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Cyclophosphamide (Cy)-facilitated adoptive immunotherapy of a Cy-resistant tumour. Evidence that Cy permits the expression of adoptive T-cell mediated immunity by removing suppressor T cells rather than by reducing tumour burden

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SUMMARY

A cyclophosphamide (Cy)-resistant immunogenic tumour, the L5178Y lymphoma, was used to demonstrate that Cy-treatment of a host bearing this tumour enables passively transferred tumour-sensitized T cells to cause complete tumour regression without any need for Cy to cause a reduction in tumour burden. It was shown that whereas infusion of tumour-sensitized T cells from immune donors had very little effect on growth of the tumour, and whereas treatment with 150 mg/kg of Cy caused appreciable enhancement of tumour growth, combination therapy with Cy plus immune T cells caused complete tumour regression and resulted in long-term survival. Evidence that Cy treatment facilitated the expression of adoptive immunity against the L5178Y lymphoma by eliminating tumour-induced suppressor T cells consisted of the demonstration that tumour regression caused by combination treatment with Cy and immune T cells could be inhibited by infusing the recipient with Cy-sensitive, L3T4$^+$ T cells from tumour-bearing but not from normal donors.

INTRODUCTION

Immunity to syngeneic murine tumours is T-cell mediated, in that it can be passively transferred from tumour-immunized donors to normal recipients with tumour-sensitized T cells (Hellstrom & Hellstrom, 1969). However, the literature shows (Rosenberg & Terry, 1977) that while passively transferred immunity can prevent the emergence and growth of a tumour cell implant, it cannot be expressed against a tumour that is already established and growing. It is apparent, therefore, that a barrier develops in a tumour-bearing recipient early in tumour growth that prevents intravenously infused tumour-sensitized T cells from expressing their anti-tumour function. Realization that this barrier exists, and that it can be eliminated by exposing recipient mice to a sublethal dose of ionizing radiation, or by treating them with Cy (North, 1985), is the reason for the large number of papers in recent years demonstrating successful adoptive immunotherapy of established murine tumours by passive transfer of tumour-sensitized T cells.

The nature of the acquired barrier to adoptive immunotherapy has been a subject of investigation in this laboratory for several years. It has been shown that the barrier can be restored in Cy-treated (North, 1982) or γ-irridiated tumour-bearing mice (North, 1984a) by infusing them with T cells from tumour-bearing but not from normal donors. These findings agree with those obtained with T-cell deficient-bearing recipients (Berendt & North, 1980; Bonventra *et al.*, 1982), and are in keeping with the hypothesis that the barrier to adoptive immunotherapy of an established tumour is due to the presence of a population of tumour-induced suppressor T cells. It was argued (North, 1984b) that successful adoptive immunotherapy of established tumours should be possible, provided steps are taken to remove tumour-induced suppressor T cells from the recipient, and provided the recipient is then infused with a sufficient number of tumour-sensitized T cells.

However, in the case of facilitation of adoptive immunotherapy by treating tumour-bearing recipients with Cy, it is not yet possible to conclude that the facilitating action of the drug is entirely dependent on its ability to remove suppressor T cells. This is because in the case of those tumours studied so far (North, 1982; Greenberg & Cheever, 1984; Evans, 1983; Bookman, Swerdlow & Matis, 1987), Cy causes extensive destruction of the tumours by itself. Consequently, infused tumour-sensitized T cells have a much smaller tumour burden with which to contend, and this may contribute wholly or partly to the success of adoptive immunotherapy. There is a need to determine, therefore, whether Cy is able to facilitate successful adoptive immunotherapy entirely on the basis of its ability to remove suppressor T cells from a tumour-bearing recipient. This could be investigated by employing a tumour that is resistant to the direct cytotoxic action of the drug.

Abbreviations: Cy, cyclophosphamide; FBS, fetal bovine serum; PBS, phophate-buffered saline.

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The purpose of this paper is to show that Cy treatment can facilitate the adoptive immunotherapy of an established Cy-resistant tumour, even though giving Cy alone causes the tumour to grow at a faster rate. It will show, in addition, that the immuno-facilitating action of Cy can be blocked by infusing the Cy-treated recipient with L3T4$^+$ T cells from tumour-bearing but not from normal donors. The results allow the conclusion that Cy can facilitate adoptive immunotherapy entirely on the basis of its ability to eliminate suppressor T cells from the tumour-bearing recipient.

