.

![Graph](image-url)
Evidence that combination therapy (arrows) with cyclophosphamide and immune spleen cells results in the complete regression of 3-, 6-, and 9-d tumors. The 9-d tumors were more than three times the size of a normal footpad. Means of five mice per group. Δ, tumor control; ψ, cyclophosphamide; ○, immune cells; □, cyclophosphamide plus immune cells.

Fig. 4. Evidence that tumor regression caused by combination therapy with cyclophosphamide and immune spleen cells (○) is completely abolished by intravenous infusion 24 h later of 1.5 × 10⁶ spleen cells from donors with established 12-d tumors (□), but not by the same number of spleen cells from normal donors (■). Means of five mice per group. ▲, control; ψ, immune cells; Δ, cyclophosphamide.

Tumor Regression Caused by Combination Therapy with Cyclophosphamide and Immune T Cells is Inhibited by Splenic T Cells from Donors with Established Tumors. The results in the preceding section, in conjunction with those previously published (2, 3) can be interpreted as showing that cyclophosphamide treatment of tumor-bearing mice facilitates adoptive immunotherapy with immune T cells by either eliminating or preventing the development of a tumor-induced mechanism that prevents intravenously infused immune T cells from expressing their antitumor function. If this tumor-induced mechanism is mediated by a population of cyclophosphamide-sensitive suppressor T cells, it should be possible to counteract the therapeutic action of cyclophosphamide and immune cells by passively transferring splenic T cells from donors with established tumors. This possibility was tested by measuring the fate of tumors in mice given combination therapy with cyclophosphamide and immune spleen cells on day 4 of tumor growth, followed 24 h later by an intravenous infusion of splenic cells from mice bearing 12-d tumors. It can be seen in Fig. 5 that passive
Evidence that the spleen cells from tumor-bearing mice, which inhibit tumor regression caused by combination therapy with cyclophosphamide and immune cells, are T cells. It can be seen that the suppressive action of tumor bearer's spleen cells was completely eliminated by treatment with anti-Thy-1.2 antibody and complement (\(\square\)). Combination therapy was given on day 4, and suppressor cells on day 5. Means of five mice per group. ○, tumor control; ▲, cyclophosphamide plus immune cells; Δ, cyclophosphamide plus immune cells plus suppressors.

Evidence that tumor regression caused by cyclophosphamide and immune cells (▲), and the inhibition of this regression by spleen cells from 12-d tumor-bearing donors can be demonstrated in syngeneic BALB/c mice. Combination therapy was given on day 4, and suppressor cells on day 5 (○). Means of five mice per group. ○, control; ▲, cyclophosphamide; Δ, immune cells.

Transfer of spleen cells from 12-d tumor-bearing donors completely inhibited tumor regression caused by combination therapy with cyclophosphamide and immune cells. The tumors behaved as though the mice had been given cyclophosphamide alone.

That the spleen cells responsible for this suppressive action were T cells is shown by the results in Fig. 6, where it can be seen that their suppressive function was totally eliminated by incubating them with anti-Thy-1.2 antibody plus complement. Fig. 5 serves to illustrate the additional important finding that an infusion of the same number of normal spleen cells had no effect on the outcome of combination therapy. Therefore, suppressor cells were generated in response to tumor growth.

The purpose of the results in Fig. 7 is to show that cyclophosphamide-facilitated adoptive T cell-mediated tumor regression and its suppression with spleen cells from tumor-bearing donors can be demonstrated in syngeneic BALB/c mice. These results serve to discount the criticism that the preceding results and those that follow are peculiar to the tumor growing in semisyngeneic CB6F1 hosts.

