ABSTRACT

We studied peripheral blood lymphocytes (PBL) from 42 patients with metastatic melanoma undergoing treatment with cyclophosphamide (CY) plus melanoma vaccine to determine whether CY immunopotentiation could be related to depletion of T-cells that function as inducers of suppression. Every 28 days, the patients were given CY, 300 mg/m² I.V., followed 3 days later by the intradermal injection of autologous, irradiated melanoma cells mixed with Bacillus Calmette-Guérin. PBL were separated by density gradient centrifugation and cryopreserved until needed for testing. They were stained with monoclonal antibodies directly conjugated to fluorescein isothiocyanate or phycoerythrin and analyzed by two-color flow cytometry. At no time after the initiation of CY plus vaccine were there any significant changes in the percentages of helper-inducer T-cells (CD4+), suppressor-cytotoxic T-cells (CD8+), or the subpopulation of CD8+ cells expressing Leu 15, a marker for suppressor cells. Treatment of melanoma patients with CY plus vaccine resulted in a progressive fall in the proportion of CD4+ T-cells expressing the HLA-DR (CD45) antigen, which identifies inducers of suppression. The reduction of CD4+, 2H4+ T-cells did not become apparent until day 28 after the first dose of CY and reached statistical significance only on days 49 (21 days after the second dose) and 105 (21 days after the fourth dose) (mean changes ± SE: day 49, -5.4 ± 1.4%, P < 0.01; day 105, -9.1 ± 2.2%, P < 0.01; t test for nonindependent samples). In contrast, the proportion of CD4+ T-cells expressing the antigen 4B4 (CDw29), which are true helper cells, increased slightly, although not significantly, following the institution of CY plus vaccine (mean changes: day 49, +2.9 ± 2.1%; day 105, +3.6 ± 2.4%). Similar results were obtained when absolute numbers of circulating cells, rather than percentages, were analyzed. Thus the number of CD4+, 2H4+ T-cells fell from a mean of 395,000/ml on day 0 to 309,000/ml on day 49 (P < 0.01) to 256,000/ml on day 105 (P < 0.05). The absolute number of CD4+, 4B4+ cells remained unchanged at the same time points. These changes were not due to progression of metastatic disease, since a comparison of patients with progressive metastases with those who were rendered disease free by surgery showed no significant differences in the reduction of the percentage of CD4+, 2H4+ T-cells. Moreover, PBL from 5 of 6 patients who had received vaccine without CY pretreatment and who had progressive metastases, exhibited slightly increased rather than decreased percentages of CD4+ cells expressing 2H4. Leu-8, another proposed marker of suppressor-inducer cells, could not be studied because of its unexpected sensitivity to cryopreservation. There was no evidence that activated suppressor or inducer cells, as defined by expression of the interleukin 2 receptor (tac) or the recently described activation antigen, Ta1, were reduced by CY plus vaccine treatment. Thus, administration of CY appears to result in progressive depletion of the suppressor-inducer subset of PBL which could be at least partially responsible for its immunopotentiating effects.

INTRODUCTION

The cytotoxic drug, CY, can augment cell-mediated immunity, especially DTH, not only in experimental animals (1, 2), but also in humans (3). Specifically, we have shown that pretreatment of tumor-bearing patients with CY augmented the development of DTH to the primary antigen, keyhole limpet hemocyanin (3, 4). Moreover, when given 3 days before injection of a whole cell vaccine, CY potentiated the development of DTH to autologous tumor cells in patients with metastatic melanoma (5, 6). In some cases, this was associated with clinically important regression of metastatic tumors.

In experimental animals, CY-mediated immunopotentiation depends on selective reduction of antigen-specific T-suppressor cell function. For example, Kaufmann et al. (7) showed that murine T-cells capable of adoptively transferring specific DTH were relatively resistant to CY, while antigen-specific suppressor T-cells were quite sensitive. Also, Berendt and North (8) reported that treatment of mice with CY permitted the successful eradication of tumor by adoptively transferred immune lymphocytes, and that this was reversed by transferring suppressor T-cells obtained from tumor-bearing animals.

In human systems, CY has been shown to inhibit the generation of concanavalin A-inducible suppressor T-cells, both in vivo (9) and in vitro (10). However, the phenotype of the CY-sensitive target cell has not been identified. Although an initial report suggested that CY selectively depleted CD8+ suppressor-cytotoxic T-cells (11), we have not been able to confirm that finding in two larger studies (9, 12).

