## *Brief Definitive Report*

# REGULATION OF SPECIFIC CELL-MEDIATED CYTOTOXIC RESPONSE AGAINST SV40-INDUCED TUMOR ASSOCIATED ANTIGENS BY DEPLETION OF SUPPRESSOR T CELLS WITH CYCLOPHOSPHAMIDE IN MICE

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Drugs are often used to suppress unwanted immune responses to antigenic stimuli. However, many clinically important immune reactions are the sum of a number of different immune factors working either synergistically or antagonistically. Attention has been given to those situations in which cell-mediated-immune responses are modulated by humoral blocking factors (1) or by suppressor cells (2–6). There is a need to find ways of selectively affecting undesirable cell-mediated or humoral immunity so that the balance can be shifted toward the direction of the reaction desired in a particular situation. The drug cyclophosphamide (cy) seems to fulfill this goal. It was first shown to enhance delayed type hypersensitivity reactions in mice and guinea pigs by damaging suppressor B cells (7–9) and later to affect suppressor T rather than B cells (10–12). Our own interest is in tumor immunology, and we have been attempting to find ways of using drugs to enhance immune responses to tumor associated antigens in the hope of achieving better immunotherapy of tumors as was suggested elsewhere (13, 14). For the past few years extensive study on immune response to SV40-induced transplantable mKSA cells of BALB/c mice has been performed in our laboratory. This tumor has been shown to possess tumor associated antigens which induce specific transplantation rejection of the tumor (15–17). Recently, we and others developed an in vitro $^{51}Cr$ release assay to measure the immune response against SV40-induced tumor associated antigens. The effector cells generated by antigen immunization were shown to be T-cell dependent (18, 19). The present report is the first demonstration that treatment of mice with cy markedly augmented the specific cell-mediated cytotoxic reactivity against tumor-associated antigens by damage of cy to T cells which suppress in vivo the differentiation of antigen-specific cytotoxic T cells. Thus treatment of mice with a single dose of cy converts their state of low to high responsiveness.

## Materials and Methods

*Mice.* Female $CBF_1$ (BALB/c × C57BL/6) $F_1$ mice, aged 8–12 wk, were obtained from the National Institutes of Health breeding colony.

*Tumor Cell Lines.* SV40 transformed cells, C57SV of C57BL/6 mice were obtained from B. B. Knowles, the Wistar Institute, Philadelphia, Pa. These cells are good targets in the in vitro $^{51}Cr$ release assay, but do not grow in vivo (18, 19). SV40 transformed cells, mKSA of BALB/c mice grow in vivo but are poor targets in the in vitro cytotoxicity assay (16). Both cell lines were shown to possess tumor-associated transplantation antigens which can immunize specifi-



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cally against the growth of mKSA cells (17, 19). The MSB cell line was established from M-MuSV-induced tumor in C57BL/6 mice (20). The cells were passed in tissue culture by exposure to 0.25% trypsin. They are good target cells for in vitro $^{51}Cr$ release assay (20).

*Removal of B Cells and Phagocytic Cells.* The technique described by Julius et al. (21) was used. About 30% of the starting spleen cell population was recovered in the nonadherent fraction; of these 1–3% (as compared to 40–45% in the unfractionated population) were surface immunoglobulin-positive cells and 0.3% phagocytic cells (as compared to about 5% in the unfractionated population) as judged by latex particle ingestion.

*Removal of T Cells.* AKR anti-Thy 1.2 C3H antibody was kindly obtained from H. Holden, National Cancer Institute, Bethesda, Md. The preparation of this serum and the treatment of lymphoid cells were performed according to the technique described in detail (22). This antiserum lysed more than 97% of C3H thymocytes and 35–40% of C3H spleen cells by trypan blue dye exclusion. The T-cell response of spleen cells to phytohemagglutinin was abolished after treatment, although the B-cell response to lipopolysaccharide was left intact.