MATERIALS AND METHODS

Mice

DBA/2 and B6D2F1 (C57BL/6 × DBA/2) mice were employed when they were 12 weeks of age. They were purchased from the Trudeau Institute Animal Breeding Facility, Saranac Lake, NY. They were known to be free of common viral pathogens, as evidenced by the results of routine testing performed by Microbiological Associates, Bethesda, MD.

Tumour

The L5178Y lymphoma, syngeneic with DBA/2 mice, was originally obtained from Dr E. F. Wheelock, Thomas Jefferson University, Philadelphia, PA. According to a recent study using flow cytometry with specific monoclonal reagents (M. Awwad and R. J. North, manuscript to be published), the L5178Y lymphoma is Thy-1.2$^+$, Ly-2$^-$, L3T4$^-$, Ia$^-$, H-2K/D$^+$. To prepare a stock of tumour cells for a complete series of experiments, the tumour was grown as an ascites in DBA/2 mice, harvested and washed in phosphate-buffered saline (PBS), and cryopreserved over liquid nitrogen in small volumes of RPMI-1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) and 10% dimethylsulphoxide. For each experiment a vial was thawed and the tumour cells expanded in number by allowing them to grow as an ascites in B6D2F1 mice. The cells were harvested, washed and resuspended in PBS for implantation into experimental B6D2F1 mice. B6D2F1 hybrid mice were used because they were less expensive and more plentiful than parental DBA/2 mice. There was no evidence of hybrid resistance against the L5178Y lymphoma. Tumour were initiated intradermally in the belly region by implanting 10^6 tumour cells in 0·05 ml of PBS. Tumour growth was followed by measuring changes against time in the mean of two perpendicular diameters with dial calipers.

T-cell deficient (TXB) mice

Mice were made T-cell deficient by thymectomy at 6 weeks of age followed 1 week later by exposure to 800 rads of γ-radiation from a ^{137}Cs source. They were infused with 10^7 syngeneic bone marrow cells 1 hr after irradiation, and employed in experiments no earlier than 6 weeks later.

Passive transfer of immunity and suppression

Donors of tumour-sensitized T cells were mice that had been injected intradermally 3 weeks earlier with an admixture of L5178Y cells and 100 μg of *propionibacterium acnes* (purchased from Trudeau Institute). Immunization by this procedure is associated with 8–9 days of progressive tumour growth followed by complete tumour regression (Dye, North & Mills, 1981). The spleens of mice so immunized were diced into small pieces and passed through a 70 mesh stainless steel screen into PBS containing 1% FBS. The resulting cell suspension was triturated to break up clumps, passed through surgical gauze to remove debris, and washed twice in PBS. The cells were then suspended in PBS for intravenous infusion. Each recipient received 1 spleen equivalent (approximately 2×10^8) donor spleen cells.

The donors of suppressor T cells were mice bearing an 18-day (1·5 cm diameter) intradermal tumour. Their spleen cells were prepared in the same way as immune spleen cells. Each recipient received 1 spleen equivalent (2×10^8) of these cells intravenously 1 hr after infusing immune spleen cells.

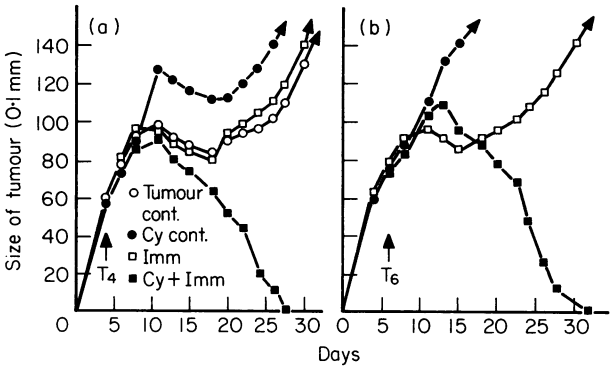

Figure 1. Treatment of mice bearing a 4-day (a) or a 6-day tumour (b) with 150 mg/kg of Cy caused increased tumour growth, and infusion of mice on either of these days with 1 organ equivalent (2×10^8) of spleen cells from immunized donor mice, had no effect on tumour growth. In contrast, treatment with Cy on Day 4 or 6 followed 1 hr later by infusion of donor immune spleen cells (Cy + IMM) resulted in complete tumour regression. Means of five mice per group.