Suppressor T Cells, but Not Immune T Cells, are Cyclophosphamide Sensitive. The results
in the preceding sections are consistent with the interpretation that treatment of the tumor-bearing host with cyclophosphamide facilitates adoptive T cell-mediated regression of its established tumor by eliminating a cyclophosphamide-sensitive population of suppressor T cells that inhibits the antitumor function of passively transferred immune T cells. If this is so, the T cells in tumor-bearing donor mice capable of suppressing cyclophosphamide-facilitated adoptive immunotherapy should be destroyed by treating the tumor-bearing donors with cyclophosphamide. This is shown in Fig. 8, where it can be seen that treating donors bearing 12-d Meth A tumors with 100 mg/kg cyclophosphamide 24 h before harvesting their spleen cells completely

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**Fig. 8.** Evidence that cyclophosphamide destroys the T cells in 12-d tumor bearers that inhibit tumor regression caused by combination therapy with cyclophosphamide and immune cells. Treating the tumor-bearing mice with 100 mg/kg of cyclophosphamide 24 h before harvesting their spleen cells completely ablated the capacity of the spleen cells to inhibit tumor regression. Means of five mice per group. (○), tumor control; (△), cyclophosphamide plus immune cells; (□), cyclophosphamide plus immune cells plus suppressor.

![Graph](image-url)

**Fig. 9.** Treating immune donors with 100 mg/kg of cyclophosphamide had no effect on the capacity of their spleen cells harvested 24 h later to cause tumor regression in cyclophosphamide-treated recipients. Means of five mice per group. (△), tumor control; (□), cyclophosphamide; (○), cyclophosphamide plus immune cells; (□), cyclophosphamide plus cyclophosphamide-treated immune cells.
ablated the capacity of their spleen cells to suppress adoptive T cell-mediated tumor regression in cyclophosphamide-treated recipients. The completeness of destruction of suppressor cells by cyclophosphamide is indicated by the additional knowledge that no suppression was observed, even though it took three spleen equivalents of cells from the cyclophosphamide-treated tumor-bearing donors to equal the number of spleen cells \(1.5 \times 10^8\) obtained from tumor-bearing control donors.

In contrast to the cyclophosphamide-sensitivity of suppressor T cells, immune spleen cells from tumor-immune donors were highly resistant to the drug. This is shown in Fig. 9, where it can be seen that the treatment of immune donor mice with 100 mg/kg of cyclophosphamide 24 h before harvesting their spleen cells had no effect on the capacity of spleen cells to cause tumor regression in cyclophosphamide-treated recipients. This result was obtained with one spleen equivalent \((5 \times 10^7)\) or with three spleen equivalents \((1.5 \times 10^8)\) of cells from cyclophosphamide-treated immune donors.

**Discussion**

This paper shows that an established methylcholanthrene-induced tumor, the Meth A fibrosarcoma, can be caused to completely and permanently regress in a syngeneic or semisyngeneic host by combination therapy consisting of intravenous injection of 100 mg/kg of cyclophosphamide followed 1 h later by intravenous infusion of splenic T cells from tumor immune donors. In contrast, infusion of immune cells alone had no effect on tumor growth, and cyclophosphamide alone caused only a temporary halt in tumor progression. Thus, because cyclophosphamide alone had only a marginal effect on tumor growth, most of the destruction of the tumor caused by combination therapy could be attributed to the antitumor action of passively transferred immune T cells. These results indicate, therefore, that cyclophosphamide facilitates adoptive immunotherapy by eliminating a mechanism from the tumor-bearing host that normally prevents passively transferred tumor-immune T cells from expressing their antitumor function. On the other hand, the success of the combination therapy did not depend on the capacity of cyclophosphamide to reduce the tumor burden below a certain critical size, because therapy was successful against small tumors, as well as against large tumors that were little affected by the direct action of cyclophosphamide.

