Cyclophosphamide-induced Immunologically Mediated Regression of a Cyclophosphamide-resistant Murine Tumor: A Consequence of Eliminating Precursor L3T4+ Suppressor T-Cells

Michel Awwad and Robert J. North
The Trudeau Institute, Saranac Lake, New York 12983

ABSTRACT

It was shown that it is possible to use cyclophosphamide (Cy) to cause immunologically mediated regression of the immunogenic, Cy-resistant L5178Y lymphoma in syngeneic and semisyngeneic mice. In order to cause tumor regression it was necessary to give Cy shortly before or shortly after tumor implantation. However, regardless of whether Cy was given before or after tumor implantation, tumor regression did not commence until 10 days of progressive tumor growth, by which time the tumor was 1 cm in diameter. Tumor regression was associated with the presence in the spleen of an increased number of Lyt-2+ T-cells capable of passively transferring immunity to tumor-bearing recipients. This augmented level of immunity was sustained throughout the period of tumor regression. In contrast, a lower level of concomitant immunity generated by control tumor bearers decayed after Day 12 of tumor growth. Because the therapeutic effect of Cy could be inhibited by passive transfer of L3T4+ T-cells from normal donor mice it is apparent that the therapeutic effect of Cy is based on its ability to preferentially destroy L3T4+ suppressor T-cells. These putative precursor suppressor T-cells were regenerated 4 days after being destroyed by Cy. Taken together the results represent a striking example of the negative regulatory influence of suppressor T-cells on the immune response to an immunogenic tumor.

INTRODUCTION

It is well documented (reviewed in Ref. 1) that an appropriate dose of the cytotoxic alkylating agent, Cy, enables mice to generate an augmented level of immunity to a variety of antigens. The additional demonstration (1) that this immunoaugmenting action can be negated by injecting Cy-treated mice with T-cells from normal donor mice represents the key evidence for postulating that Cy augments immune responses by preferentially destroying suppressor T-cells. This interpretation is in keeping with the view (2–4) that the magnitude of immune responses to certain antigens is predetermined by the ratio of helper T-cells to suppressor T-cells that respond to those antigens, with helper and suppressor cells probably responding to different epitopes on the same antigens (3). The ability of Cy to augment immune responses by preferentially eliminating suppressor T-cells also applies to the in vivo production of cytolytic T-cells. It has been shown that the cytolytic T-cell response to hapten-coupled syngeneic cells (5), or to nonreplicating cells of a virus-induced syngeneic tumor (6), is substantially enhanced by pretreating mice with Cy. This immunoaugmenting action of Cy also can be negated by an infusion of T cells from normal donor mice.

It might be expected, in view of these last-mentioned publicated findings, that it should be possible to use Cy to cause the immunologically mediated regression of immunogenic murine tumors, particularly those tumors against which a host is known to generate a subtherapeutic number of effector T-cells. However, there appears to be only one convincing example of the use of Cy to cause the immunologically dependent regression of an established murine tumor, namely, Cy-induced regression of the MOPC-315 plasmacytoma in BALB/c mice, as described by Mokyr and colleagues (7, 8). However, although there is no doubt that host immunity is involved to some extent in Cy-induced regression of this tumor, most of the tumor is destroyed by the direct cytotoxic action of the drug. It is not known at this stage, therefore, whether complete regression of the MOPC-315 tumor depends on the elimination of suppressor T-cells, or whether regression is more easily explained in terms of the ability of an existing subtherapeutic level of concomitant immunity to destroy a tumor burden substantially reduced by Cy. Obviously, the role of antitumor immunity in Cy-induced regression of immunogenic tumors would be much easier to analyze with tumors that are resistant to the direct cytotoxic action of the drug.

The purpose of this paper is to show that Cy can cause immunologically mediated regression of a Cy-resistant tumor (the L5178Y lymphoma) by preferentially destroying L3T4+ suppressor T-cells, thereby allowing the host to generate an increased number of Lyt-2+ effector T-cells. It will show, in addition, that in order for Cy to cause tumor regression, it needs to be given before the underlying antitumor immune response is induced, because antigen-activated effector T-cells are just as sensitive to Cy as suppressor T-cells.

MATERIALS AND METHODS

Mice. B6D2F1 (C57BL/6 × DBA/2) female mice were used when they were 12–14 weeks old. They were obtained from the Trudeau Institute Animal Breeding Facility, and were known to be free of most common viral pathogens, as evidenced by the results of routine serological testing performed by Charles River Professional Services, Wilmington, MA.

