Effect of Low Dose Cyclophosphamide on the Immune System of Cancer Patients: Reduction of T-Suppressor Function without Depletion of the CD8+ Subset

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ABSTRACT

Low dose cyclophosphamide (CY) can augment the development of delayed-type hypersensitivity to primary antigens in patients with advanced cancer. In this paper, we have considered the hypothesis that the immunosuppression is related to reduction of T-suppressor activity. Peripheral blood lymphocytes were collected and cryopreserved from 45 patients with metastatic malignancy before and then 3, 7, and 19 days after administration of CY, 300 mg/m² i.v. The peripheral blood lymphocytes were tested for generation of concanavalin A-inducible suppressor activity, proliferative response to phytohemagglutinin, and phenotype using monoclonal antibodies to CD4 and CD8. Concanavalin A-inducible suppression was significantly reduced by day 3 and declined progressively through day 19. The mean percentage changes in suppression were: day 3, -23.4 ± 6.8 (SE) (P < 0.01); day 7, -33.1 ± 14.3 (P = 0.052); day 19, -43.1 ± 10.7 (P < 0.01). In contrast, CY caused no significant changes in phytohemagglutinin proliferation (mean percentage changes: day 3, -4.7 ± 6.1; day 7, -15.6 ± 7.5; day 19, -5.5 ± 8.1), indicating that the reduction in concanavalin A-inducible suppression was not merely a reflection of a general reduction in peripheral blood lymphocyte function. The total number of circulating lymphocytes was not affected by low dose CY. Moreover, flow cytometric analysis showed no significant changes in the percentage of circulating CD8+ or CD4+ T-cells or in the CD4/CD8 ratio at any time point after CY. Thus, administration of low dose CY to these patients caused impairment of nonspecific T-suppressor function without selective depletion of the CD8+ subset that is generally associated with that function. Several immunoregulatory models that are consistent with these observations are discussed.

INTRODUCTION

Cyclophosphamide is a potent immunosuppressive agent that can also augment a variety of immune responses (1). The major determinant of whether CY suppresses or potentiates immunity is the time of its administration in relationship to the presentation of antigen. Thus, when given following antigen, CY can suppress the immune response but often augments the response when given 1–3 days prior to antigen (2).

In experimental systems, CY has been shown to augment immunity by its selective toxicity to T-lymphocyte-mediated suppressor function (3). This finding is of more than theoretical interest, because reduction in T-suppression may be an essential component of successful immunotherapy, as indicated by several murine tumor systems (4). For example, Berendt and North (4) have shown that depletion of T-suppressor cells by CY markedly facilitates the eradication of an established tumor by adoptively transferred immune lymphocytes.

For the past several years, we have been attempting to apply these principles to the treatment of human cancer. We observed that pretreatment of patients with CY, 300 mg/m² i.v., 3 days before injection of antigen augmented the development of delayed-type hypersensitivity, both to keyhole limpet hemocyanin (6) and to autologous tumor cells (7). With this publication, we have begun to investigate the immunological basis of these findings. The data will show that administration of CY is followed by reduction in Con A-inducible T-suppressor function of peripheral blood lymphocytes without a corresponding depletion of the suppressor-cytotoxic (CD8+) subset.

MATERIALS AND METHODS

Patients. The study population consisted of patients with surgically incurable, metastatic cancer. Initially, PBL from 13 patients were studied for Con A-inducible suppression, PHA stimulation, and surface phenotype. Subsequently, the surface phenotype studies were expanded by the addition of 32 patients to make a total of 45 study subjects.

A summary of the clinical characteristics of these patients is shown in Table 1. Thirty-seven of the study subjects had malignant melanoma; of the other 8, 7 had colorectal carcinoma and 1 had breast adenocarcinoma. Despite the presence of advanced visceral metastases in most of these patients, their performance status was good (median Karnovsky score, 80). Although 34 patients had been treated previously with chemotherapy and 7 had received radiation therapy, all such cytotoxic therapy had been discontinued for at least 4 weeks prior to entering this study. No patients were given corticosteroids while under study.

Treatment. The patients for this study were drawn from two previously published studies: study 1, the effect of low dose CY on the immunological response to primary, non-tumor-related antigens (6) (15 patients); and study 2, the effect of low dose CY on the development of delayed-type hypersensitivity to autologous tumor cells (7) (30 patients). CY was given at a dose of 300 mg/m² by rapid i.v. infusion. Three days after CY, the patients from study 1 were sensitized with either dinitrochlorobenzene or keyhole limpet hemocyanin, and the patients on study 2 were given i.d. injections of irradiated, autologous tumor cells mixed with Bacillus Calmette-Guérin. Heparinized blood was obtained from all patients just prior to CY infusion (day 0) and then 3, 7, and 19 days afterwards. No additional treatment was given during the study period.