Elimination of T cells and T-cells subsets

Thy-1.2$^+$ and Lyt-2$^+$ T cells were removed from spleen cell suspensions by treating the suspensions with monoclonal anti-Thy-1.2 antibody or anti-Ly-2.2$^+$ antibody, respectively (clones 30-H12 and TIB-150, from the American Type Culture Collection, Rockville, MD). L3T4$^+$ T cells were removed by treating the suspensions with anti-L3T4 antibody (clone GK1.5 from Dr Frank Fitch, Department of Pathology, University of Chicago, IL). Treatment with each antibody was followed by treatment with rabbit complement, as described previously (North, 1986).

Cyclophosphamide

This was purchased from Mead Johnson, Evansville, ID. It was dissolved in physiological saline, and injected intravenously in the doses indicated.

RESULTS

Cy facilitates the expression of adoptive immunotherapy even though it enhances tumour growth when given alone

It became apparent from pilot studies with the L5178Y lymphoma that this tumour is resistant to the direct cytotoxic action of Cy, in that it grows faster in Cy-treated mice. Figure 1 shows the results of an experiment that compared the effect against a 4-day or a 6-day L5178Y lymphoma of giving Cy alone, immune spleen cells alone, or Cy plus immune spleen

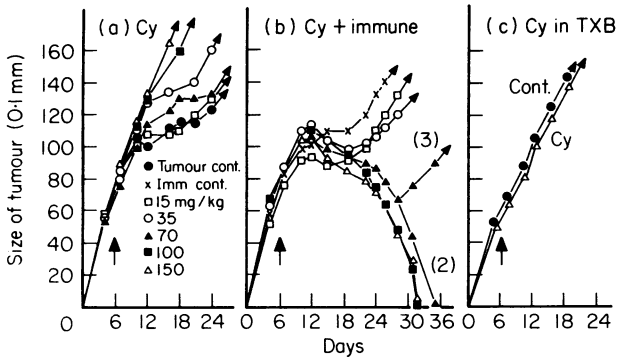

Figure 2. Evidence that doses of Cy above 70 mg/kg are tumour promotive (a), but that these doses are needed to facilitate the expression of adoptive immunity against an established tumour (b). (c) Shows that the tumour grew much faster in T-cell deficient mice, and that the rate of tumour growth was not affected by a 150 mg/kg dose of Cy. In this experiment the doses of Cy shown were given alone on Day 6 of tumour growth, or in combination with an infusion of 1 organ equivalent of donor immune spleen cells 1 hr later. Means of five mice per group.

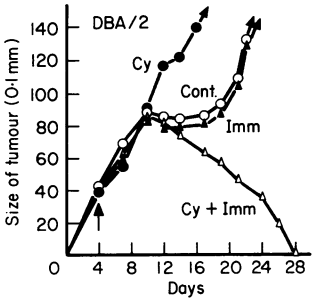

Figure 3. Evidence that the basic result shown in Fig. 1 obtained with B6D2F1 mice can also be obtained with parental DBA/2 mice. Whereas Cy alone caused increased tumour growth in DBA/2 mice, and immune cells alone had no effect on tumour growth, Cy plus immune spleen cells caused complete tumour regression. Means of five mice per group.

cells. It can be seen that giving Cy alone in a dose of 150 mg/kg caused significant enhancement of tumour growth, whereas giving immune cells alone had no effect on tumour growth. However, when injection of 150 mg/kg of Cy was followed 1 hr later by infusion of immune spleen cells, the tumour in all mice underwent complete regression. There can be little doubt, therefore, that Cy facilitates the expression of adoptive anti-tumour immunity against the L5178Y lymphoma without causing any direct destruction of the tumour by itself. On the contrary, the doses of Cy that needed to be given to facilitate the expression of adoptive immunity caused the recipient tumour to grow much larger before immunological mediated regression commenced.