Tumors. The chemically induced, immunogenic L5178Y lymphoma, syngeneic in DBA/2 mice, was grown and prepared as described previously (9). All experiments were performed with a single stock of cryopreserved tumor cells. According to the results of a recent flowcytometric study (10) the surface phenotype of the L5178Y lymphoma is Thy-1.2+, Lyt-2-, L3T4+, Ia+, and H2K/D+. The L5178Y lymphoma is known to be resistant to the cytotoxic action of Cy (11).

Before each experiment a vial of tumor cells was thawed and the cells expanded in number by growing them as an ascites in the peritoneal cavity of syngeneic mice. They were then harvested, washed, and resuspended in PBS for implantation. Tumors were initiated by injecting 10⁶ tumor cells in 0.05 ml of PBS intradermally in the belly region. Tumor growth was monitored by measuring changes against time in the mean of two perpendicular diameters with dial calipers.
mm diameter) tumor in recipient mice. Spleen cells were obtained by cutting spleens into small pieces and forcing the pieces through a 60-mesh screen into PBS. The cell suspension was triturated with a pasteur pipet to break up clumps, and passed through surgical gauze to remove debris. The cells were then washed twice in PBS and resuspended in PBS for i.v. infusion. The recipient mice were bearing an intradermal tumor initiated 4 days earlier, and were injected with a 150 mg/kg dose of Cy 1 h before receiving donor spleen cells to remove a suppressor barrier to adoptive immunity as described previously (11, 12).

Deleting T-Cell Subsets. Anti-L3T4 MAb was generated by hybridomas GK-1.5 (Dr. Frank Fitch, University of Chicago, IL), and anti-Ly-2.2 MAb by hybridoma TIB-210 (American Type Culture Collection, Rockville, MD). The hybridomas were grown to 10^6 per ml in RPMI 1640 medium (GIBCO Laboratories, Grand Island, NY) containing 10% fetal bovine serum, 2 mM glutamine and antibiotics. The cells were removed by centrifugation and the supernatants (containing between 10 and 20 µg of antibody/ml) used without dilution to treat spleen cells at 2 x 10^7/ml for 30 min at 4°C. The cells were then treated with an equal volume of a 1:10 dilution of rabbit serum as a source of complement for 30 min at 37°C, as described previously (9). T-cell subset depletions were better than 95% as measured by cytofluorometry with a FACScan cytofluorograph (Becton Dickinson, Sunnyvale, CA).

Cyclophosphamide. This was purchased from Mead Johnson, Evansville, ID, as a lyophilized preparation for human use. It was reconstituted with distilled water and injected i.v. in doses indicated under “Results.”

RESULTS

Early, But Not Late, Treatment with Cy Causes Delayed Regression of the L5178Y Lymphoma. Fig. 1 shows that treating B6D2F1 mice with 150 mg/kg of Cy either 1 h before, or 1 h after, implanting 10^6 tumor cells intradermally resulted, after about 10 days of progressive tumor growth, in complete tumor regression. This means that by the time regression began, the tumor had grown to about 1 cm in diameter. The same result was obtained with parental strain DBA/2 mice (results not shown). However, B6D2F1 mice were employed in all experiments because they were more plentiful and less expensive.

Fig. 1 also shows that in order to obtain tumor regression it was necessary to give Cy in a dose of between 125 and 200 mg/kg body weight. Giving a 100-mg/kg dose was without effect on tumor growth, whereas a 250-mg/kg dose caused increased tumor growth.

As to the time that Cy needed to be given to cause tumor regression, Fig. 2 show that the drug was capable of causing complete tumor regression in all mice, provided it was given between 4 days before tumor implantation and 2 days after. It will be noted that in every case, the tumor grew progressively for about 10 days before regression commenced. It will also be noted that when Cy was given 6 days after tumor implantation (when the tumor was 5–6 mm in diameter) there was a striking enhancement of tumor growth.

Early Treatment with Cy Results in Long Term Survival, Whereas Later Treatment Results in Earlier Death. The foregoing results show that injecting Cy i.v. as early as 2–4 days before, or 2 days after implanting L5178Y tumor cells intradermally, resulted in complete regression of the tumor that emerged, but not until after the tumor had been growing for 10 days. Because the L5178Y lymphoma disseminates to cause systemic disease, it was important to determine next whether Cy-induced regression of the primary tumor resulted in long-term survival of the host. Fig. 3 shows that regression of a 10-day tumor caused by giving Cy 1 h after tumor implantation enabled mice to live well beyond a 90-day period of observation. In contrast, control mice all died by Day 50. On the other hand, giving Cy 4 days after tumor implantation caused a marginal decrease in survival time, and giving it 2 days later resulted in a substantial decrease in survival time.