Preparation of PBL. As described previously (6), PBL were separated by density gradient centrifugation on Ficoll:metrizoate, suspended in freezing medium (consisting of RPMI 1640 (Grand Island Biological Co., Grand Island, NY), penicillin, streptomycin, 10% pooled human AB+ serum (Biocell Laboratories, Carson, CA), and 10% dimethyl sulfoxide), frozen in a controlled-rate freezer, and stored in liquid nitrogen. When needed for testing, MNC were thawed rapidly, diluted slowly to gradually reduce the concentration of dimethyl sulfoxide, and washed twice.

Lymphocyte Phenotype Studies. The MNC were stained by incubat-
Table 1  Clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>37</th>
</tr>
</thead>
<tbody>
<tr>
<td>Melanoma</td>
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<tr>
<td>Other</td>
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<tr>
<td>Sex</td>
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<td>16</td>
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<tr>
<td>Age</td>
<td>59 (27-79)*</td>
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<tr>
<td>Karnovsky status</td>
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<tr>
<td>Metastases</td>
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<td>Visceral</td>
<td>36</td>
</tr>
<tr>
<td>Skin or nodal only</td>
<td>9</td>
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<td>Prior chemotherapies</td>
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<td>0</td>
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</tr>
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<td>1</td>
<td>14</td>
</tr>
<tr>
<td>&gt;1</td>
<td>20</td>
</tr>
<tr>
<td>Prior radiation therapy</td>
<td>7</td>
</tr>
<tr>
<td>Survival (mo)</td>
<td>7 (1-50+)</td>
</tr>
</tbody>
</table>

* Median (range).

...ing 0.5 × 10^6 cells with one of the following antibodies on ice for 20 min: anti-Leu 3a (anti-CD4; helper-inducer T-cells) (8); anti-Leu 2a (anti-CD8; suppressor-cytotoxic T-cells) (8); and anti-Leu M3 (monocytes) (9); all were directly conjugated to fluorescein isothiocyanate (green fluorescence) or phycoerythrin (red fluorescence) (Becton-Dickinson, Mountainview, CA). Negative fluorescence controls were obtained by using fluorescein isothiocyanate-conjugated mouse IgG of irrelevant specificity.

Analysis of cell surface markers was performed with a Coulter EPICS C flow cytometer (Coulter Electronics, Hialeah, FL). Lymphocytes were examined by setting bit map gates on a plot of forward versus 90-degree light scatter, which excluded monocytes (10); absence of monocytes in the bit map was confirmed by lack of staining with anti-Leu M3.

Blood leukocytes were enumerated in a standard manner with a Coulter Counter (Coulter Electronics), and differential counts were performed by examination of Giemsa-stained blood smears. Total MNC was calculated by adding the proportions of lymphocytes and monocytes, as determined by Giemsa smears, and multiplying the sum by the total blood leukocyte count. The lymphocyte count was determined by subtracting monocytes (% Leu M3+)/100 × total MNC) from total MNC.

Con A-inducible Suppression. This was performed by a modification of the method of Shou et al. (11), which has been described previously (6). MNC (2 × 10^6) were incubated in 1 ml of culture medium with or without Con A (25 μg/ml) in plastic tubes in a 37°C incubator for 3 days. Then they were washed and exposed to mitomycin C, 25 μg/ml (Sigma Chemical Co., St. Louis, MO), for 30 min in a 37°C water bath. After 3 washes, they were suspended in culture medium, and 5 × 10^6 cells were added to 1 × 10^5 “indicator” cells in microtiter wells. The latter were MNC from a normal donor, and the same donor was used in all the experiments described herein. Then PHA was added to all the wells at a predetermined suboptimal concentration (0.1 μg/ml). The mixtures were incubated at 37°C in 5% CO_2 for 3 days and then pulsed with [3H]thymidine (New England Nuclear, Boston, MA) and harvested with an automatic harvester. The percentage of suppression was calculated as

\[ 100 \times \frac{cpm (I + UN) - cpm (I + Con A)}{cpm (I + UN)} \]

where cpm (I + UN) are the uptake of [3H]thymidine by indicator cells in the presence of unstimulated patient’s cells, and cpm (I + Con A) are the uptake by indicator cells in the presence of Con A-stimulated patient’s cells.