Figure 2 shows the results of an experiment that tested the anti-tumour effect of giving different doses of Cy either alone or in combination with 1 spleen equivalent of immune spleen cells. It can be seen that doses of Cy above 70 mg/kg had to be given to ensure that passively transferred immune spleen cells would cause complete tumour regression in all mice. Giving these doses of Cy alone caused enhancement of tumour growth.

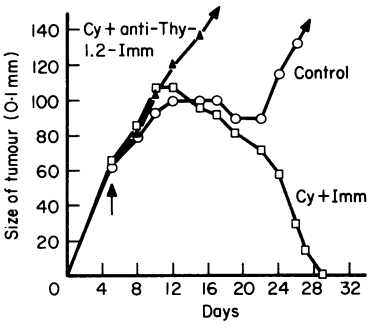

Figure 4. Evidence that the donor spleen cells that mediate tumour regression in Cy-treated recipients were T cells. Treating the spleen cells with anti-Thy-1.2 antibody and complement (Cy + anti-Thy-1.2-Imm) completely eliminated their capacity to cause tumour regression. Means of five mice per group.

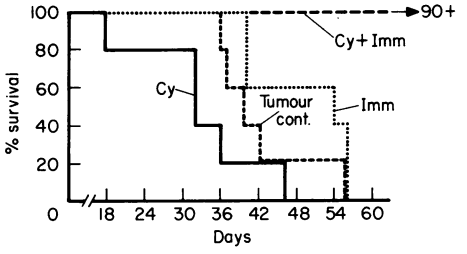

Figure 5. Evidence that giving tumour-bearers 150 mg/kg of Cy plus 1 organ equivalent of immune spleen cells on Day 4 of tumour growth resulted in long-term survival. None of the mice so treated had died of their tumour at the termination of the experiment on Day 90. In contrast, mice treatment with Cy alone had a shortened survival time, and mice given an infusion of immune cells died at about the same time as control mice.

Figure 2 also provides convincing additional evidence that the L5178Y lymphoma is resistant to Cy *in vivo*. It shows that the tumour grew at the same rate in T-cell deficient (TXB) mice, regardless of whether or not they were given a 150 mg/kg dose of Cy on Day 6 of tumour growth. This means, almost certainly, that Cy caused increased tumour growth in immunocompetent mice by ablating concomitant anti-tumour immunity. This will be the subject of a forthcoming publication (M. Awwad and R.J. North, manuscript in preparation).

With one exception, all of the experimental results presented in this paper were obtained with the L5178Y lymphoma growing in B6D2F1 mice. The exception is shown in Fig. 3, which represents the results of an experiment performed in parental DBA/2 mice. It can be seen that the results obtained with DBA/2 were essentially the same as those obtained with B6D2F1 mice. Whereas an injection of 150 mg/kg of Cy caused enhanced tumour growth, and whereas an infusion of immune cells alone had no effect on tumour growth, injection of 150 mg/kg of Cy followed 1 hr later by infusion of donor immune cells caused complete regression of the tumour in all recipients.

That the donor spleen cells that passively transferred immunity from immunized donors to Cy-treated recipients were T cells is shown in Fig. 4, where it can be seen that incubating the cells with anti-Thy-1.2 and complement completely eliminated their ability to cause regression of the tumour in recipients treated with Cy 1 hr earlier.

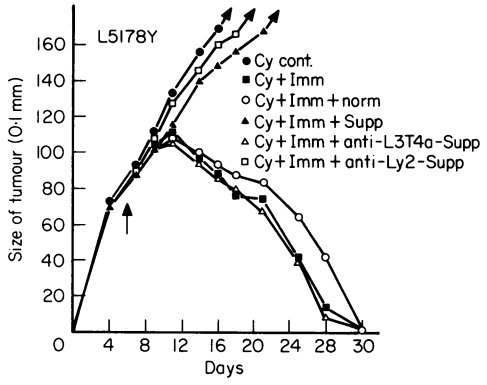

Figure 6. Demonstration that tumour regression caused by treatment with Cy plus immune cells (Cy+Imm) could be inhibited by infusion 1 hr later of 1 organ equivalent (2×10^8) of spleen cells from donor bearing an 18 day tumour (Cy+Imm+Supp), but not by infusing the same number of spleen cells from normal donors (Cy+Imm+norm). Moreover the spleen cells from tumour bearers that inhibited tumour regression could be functionally eliminated by treatment with anti-L3T4 antibody and complement (Cy+Imm+anti-L3T4-Supp), but not treatment with anti-Ly-2.2 antibody and complement (Cy+Imm+anti-Ly-2-Supp). Means of five mice per group.