Onset of Cy-Induced Tumor Regression Is Associated with an Increased Level of T-Cell-mediated Immunity. It was found that Cy-induced regression of the L5178Y lymphoma is immunologically mediated in that Cy treatment failed to cause regression of the tumor growing in mice that were incapable of generating immunity because of having been made T-cell-deficient by thymectomy and irradiation, and restored with bone marrow (results not shown). Direct evidence that early treatment with Cy causes the host to generate an increased level of antitumor immunity was obtained by determining whether spleen cells harvested from Cy-treated mice at the time of onset of tumor regression (Day 10) were capable, on passive transfer, of expressing more antitumor immunity than spleen cells harvested from control tumor bearers on the same day of tumor growth. In this experiment, the recipient mice were carrying a 4-day tumor, and were given Cy 1 h before receiving donor spleen cells in order to remove a T-cell barrier to adoptive immunity (11, 12). Donor mice were given Cy 1 h after tumor implantation.
CYCLOPHOSPHAMIDE-INDUCED REGRESSION OF CY-RESISTANT TUMOR

Fig. 2. Evidence that in order (150 mg/kg) to cause tumor regression in all mice Cy needed to be administered no earlier than 2 days before tumor implantation (A), and no later than 2 days after (B). Treating mice with Cy 4 days before tumor implantation (A) caused regression of the tumor in three of five mice, whereas Cy given 6 days before tumor cells had practically no effect on tumor growth. On the other hand, giving Cy on Day 4 and particularly on Day 6 after tumor implantation (B) caused enhanced tumor growth. Means of five mice per group.

Fig. 3. Whereas, treating mice with 150 mg/kg Cy 1 h after tumor implantation resulted in their surviving more than 90 days, treating them on Day 6 (T6) resulted in a significantly reduced time of survival. Five mice per group.

It can be seen in Fig. 4 that spleen cells from control tumor bearers, as well as spleen cells from Cy-treated tumor bearers, were capable of causing regression of an established tumor in recipient mice. However, whereas it took two organ equivalents (4 x 10^6) of spleen cells from control tumor bearers to cause regression of the recipient tumor, it took only 0.5 organ equivalents (10^6) of spleen cells from Cy-treated tumor bearers to achieve the same result. Therefore, it seems reasonable to conclude that the spleens of Cy-treated tumor bearers contained a much larger number of effector cells than the spleens of control tumor bearers. In these experiments the number of spleen cells from 10-day tumor bearers treated with Cy 1 h after tumor implantation, was equal to that of untreated control tumor bearers (results not shown). It has been shown elsewhere (10) that the cells that passively transfer immunity to the L5178Y lymphoma are Ly-2+ T-cells.

Cy-induced Tumor Regression Is Associated with the Sustained Presence of Immune T-Cells. The preceding findings show that early treatment with Cy results in the generation of an augmented level of T-cell-mediated concomitant immunity. It was considered important to determine next whether this increased level of immunity is sustained during the period of tumor regression.

It can be seen in Fig. 5 that, whereas 2 organ equivalents of spleen cells from control tumor bearers harvested on Days 9 or 12 of tumor growth were capable, on passive transfer, of causing regression of a tumor in recipient mice, the same number of spleen cells harvested on Day 15 or 18 was incapable of mediating an antitumor effect. In contrast, it was possible to cause regression of the recipient tumor with the same number of spleen cells harvested from Cy-treated tumor bearers any time between Day 9 and 21. Therefore, while progressive growth of the L5178Y lymphoma in control mice was associated with the generation and subsequent loss of concomitant immunity, tumor regression in Cy-treated mice was associated with the generation of an increased level of concomitant immunity that was sustained throughout the period of tumor regression, and almost certainly beyond. It should be noted, however, that
Cyclophosphamide-Induced Regression of CY-Resistant Tumor

Fig. 5. Treating mice with 150 mg/kg Cy 1 h after tumor implantation resulted in sustained production of effector cells. In A, 2 organ equivalents of spleen cells from Cy-treated mice donors harvested on Days 9, 12, 15, 18, or 21 of the experiment were capable, in all cases of causing complete regression of a tumor in recipients. In B, 2 organ equivalent spleen cells from untreated tumor bearers were capable of causing complete regression of the recipient's tumor only if the spleen cells were harvested on Day 9 or 12. Spleen cells from these mice on days 15, 18, or 21 had no effect on the recipients' tumor. The recipient mice were treated as described in Fig. 4. Means of five mice per group.

spleen cells harvested from Cy-treated tumor bearers on Day 9 or 12 caused regression of the recipient tumor after a shorter delay than spleen cells harvested on Day 15 or 18.