Proliferative Response to PHA. This was performed by a standard technique, described previously (6). The concentration of PHA was 1.0 μg/ml.

Analysis of Data. Lymphocyte functional assays were performed in triplicate and the mean was calculated. Replicate values never varied by more than 20% For each patient and for each parameter, we calculated the change between the pre-CY sample and each post-CY sample. Then for a given parameter, we determined whether the mean change, or percentage change, of the whole group was significantly different from zero at each time point by using the Student t test for nonindependent variables (2-tailed). This approach avoids the potential problem of mistaking spontaneous fluctuations for CY-induced changes.

RESULTS

Con A-inducible Suppression. The generation of nonspecific suppressor T-cells was studied in 13 patients before and 3, 7, and 19 days after low dose CY. Suppressor function was significantly reduced by day 3 and declined progressively through day 19. The mean values (percentage of suppression) were: day 0, 42.4%; day 3, 33.4%; day 7, 30.4%; day 19, 26.8%. The data were analyzed by an independent t test (2-tailed) after calculating the percentage change in suppression at each time point compared with the pretreatment (day 0) value (Fig. 1). The mean percentage changes were: day 3, -23.4 ± 6.8 (P < 0.01); day 7, -33.1 ± 14.3 (P = 0.052); day 19, -43.1 ± 10.7 (P < 0.01). The percentage change in suppression on day 19 compared with day 3 was also significant (mean percentage change, -27.3 ± 10.8, P < 0.025). A similar degree of impairment of Con A-inducible suppression was observed whether patients were sensitized on day 3 with non-tumor antigens or with tumor cells (Fig. 1, *, O, respectively).

PHA Proliferation. In parallel, PBL from this group of patients were tested for their proliferative response to an optimal concentration of Con A. In contrast to its effect on Con A suppressors, CY caused no significant changes in PHA reactivity. The mean responses (cpm) for the group were as follows: day 0, 101, 949; day 3, 98,439; day 7, 104,415; day 19, 89,415. The percentage changes at each time point were analyzed as described above: day 3, -4.7 ± 6.1; day 7, -15.6 ± 7.5; day 19, -5.5 ± 8.1 (P > 0.05 for each) (Fig. 2). Similar results were obtained when a suboptimal concentration of PHA (0.1 μg/ml) was used (data not shown). Thus, the progressive reduction in Con A-inducible suppression seen after CY was not merely a reflection of a general reduction in peripheral blood lymphocyte function.

Blood Lymphocyte Composition. Initially, PBL from 10 of...
the original 13 patients were studied. To reduce the possibility that small changes in lymphocyte composition would be missed because of inadequate sample size, PBL that had been obtained and cryopreserved from an additional 32 patients were studied, and the results were analyzed compositely. The number of data points at each time point was: day 0, 42; day 3, 40; day 7, 27; day 19, 12.

The total number of circulating lymphocytes was not affected by a single administration of low dose CY; the values (number of cells per mm$^3$) were: day 0, 1620 ± 112; day 3, 1681 ± 132; day 7, 1602 ± 152; day 19, 1576 ± 135. Prior to CY, the T-cell composition (percentage) measured by flow cytometric analysis, was: CD4+ T-cells, 46.6 ± 2.0; CD8+ T-cells, 19.4 ± 1.3; CD4/CD8 ratio, 3.2 ± 0.4. Fig. 3 shows the percentage change in circulating CD8+ and CD4+ T-cells for each of the study patients following administration of low dose CY. For a particular patient, there was sometimes considerable variation in the proportion of cells in each subset from one time point to another. However, analysis of the group demonstrated that administration of CY was not followed by any significant changes in circulating CD8+ or CD4+ T-cells or in the CD4/CD8 ratio, despite a significant change in Con A suppression and, clinically, an augmented induction of DTH to keyhole limpet hemocyanin or autologous melanoma cells.

DISCUSSION

The original observation, some 20 years ago, that CY could potentiate immune responses (12) had been corroborated and extended by a number of investigators. Most studies have demonstrated augmentation of cell-mediated immunity, usually measured as delayed-type hypersensitivity, to an array of antigens, varying from microbial products to syngeneic testicular tissue (13, 14). Under some circumstances antibody responses have also been heightened (15).