*$^{51}Cr$ Release Assay.* The assay was performed in the wells of flat bottom microplates. C57SV target cells were trypsinized and $1 \times 10^4$ cells and 5 $\mu$Ci $Na^{51}CrO_4$ in 0.2 ml RPMI-1640 medium containing 5% fetal bovine serum were added to each well. After 18 h at 37°C in a $CO_2$ incubator, the target cells were washed and effector cells were added in 0.2 ml vol in various lymphocyte:target cell ratios. After 6 h incubation at 37°C in a $CO_2$ incubator, the plates were centrifuged at 350 *g* for 5 min. 0.1 ml of the supernate was removed for counting. Results are calculated from the mean of triplicate samples and expressed as:

$$\text{Per cent-specific lysis} = \frac{\text{cpm } ^{51}\text{Cr released from cells in the presence of immune cells} - \text{cpm } ^{51}\text{Cr released from cells in the presence of normal cells}}{\text{cpm } ^{51}\text{Cr released from cells in the presence of 1\% SDS}} \times 100.$$

The $^{51}Cr$ released in the presence of normal cells was always either very close or identical to the release in medium and ranged from 5 to 15%. Representative experiments are shown in Tables I–III. Each experiment was repeated three to four times with similar results.

*Hydrocortisone Treatment.* 2 mg of hydrocortisone acetate (The Upjohn Co., Agricultural Prods. Mkt., Kalamazoo, Mich.) were injected i.p. into normal $CBF_1$ mice. The mice were used as lymphocyte donors 2 d later.

## Results

The data of the first experiment (Table I) demonstrate that after i.p. immunization of $CBF_1$ mice with $2 \times 10^7$ C57SV cells, cell-mediated cytotoxic response was generated in the spleens with peak activity at day 8, and this activity decreased to low levels by day 14. This finding is in accordance with earlier reports (18, 19). $CBF_1$ mice treated i.p. with cy 2 d before C57SV cell immunization generated markedly higher levels of cytotoxic response which was still detected at day 20. Cy at a concentration of 100 mg/kg had an optimal augmenting effect, whereas higher concentrations were less effective. These results implied that cy administered in vivo before antigenic immunization can augment the magnitude and kinetics of cell-mediated cytotoxic response against SV40 transformed cells. In Table II the relationship between cy administration before or after antigen immunization and the cytotoxic response generated in vivo is observed. $CBF_1$ mice were treated i.p. with 100 mg/kg of cy at various times before or after i.p. immunization with $2 \times 10^7$ C57SV cells. The cytotoxicity assay was performed on day 8 after antigen inoculation. The optimal time for cy treatment was 2 d before in vivo antigen immunization. The effect of cy decreased with an increase in time interval between cy administration before antigen inoculation and C57SV cell immunization. Drug application simultaneously with, or 2 or 4 d after antigen immunization had no augmenting effect, whereas, drug

TABLE I

*The Effect of Treatment of $CBF_1$ Mice with Cyclophosphamide on the In Vivo Generation of Cell-Mediated Cytotoxic Response against SV40 Transformed Cells*

| Dose of Cy* | After C57SV cell immunization‡ | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 4 | 6 | 8 | 10 | 14 | 20 |
| *(mg/kg)* | *days* | | | | | | |
| 0 | 5§ | 13 | 24 | 31 | 25 | 8 | 4 |
| 25 | 5 | 12 | 26 | 33 | 21 | 10 | 5 |
| 50 | 7 | 19 | 36 | 45 | 32 | 18 | 15 |
| 100 | 8 | 23 | 48 | 62 | 53 | 46 | 34 |
| 200 | 4 | 10 | 21 | 34 | 23 | 6 | 3 |

* Cy was inoculated i.p. 2 d before C57SV cell immunization.

‡ $CBF_1$ mice were inoculated i.p. with $2 \times 10^7$ C57SV cells 2 d after cy treatment. At various times thereafter spleens were removed and the cells were tested for cytotoxic activity against $^{51}Cr$-labeled C57SV target cells.

§ Percent cytotoxicity.

TABLE II

*The Effect of Treatment of $CBF_1$ Mice with Cyclophosphamide at Various Intervals on the In Vivo Generation of Cell-Mediated Cytotoxic Response against SV40 Transformed Cells*

| Cy administration in relation to antigen immunization‡ | Cytotoxicity against: | | | | | |
|---|---|---|---|---|---|---|
| | C57SV | | | MSB* | | |
| | 100:1§ | 30:1 | 10:1 | 100:1 | 30:1 | 10:1 |
| *days* | *Percent* | | | | | |
| +6 | 5 | 4 | 4 | 4 | 4 | 3 |
| +4 | 20 | 13 | 6 | 4 | 6 | 4 |
| +2 | 24 | 10 | 5 | 6 | 4 | 5 |
| 0 | 21 | 12 | 4 | 4 | 3 | 4 |
| −2 | 54 | 31 | 17 | 7 | 5 | 4 |
| −4 | 36 | 20 | 11 | 6 | 4 | 3 |
| −6 | 28 | 19 | 9 | 5 | 5 | 2 |

* The conditions for the cytotoxicity assay are the same as for C57SV (see details in Table I.)