Cy-induced Tumor Regression Can Be Inhibited by Infusion of L3T4+ T-Cells from Normal Mice. Taken together, the foregoing results show that early treatment with Cy causes spontaneous regression of the Cy-resistant L5178Y lymphoma after an appreciable period of tumor growth, by augmenting the level of host concomitant immunity. To determine whether Cy-induced augmentation of immunity depends on the elimination of Cy-sensitive suppressor T-cells, an attempt was made to prevent Cy-induced tumor regression by infusing Cy-treated mice with splenic T-cells from normal mice. It can be seen in Fig. 6 that if mice implanted with 10⁶ L5178Y lymphoma cells were given Cy 1 h later, and infused after an additional hour with 2 organ equivalents of spleen cells from normal mice, Cy-induced tumor regression that normally begins after 10 days of tumor growth, failed to occur. Instead, the tumor grew at the same rate as in control mice. Fig. 6 shows, in addition, that the normal spleen cells that blocked Cy-induced tumor regression were of the L3T4+, Lyt-2- phenotype, as evidenced by their susceptibility to treatment with anti-L3T4 antibody and complement, and their resistance to anti-Lyt-2 antibody and complement.

Suppressor T-Cells in Normal Mice Are Destroyed and Then Regenerated after Giving Cy. The preceding results show clearly that the therapeutic effect of Cy against the L5178Y lymphoma can be negated by an infusion of T-cells from normal mice. It was considered necessary to determine next whether these putative suppressor T-cells are destroyed by the standard therapeutic dose of Cy used to cause tumor regression, and whether they are then regenerated.

Fig. 7 shows the results of an experiment in which an attempt was made to inhibit the therapeutic effect of Cy with 2 × 10⁶ spleen cells harvested from normal donor mice 1 h, 2 days, 4 days, or 8 days after giving a single 150-mg/kg dose of Cy. It can be seen that Cy ablated the ability of normal spleen cells to inhibit Cy-induced regression of the L5178Y lymphoma in Cy-
treated recipients. However, it can also be seen that the suppressor T-cells were regenerated between 4 and 8 days after Cy was given.

DISCUSSION

This study provides additional evidence for the view (13-15) that the negative regulatory influence of suppressor T-cells is responsible for the inadequacy of the immune response to certain immunogenic murine tumors. The results show that a single 150-mg/kg dose of Cy given i.v. shortly before, or shortly after, implanting 10⁶ cells of the Cy-resistant LS178Y lymphoma intradermally, results, after 10 days of progressive tumor growth, in complete tumor regression and in long-term host survival. Evidence that this impressive therapeutic action of Cy is based on its ability to selectively eliminate suppressor T-cells is provided by the demonstration that Cy-induced tumor regression could be completely inhibited by an infusion of splenic T-cells from normal mice. These T-cells of normal mice were themselves susceptible to Cy, but were regenerated 4 days after giving a 150-mg/kg dose of the drug i.v. This explains why Cy failed to cause tumor regression if given earlier than 4 days before tumor implantation. It has been shown by others (1) that the immunoaugmentative action of Cy depends on it being given no earlier than 2-3 days before antigen.

The T-cells from normal mice that negated the therapeutic effect of Cy in tumor-bearing recipients were shown to be of the L3T4⁺ surface phenotype. Thus they are of the same surface phenotype as tumor-induced suppressor T-cells induced after 9-15 days of progressive growth of the LS178Y lymphoma (11) and other tumors (16, 17). Tumor-induced suppressor T-cells are also Cy-sensitive (11, 12) and have the capacity to suppress the ability of immune T-cells to cause regression of an established tumor in T-cell-deficient recipient mice (16, 17). The fact that Cy-sensitive preexisting T-cells from normal mice, as described here, fail to suppress in this assay will be dealt with in a forthcoming publication. It is apparent at this stage, however, that these T-cells are the precursors of tumor-induced suppressors.