An explanation for the mechanism of cyclophosphamide-facilitated adoptive immunotherapy requires a consideration of the results of studies of tumor-induced, T cell-mediated immunosuppression already published from this laboratory (2). It was shown that progressive growth of the Meth A fibrosarcoma (2) and P815 mastocytoma (3) in their syngeneic hosts is associated with the generation of a mechanism of T cell-mediated immunosuppression that blocks attempts to cause the regression of these tumors by adoptive immunotherapy with tumor-sensitized T cells. Indirect evidence for this conclusion consisted of the demonstration that passive transfer of sensitized T cells failed to cause the regression of established tumors, unless the tumors were growing in recipient mice made T cell deficient by thymectomy and irradiation. This indicated that normal tumor-bearing mice generate a T cell-dependent mechanism that prevents intravenously infused immune cells from expressing their antitumor function. Direct evidence for the existence of this mechanism of T cell-mediated immunosuppression in normal tumor bearers was revealed by an experiment which showed that prior intravenous infusion of splenic T cells from these mice prevented passively transferred immune T cells from causing the regression of tumors in T cell-
deficient recipients. It was hypothesized, therefore, that progressive growth of an immunogenic tumor eventually results in the generation of a state of immunosuppression that is mediated by suppressor T cells. It was hypothesized more recently (4), that this state of immunosuppression represents negative regulation of a concomitant antitumor immune response that undergoes decay before enough effector T cells are generated to cause regression of the tumor.

The experiments reported in this paper were designed to further investigate this hypothesis. It was predicted that, because passively transferred immune T cells are capable of causing tumors to regress in T cell-deficient recipients, any treatment that either temporarily or permanently prevents the generation of T cell-mediated concomitant immunity and its negative regulation should allow passively transferred immune T cells to express their antitumor function. The results presented here leave little doubt that cyclophosphamide facilitates adoptive immunotherapy in this way. They show that tumor regression caused by combination therapy with cyclophosphamide and immune T cells can be completely inhibited by the intravenous infusion of splenic T cells from tumor-bearing donors, but not from normal donors. They also show that this suppressor activity of the splenic T cells from tumor-bearing donors can be completely ablated by treating the donors by cyclophosphamide. This represents direct evidence that mice bearing the Meth A fibrosarcoma contain a tumor-induced population of cyclophosphamide-sensitive T cells which suppresses the antitumor action of passively transferred immune T cells.

In contrast, cyclophosphamide treatment of immune donors had no effect whatsoever on the capacity of their splenic T cells to cause tumor regression. This could be taken as evidence that cyclophosphamide selectively destroys suppressor cells. It is important to realize, however, that the model used in this study is artificial with regards to the immune response to a growing tumor, because the immune cells were obtained, not from mice responding to a progressive tumor, but from immunized donors, the tumors of which had been caused to regress 3–4 wk earlier by intrallesional C. parvum therapy (5). It then seems safe to assume that the immune cells were memory or helper cells, and that they were resistant to cyclophosphamide treatment because they were nonreplicating. This cannot be used as evidence, therefore, that cytolytic effector T cells are cyclophosphamide resistant. The possibility remains that replicating cytolytic T cells that might be generated as part of the concomitant immune response to a growing tumor (6) will be susceptible to the dose of cyclophosphamide used to eliminate suppressor T cells. In this connection, we have failed to find a dose of cyclophosphamide that will cause the complete regression of the Meth A fibrosarcoma in mice that are expressing concomitant immunity to an implant of this tumor.