‡ Cy at 100 mg/kg was administered i.p. at the time intervals listed in relation to i.p. inoculation of $2 \times 10^7$ C57SV cells. The cytotoxicity assay was performed 8 d after antigen immunization.

§ Effector:target cell ratio.

administration 6 d after immunization resulted in suppression of cytotoxic response. These results implied that cy administered 2 d before antigen had an optimal augmenting effect on cell-mediated cytotoxic response, whereas cy administration 6 d after antigen had a suppressive effect. In the last experiment $CBF_1$ mice were treated i.p. with 100 mg/kg of cy and 2 d later were injected i.v. with various cell populations from normal $CBF_1$ mice and then inoculated i.p. with $2 \times 10^7$ C57SV cells. The cytotoxicity assay was performed 8 d later. The results (Table III) indicate that transfer of T cells abolished the cy-induced augmentation of cytotoxic response, implying that cy-sensitive T cells suppressed the in vivo generation of specific cytotoxic T lymphocytes against SV40 transformed cells.

## Discussion

The new aspects of the present study are that pretreatment of mice with cyclophosphamide before immunization with syngeneic tumor-associated antigens results

TABLE III

*The Effect of Transfer of Different Cell Populations to Cyclophosphamide-Treated Mice on the Ability to Generate In Vivo Cell-Mediated Cytotoxicity against SV40 Transformed Cells*

| Cells transferred ($60 \times 10^6$) | Cytotoxicity | | |
|---|---|---|---|
| | 100:1* | 30:1 | 10:1 |
| | *Percent* | | |
| None | 50 | 38 | 23 |
| Unfractionated spleen | 23 | 12 | 4 |
| Nylon wool column spleen | 20 | 10 | 6 |
| Anti-Thy 1.2 serum and complement-treated spleen | 47 | 35 | 21 |
| Complement-treated spleen | 18 | 11 | 7 |
| Cortisone-resistant thymus | 23 | 14 | 8 |
| Anti-Thy 1.2 serum and complement-treated bone marrow | 52 | 36 | 19 |

* Effector:target cell ratio.

in marked augmentation of their ability to generate in vivo antigen-specific cell-mediated cytotoxic response. Because the in vitro cytotoxic reactivities observed in syngeneic tumor systems are often weak or even undetectable, cy treatment may be used to convert such state of low to high responsiveness. Moreover, by such manipulation, it is hoped to achieve more effective ways for immunotherapy of tumors (13, 14). We are currently studying the possible augmenting effect of cy on in vivo rejection of lethal syngeneic tumors. The conversion to high responsiveness by cy can be reverted to a state of low responsiveness by reconstituting the cy-treated animals with normal T cells, suggesting that in vivo cy-sensitive T cells suppress the in vivo generation of T cells cytotoxic against SV 40 transformed cells. An interesting observation in the present study is that cy treatment of mice 6 d after antigen immunization resulted in suppression of cell-mediated cytotoxic response. This phenomenon which is in accord with the one reported in an allogeneic system (23) is now under study. Our results of augmentation of cell-mediated immunity against tumor-associated antigens are similar to those demonstrating that cy enhanced delayed type hypersensitivity reactions by elimination of suppressor B cells (7–9) and recently augmented cell-mediated cytotoxic response against alloantigens by elimination of suppressor T cells (10). These reports together with the data presented here favor the concept that T-cell-immune responsiveness in vivo against a variety of antigens is under the control of cy-sensitive suppressor T cells.

## Summary

When cyclophosphamide was administered to mice before immunization with syngeneic SV40 transformed cells, the specific immune response elicited, as was measured by in vitro $^{51}Cr$ release assay was stronger and lasted longer when compared to the response generated in noncyclophosphamide-treated mice. The augmentation effect of the drug was dependent on cyclophosphamide concentration being optimal at 100 mg/kg and on the time of drug administration in relation to antigen immunization being optimal at 2 d before antigen administration. Transfer of T cells from normal syngeneic mice to drug-treated animals abolished the cyclophosphamide-induced augmentation of immune response. These results implied that cyclophosphamide sensitive T cells suppressed the in vivo generation of specific effector T cells against SV40-induced tumor-associated antigens.

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