The finding that Cy-sensitive precursor suppressors and tumor-induced suppressors are of the CD4⁺ phenotype should not be considered unusual, because all “suppressor inducer” T-cells that function to suppress the induction of immunity are of the CD4⁺ phenotype. There is evidence (18) in this connection, that L3T4⁺ tumor-induced suppressors function to suppress the generation, rather than the expression, of antitumor immunity. A role for CD8⁺ suppressor T-cells cannot be ruled out in this suppression, although there is no compelling reason at this time to invoke their participation. CD4⁺ suppressor T-cells have been shown to be involved in suppressing immunity to UV-induced tumors (19, 20) and the production of cytolytic T-cells to minor histocompatibility antigens (21-23). CD4⁺ suppressor cells have also been shown to be capable of transferring acquired tolerance to cardiac allografts in rats (24), and CD4⁺ human suppressor T-cells have been described (25, 26).

The function of the preexisting Cy-sensitive suppressor T-cells revealed by this study undoubtedly is to cause abridgement of the concomitant immune response to the LS178Y lymphoma, and to thereby prevent immunity from reaching a high enough level to cause tumor regression. This is evidenced by the finding that the elimination of suppressor T-cells with Cy resulted, at the time of onset of tumor regression, in an increased number of splenic T-cells capable of causing regression of a small tumor in recipients. This Cy-augmented therapeutic number of tumor-sensitized T-cells was maintained for at least the period of tumor regression. In contrast, the lower level of immunity in control tumor bearers rapidly decayed after Day 12 of tumor growth.

Obviously, the ability of Cy to augment immunity depended not only on the elimination of suppressor T-cells, but also on the sparing of effector T-cells. In order for effector T-cells to be spared, however, Cy needed to be given no later than 2 days after tumor implantation. Giving it 6 days later resulted in greatly enhanced tumor growth, and results to be published show that this is associated with a failure of the host to generate T-cells capable of passively transferring immunity. This resulted in the tumor growing at the same rate as it did in T-cell-deficient immunoincompetent mice (11). These results indicate that antigen-activated effector T-cells are highly sensitive to Cy, in contrast to precursor effector T-cells which are relatively Cy-resistant. Therefore, Cy is not useful as a therapeutic agent after the antitumor immune response has been induced. This is in keeping with the general finding (1) that Cy is immunoaugmentative if given before, but not after a variety of antigens. Thus the results presented here hardly serve to promote the use of Cy as an immunotherapeutic agent. Indeed, it has been the general finding (12, 27-30) that treatment of tumor-bearing mice with Cy fails to cause complete tumor regression, unless the mice are also infused with immune T-cells from immunized donors, even though Cy by itself causes a substantial reduction in tumor burden. There is evidence in the case of the LS178Y lymphoma (11) and Meth A fibrosarcoma (27) that Cy treatment needs to be given for successful adoptive immunotherapy of relatively large tumors, because it serves to remove a suppressor T-cell barrier to adoptive immunity.

The exception to this rule is the MOPC-315 plasmacytoma (7, 8) which undergoes complete regression in response to Cy given at a late, but not an early, stage of tumor growth. There can be little doubt that complete regression of this tumor depends on host immunity, although most of the tumor is destroyed by the direct action of Cy. We have similarly observed that the LS178Y lymphoma undergoes partial or complete regression in response to Cy given after, but not before, Day 10 of tumor growth. The possibility that this is because the Cy-sensitive effector T-cells generated between days 2 and 10 of tumor growth are converted to Cy-resistant memory T-cells after the onset of dominant T-cell-mediated suppression will be dealt with in a forthcoming publication. Finally, it needs to be stressed that the ability of Cy given just before, or just after, tumor implantation to destroy the LS178Y lymphoma will not apply to all immunogenic tumors. This will be the subject of a forthcoming publication.

ACKNOWLEDGMENTS

We would like to express our appreciation to Ronald LaCourse, Lynn Ryan, and Debra Duso for expert technical support and to Mary Durett for typing the manuscript.

REFERENCES

3. Sercarz, E. Ir gene regulation: past failures to present cogent mechanisms

* M. Awwad and R. J. North, to be published.
Cyclophosphamide-induced Immunologically Mediated Regression of a Cyclophosphamide-resistant Murine Tumor: A Consequence of Eliminating Precursor L3T4+ Suppressor T-Cells

Michel Awwad and Robert J. North


Updated version Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/49/7/1649

E-mail alerts Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.