CY-mediated immunopotentiation has been achieved in experimental animals with a remarkably wide range of dosages from 60 to 2100 mg/m$^2$ (2, 16). However, both in vivo and in vitro evidence suggests that lower concentrations of CY may be advantageous in that they are selectively toxic to the T-suppressor system and sparing of other T-cell functions. Thus, Kauffman et al. (3) reported that T-cells capable of adoptively transferring DTH were resistant to 4-hydroperoxy-CY (which does not require enzymatic activation) at a concentration of 3 μg/ml, while suppressor T-cells were inactivated at a concentration of 1 μg/ml or less.

Our laboratory was the first to demonstrate that CY could augment the human immune response in vivo (17). We showed that the administration of a conventional cytotoxic dose of CY, 1000 mg/m$^2$, to patients with advanced cancer 3 days before sensitization with antigen resulted in augmentation of DTH to the primary antigen, keyhole limpet hemocyanin, with no effect on the antibody response to that antigen. In a subsequent study (6), we reported that a much lower dose of CY, 300 mg/m$^2$, was just as effective in augmenting the acquisition of DTH and heightened the antibody response as well. More recently, we have shown that "low dose" CY potentiated the acquisition of DTH to autologous melanoma cells, which in two cases was followed by regression of metastatic tumor (7).

In the present study, we have tested the hypothesis that reduction of nonspecific T-suppressor function might be at least partially responsible for the immunopotentiating effects of low dose CY. Using cryopreserved PBL from patients with advanced cancer who had been treated with CY in one of our previous studies, we observed a striking impairment of Con A-inducible suppressor activity. This reduction was detectable by the third day after CY administration and increased progressively through day 19. The reduction in suppressor function on
day 3 is consistent with the immunopotentiating studies in both humans and experimental animals (2, 6, 7). Thus, sensitization with antigen on day 3, a time when nonspecific suppressor function is low, might result in impaired generation of antigen-specific suppression as well, and thus more efficient production of T-cells that mediate DTH and provide help for antibody production. The persistence of the impairment of Con A suppressor through day 19 is more difficult to correlate, since in the few human studies and in most of the published animal experiments the interval between CY and immunization has been no more than 3 days. However, at least one laboratory (18) has shown immunopotentiating can be obtained in mice when antigen is given as long as 7 days after CY, and in humans the potentiation window may be considerably wider.

Since Con A-inducible suppression is generally associated with the CD8+ subset of T-cells (19), it is noteworthy that the impairment of T-suppressor activity was associated neither with a fall in the percentage of circulating CD8+ cells nor with a change in the so-called "helper/suppressor" ratio, i.e., (%CD4+/%CD8+). Previously, we found that high dose (1000 mg/m²) CY inhibited Con A-inducible suppression without changing the absolute proportions of CD4+ and CD8+ T-cells, but in that study, there was a marked reduction in the total blood lymphocyte concentration and thus a fall in the absolute number of CD8+ T-cells (20). In contrast, in the current study low dose CY reduced suppressor function without affecting either the relative or the absolute numbers of this subset.

These results are in conflict with data published by Bast et al. (21) who reported that low doses of CY (200–400 mg/m²) did produce selective depletion of circulating CD8+ T-lymphocytes in patients with melanoma. However, their conclusion was based on the study of only 4 patients, 3 of whom exhibited a significant change. Moreover, they administered multiple, escalating doses of CY to each patient and thus could have been measuring a cumulative effect rather than the effect of a single dose. We believe that our study (testing a single administration of CY, using a much larger sample size, and performing statistical analysis by group) provides a more accurate assessment of the effects of low dose CY on peripheral blood T-cell composition.

There are several immunoregulatory models that are consistent with the observation that CY reduces Con A-inducible suppression without depleting CD8+ T-cells. Ozer et al. (22) have shown that in vitro exposure of human lymphocytes to 4-hydroperoxy-CY prevents the generation of suppressor T-cells by Con A but does not inhibit the suppressor cells after they have been induced. They postulated the existence of a minor, extremely CY-sensitive, population of "presuppressor" cells. Secondly, Damle et al. (23) have studied a T-cell with the phenotype CD4+, Leu 8+, which is not a suppressor but is necessary for the induction of CD8+ suppressors. Finally, evidence has accumulated that CD4+ T-cell themselves can become potent suppressors after stimulation with Con A (24). Morimoto et al. (25) have suggested such cells bear the newly recognized surface molecule, 2H4. Partial depletion of, or sublethal damage to, one of these subpopulations of CD8+ lymphocytes by CY could be reflected in reduction of T-suppressor activity and, consequently, in augmentation of immune responses.

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