There are numerous publications showing that appropriately timed pretreatment with cyclophosphamide can result in the generation of augmented levels of cell-mediated immunity to a variety of antigens (7). Only in a few cases, however, has directed evidence been supplied to show that the augmenting action of the drug depends on its ability to preferentially eliminate suppressor T cells. For example, it has been demonstrated that cyclophosphamide-augmented production of T cells cytolytic for SV40-transformed syngeneic cells (8) and for TNP-coupled syngeneic cells and allogeneic spleen cells (9) can be abolished by passively transferring normal splenic T cells before giving the immunogens. Because these are examples of the elimination of precursors of suppressor T cells, their exact relevance to the T cells that
suppress the cyclophosphamide-facilitated expression of adoptive antitumor immunity described here is as yet unknown. It seems unlikely, however, that the drug facilitates the expression of adoptive antitumor immunity by eliminating suppressor precursors, because its facilitating effect was not abolished by infusing normal T cells, but only by infusing cyclophosphamide-sensitive T cells from donors with established tumors. The relationship between cyclophosphamide-sensitive suppressor precursors and antigen-differentiated suppressor T cells is currently under study. Consideration also is being given to the possibility that the suppression of antitumor immunity is achieved by two types of suppressor T cells, only one of which is destroyed by cyclophosphamide, as appears to be the case of the suppression of contact sensitivity (10) and delayed sensitivity to certain haptens (11). Moreover, discovering the mode of action of suppressor T cells will depend on a more detailed knowledge of the events that need to occur in the tumor-bearing recipient of tumor-immune T cells before adoptive T cell-mediated tumor regression is expressed. In this regard, there is evidence (4) that the passively transferred tumor-immune T cells used in this study, being memory cells, do not themselves cause tumor regression, but impart to the tumor-bearing recipient the capacity to generate a secondary cytolytic T cell response. Experiments performed thus far (4) indicate that it is this adoptive secondary cytolytic response that fails to occur when immune T cells are infused into normal tumor bearers, and when immune T cells are infused into T cell-deficient or cyclophosphamide-treated tumor bearers that are also infused with suppressor T cells from normal tumor bearers.

The model of cyclophosphamide-facilitated, adoptive T cell-mediated tumor regression described in this paper is reminiscent of the model described some years ago by Fefer (12) who passively transferred immunity to the highly immunogenic MSV-induced sarcoma growing progressively in mice that were immunosuppressed by cyclophosphamide. This is immunologically more appealing than combination therapy models, which consist of injecting sensitized T cells locally into ascites tumors that have been substantially destroyed to an unknown degree by cyclophosphamide or other agents. Because the Meth A fibrosarcoma growing as a solid tumor is only slightly susceptible to the direct cytotoxic action of the drug, immunologically mediated regression can be measured in terms of the destruction of a known tumor mass. Moreover, models of immunotherapy that consist of injecting immune cells directly into a tumor site fail to investigate one of the most important functions that immune T cells or their progeny must perform to be useful in tumor immunotherapy: the capacity to migrate from the intravascular compartment to an extravascular site of tumor growth. It would seem obvious that tumor-sensitized T cells generated in large numbers in vitro would have little therapeutic value unless they retained the capacity to perform this essential in vivo function. In this regard, a recent publication (13) demonstrating that mice can be cured of a peritoneal Friend virus-induced leukemia by treatment with cyclophosphamide followed by intraperitoneal infusion of Ly-1+ T cells from immune donors is therapeutically convincing, because the tumor had spread to blood and spleen at the time of therapy.

Summary

On the basis of preceding studies showing that tumor-induced, T cell-mediated immunosuppression serves as an obstacle to adoptive immunotherapy of the Meth A fibrosarcoma, it was predicted that cyclophosphamide treatment of tumor bearers
would remove this obstacle and allow passively transferred immune T cells to cause tumor regression. It was found that infusion of immune spleen cells alone had no effect on tumor growth, and cyclophosphamide alone caused a temporary halt in tumor progression. In contrast, combination therapy consisting of intravenous injection of 100 mg/kg of cyclophosphamide followed 1 h later by intravenous infusion of tumor-immune spleen cells caused small, as well as large tumors, to completely and permanently regress. Tumor regression caused by combination therapy was completely inhibited by intravenous infusion of splenic T cells from donors with established tumors, but not by spleen cells from normal donors. These suppressor T cells were eliminated from the spleen by treating the tumor-bearing donors with 100 mg/kg of cyclophosphamide. Immune T cells, in contrast, were resistant to this dose of cyclophosphamide. These results show that failure of intravenously-infused, tumor-sensitized T cells to cause regression of the Meth A fibrosarcoma growing in its syngeneic or semi-syngeneic host is caused by the presence of a tumor-induced population of cyclophosphamide-sensitive suppressor T cells.

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References

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