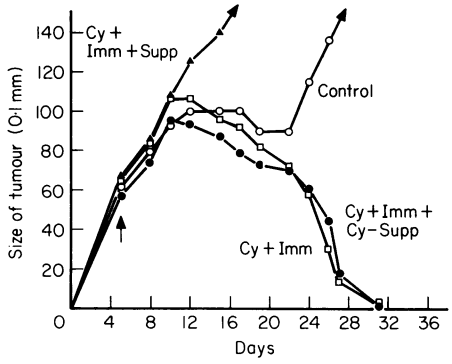

Figure 7. Spleen cells from tumour-bearing donors that inhibited tumour regression caused by Cy plus immune cells (Cy+Imm+Supp) were functionally eliminated by treating the tumour-bearing donors with 150 mg/kg of Cy 1 hr before harvesting their spleen cells (Cy+Imm+Cy-Supp). Cy-treated tumour bearers given only spleen cells from Cy-treated tumour-bearing donors failed to cause regression of their tumour (not shown). Means of five mice per group.

Combination therapy with Cy and immune cells results in long-term survival

The L5178Y lymphoma metastasizes from its site of intradermal or subcutaneous implantation predominantly to the liver where it grows to kill its host in about 35–50 days. It was important to determine, therefore, whether combination therapy with Cy and immune cells, besides causing regression of the primary tumour, also causes destruction of metastases as evidenced by long-term host survival. That this was the case is shown in Fig. 5 where it can be seen that, while treatment of mice bearing a 4-day tumour with Cy alone marginally shortened survival time, and while giving immune cells alone only marginally extended survival time, combination therapy with Cy plus immune cells enabled all mice to live beyond a 90-day period of observation. When the same experiment was performed with mice bearing a 6-day tumour treatment with Cy alone resulted in much shorter survival (results not shown), as would be predicted from Fig. 1 where it can be seen that giving Cy on Day 6 caused much more growth of the primary tumour.

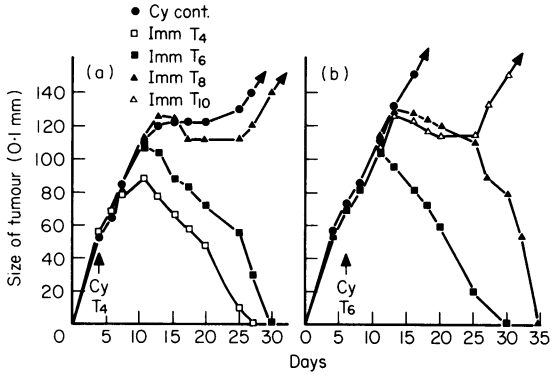

Figure 8. Evidence that the ability of 150 mg/kg dose of Cy on Day 4 (a) or Day 6 (b) of tumour growth to facilitate the expression of adoptive immunity by tumour-sensitized T cells lasts for only 2–3 days. Cy given on Day 4 of tumour growth enabled immune T cells given the same day or 2 days later, but not 4 days later, to cause tumour regression. Cy given on Day 6 enabled immune T cells given on Day 6 or 8, but not on Day 10 to cause tumour regression. Means of five mice per group.

The immuno-facilitating action of Cy can be blocked by Cy-sensitive L3T4$^+$ suppressor T cells from tumour-bearing donors

The foregoing results suggest that Cy treatment of mice bearing the L5178Y lymphoma removes a barrier that functions to prevent passively transferred L5178Y-sensitized donor T cells from expressing their anti-tumour function. Evidence generated previously from this laboratory with other tumours (North, 1984b) suggests that the barrier to adoptive immunotherapy is due to the presence of tumour-induced suppressor T cells. To determine whether this is the case for mice bearing the L5178Y lymphoma, an attempt was made to restore the barrier in Cy-treated tumour-bearing recipients by infusing them with spleen cells from donor mice bearing a large (1·5 cm diameter) L5178Y lymphoma.

Figure 6 shows that the ability of immune T cells to cause regression of the L5178Y lymphoma in Cy-treated mice could be completely inhibited by infusing the mice 1 hr later with spleen cells from tumour-bearing donors. Figure 6 shows, in addition, that the donor spleen cells that suppressed the expression of adoptive immunity were L3T4$^+$ T cells, in that they were functionally eliminated by treatment with anti-L3T4 antibody and complement, but not by treatment with anti-Ly-2 antibody and complement.

Evidence that the suppressor T cells from tumour-bearing donors were sensitive to the dose of Cy used to facilitate adoptive immunotherapy is shown in Fig. 7. It can be seen that spleen cells from tumour-bearing donors failed to inhibit adoptive immunotherapy, if the donors were given a 150 mg/kg dose of Cy 1 hr before their spleen cells were harvested. Because there was no reduction in the number of cells in the spleens of Cy-treated donors at the time of cell harvest (results not shown), Cy-sensitive suppressor T cells presumably died some time after they were passively transferred to recipients.

It is important to point out at this stage that, because the L5178Y lymphoma is L3T4$^+$ and Cy resistant, these results show that suppression was not caused by the presence of contaminating tumour cells in the suppressor spleen cell preparation.

Immuno-facilitating effect of Cy lasts for 2 days

If Cy facilitates the expression of adoptive anti-tumour immunity by eliminating tumour-induced suppressor T cells from the recipient, it is important for the future analysis to know how long after giving Cy that suppressor T cells remain absent.

Figure 8 shows that a 150 mg/kg dose of Cy given on Day 4 or Day 6 of growth of the L5178Y lymphoma allowed donor immune cells given 2 days later, but not 4 days later, to cause complete regression of the tumour. Therefore, the suppressor barrier to adoptive immunotherapy returns between 2 and 4 days after Cy is given. It is obvious from Fig. 8, moreover, that the inability of immune cells to cause regression when given 4 days after injecting Cy on Day 4 of tumour growth was not caused by the tumour having grown too large to be destroyed. The same size tumour was destroyed if Cy was given on Day 6 and immune cells were given on Day 8.

DISCUSSION

Complete regression of immunogenic tumours in syngeneic mice by combination therapy with Cy and tumour-sensitized T cells has been reported previously from several laboratories (North, 1982; Greenberg & Cheever, 1984; Evans, 1983; Bookman *et al.*, 1987). It was shown in all cases that treatment with Cy plus immune T cells caused complete tumour regression, treatment with immune T cells alone had no significant effect on tumour growth, and treatment with Cy alone caused various degrees of tumour regression, depending on the tumour. In the case of some of these models of Cy-facilitated adoptive immunotherapy (Greenberg & Cheever, 1984; Evans, 1983; Bookman *et al.*, 1987), the degree of tumour regression caused by Cy alone was extensive enough to make it highly likely that Cy facilitated adoptive immunotherapy, not by removing suppressor cells, but by making the tumour burden small enough for the passively transferred immune T cells to destroy. Therefore, in these particular models of chemo-immunotherapy there seems to be no reason to invoke the function of Cy-sensitive suppressor T cells to explain why Cy facilitates the expression of adoptive immunity. The same might be said for more recently published results (Rosenberg, Spress & Lafraniere, 1986) showing that recipient mice bearing artificial lung metastases need to be treated with Cy in order for passively transferred tumour-infiltrating donor lymphocytes to cause tumour destruction.

However, in another model of combination therapy with Cy and donor immune T cells (North, 1982), Cy treatment alone caused only partial regression of the recipient's tumour, thereby leaving an appreciable tumour burden for passively transferred immune T cells to destroy. Moreover, with this model of adoptive immunotherapy it was possible to inhibit the expression of Cy-facilitated adoptive immunotherapy by infusing the recipient with Cy-sensitive T cells from mice bearing an established tumour. This was taken to mean that tumour regression in this model depends, at least in part, on the ability of Cy to eliminate a population of tumour-induced suppressor T cells that otherwise would function to inhibit the ability of tumour-immune donor T cells to express their anti-tumour function. Even with this model, however, the possibility remained that partial destruction of the tumour by Cy needed to occur before passively transferred immune T cells could cause complete tumour regression.

The results presented here with the L5178Y lymphoma represent convincing evidence that reduction of tumour burden by Cy is not necessary in order for Cy to facilitate the expression of passively transferred immunity against an established immunogeneic tumour. Because the L5178Y lymphoma was completely resistant to the direct cytotoxic action of Cy, there can be little doubt that the role of the drug in facilitating the expression of adoptive immunity was to remove a barrier that prevents intravenously infused tumour-sensitized donor T cells from expressing their anti-tumour function. Moreover, because it was shown that the barrier could be replaced by infusing Cy-treated recipients with L3T4$^+$ T cells from tumour-bearing, but not from normal donors, it is almost certain that the barrier is mediated by a population of tumour-induced suppressor T cells. The production in tumour-bearing mice of suppressor T cells with the same surface phenotype was revealed during the course of previous studies in this laboratory (North & Bursuker, 1984; North & Dye, 1985). It was hypothesized on the basis of these findings (North, 1985) that the emergence of suppressor T cells after an immunogeneic tumour grows beyond a certain size is the reason why underlying, concomitant anti-tumour immunity fails to develop sufficiently to cause tumour regression. Recent causal evidence in support of this hypothesis consists of the demonstration (North, 1986) that preferential elimination of suppressor T cells from mice bearing the L5178Y lymphoma, or certain other immunogenic tumours, by whole-body exposure to sublethal γ-radiation results in spontaneous immunologically mediated tumour regression.

It is important to point out, however, that no evidence was generated in the present study to show that Cy treatment preferentially eliminates suppressor T cells. On the contrary, because treatment with Cy alone on Day 4 or 6 of tumour growth caused the tumour to grow faster, it is likely that effector T cells and their precursors were also destroyed by Cy. Direct evidence that this is the case will be presented in a forthcoming publication (M. Awwad and R. J. North, manuscript to be published) from this laboratory. It can be mentioned here, however, that the onset of expression of concomitant immunity to the L5178Y lymphoma in tumour-bearing control mice is indicated by a flattening of the tumour growth curve at Day 10 of tumour growth, as shown by most of the figures of this paper. This reduction in the rate of tumour growth was either substantially delayed or prevented from occurring if the mice were given Cy.

It needs to be mentioned, with regard to this immunodepressive action of Cy, that the drug has been used to augment immune responses on numerous occasions (discussed by Turk & Parker, 1982; Goto *et al.*, 1981). However, the ability of Cy to augment the generation of delayed sensitivity (Askenase, Hayden & Gershon, 1975; Mitsuoka, Baba & Morikaw, 1976) or cytolytic T cells (Rollinghoff *et al.*, 1977; Glaser 1979), for example, requires that it be injected before antigen is given. This is different from the way Cy was used in the study presented here, in that Cy was injected well after antigen was given, and at a time when the T cells that mediate concomitant immunity

would be expected to be in the process of being generated and activated (North & Bursuker, 1984; North & Dye, 1985). Activated effector T cells are known to be susceptible to the doses of Cy used here (Glaser, 1979; Dye & North, 1984).

In any case, regression of the L5178Y lymphoma did not automatically occur after injecting Cy, but required that the host be infused with a sufficient number of donor immune T cells within 2–3 days after Cy was given, presumably because suppressor T cells were regenerated. As to the substantial delay after giving Cy and immune T cells before adoptive immunity was expressed, this was probably due to the need for passively transferred immune T cells to expand to a number large enough to cause tumour regression. According to previous studies (Mills & North, 1985) it is likely that the donor immune T cells employed were memory T cells that possessed no immediate capacity of their own to cause tumour regression, but needed time to give rise to activated effector T cells by way of a secondary active immune response in the recipient. Presumably the removal of suppressor T cells by Cy allowed this secondary adoptive immune response to proceed. According to this interpretation suppressor T cell suppress the induction of immunity, and this is in keeping with them having the L3T4$^+$, Ly-2$^-$ surface phenotype like the suppressor inducers T cells in other models of suppression (Claman *et al.*, 1980; Greene, 1980). T cells with this phenotype also have been shown to be generated during the growth of other immunogenic tumours (North, 1985), to suppress the generation of cytolytic T cells to minor histocompatability antigens (Macphail & Stutman, 1982; Holan & Mitchison, 1984), and to pasively transfer tolerance to heart allografts in rats (Hale *et al.*, 1985).

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