Recently, a subpopulation of CD4+ T-cells has been described which is required for induction of suppressor activity in CD8+ T-cells. This subpopulation can be identified by expression of two surface antigens: Leu-8 (13), and 2H4 (CD45) (14). Moreover, CD4+ T-cells that function as helpers are 2H4(−), but do express the antigen, 4B4 (CDw29) (15).

We hypothesized that administration of CY could cause depletion of CD4+, 2H4+ suppressor-inducer T-cells with relative sparing of CD4+, 4B4+ cells, thus shifting the immunoregulatory balance in the direction of less suppression and more help. Indeed, there is precedent for such a model in a murine system (16). In this paper, we have tested that hypothesis in a group of cancer patients being treated with CY plus an autologous tumor vaccine.

MATERIALS AND METHODS

Patients. The study population consisted of 42 patients with malignant melanoma. A summary of the clinical characteristics of these patients is shown in Table 1. Twenty-three patients had visceral metastases (mainly lung). Of the 31 patients with only s.c. and/or nodal metastases, 12 had gross surgical resection of their tumors prior to entry on this study. As indicated in Table 1, these patients were selected for good performance status (median Karnovsky score, 90) and minimal exposure to chemotherapy (29 of 42 had received none, or only one cytotoxic drug). All patients were given corticosteroids or other known immunomodulating drugs while on study. For some analyses, we utilized a comparison group of 11 age-matched patients who had undergone curative resection of superficial melanomas at least 5 years previously.
DEPLETION OF 2H4+ T-CELLS BY CY

Treatment. Patients were treated with CY plus melanoma vaccine as previously described (5, 6). CY 300 mg/m² was given by rapid i.v. infusion. Three days after CY, the patients were given injections intra-dermally of a vaccine, consisting of irradiated, autologous tumor cells mixed with Bacillus Calmette-Guérin. As shown in the schema (Fig. 1), this treatment sequence was repeated every 28 days, and heparinized blood was obtained at the following time points: day 0, 42 patients; day 3, 30 patients; day 7, 21 patients; day 28 (just before the second CY infusion), 17 patients; day 49, 29 patients; and day 105, 21 patients.

Preparation of PBL. As previously described (9), 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 sulphoxide; frozen in a controlled-rate freezer; and stored in liquid nitrogen. When needed for testing, PBL were thawed rapidly, diluted slowly to gradually reduce the concentration of dimethyl sulphoxide, and washed twice.

Monoclonal Antibodies. All of the monoclonal antibodies used were directly conjugated either to FITC (green fluorescence) or to PE (red fluorescence). The following antibodies were obtained from Becton-Dickinson (Mountain View, CA): anti-Leu-4 (FITC) (anti-CD3; pan T-cell); anti-Leu-2a (FITC or PE) (anti-CD4; helper-inducer T-cells) (17); anti-Leu-2a (FITC or PE) (anti-CD8; suppressor-cytotoxic T-cells) (17); anti-Leu-8 (FITC) (13); anti-Leu-15 (PE) (18); anti-IL2 receptor (FITC); and anti-Leu-M3 (monocytes) (19). The following antibodies were obtained from Coulter Immunology (Hialeah, FL): anti-2H4 (PE) (15); and anti-Tal (PE) (20). Negative fluorescence controls were obtained by using fluorescence-conjugated, purified mouse immunoglobulin of the same isotype as the monoclonal antibodies.

Analysis of PBL Surface Antigens. PBL were stained with one or two monoclonal antibodies on ice for 30 min. Then they were washed and analyzed with a Coulter EPICS C flow cytometer (Coulter Electronics, Hialeah, FL), equipped with a 488 nm argon laser (used at 500 mW). Lymphocytes were examined by setting bit map gates on a plot of forward versus 90° light scatter, which excluded monocytes (21); absence of monocytes in the bit map was confirmed by lack of staining with anti-Leu-M3. By using the appropriate optical filters, green fluorescence was measured at 510 to 530 nm and red fluorescence at >610 nm. Both green and red signals were displayed on a logarithmic scale. Cross-over of green fluorescence into the red detection window was compensated by analogue subtraction at the preamplifier stage. Statistical analysis of two-color staining was performed by using the "quad stat" program distributed by Coulter Electronics.

Analysis of the Data. For each patient and for each parameter, we calculated the change between the pretreatment sample and each post-treatment sample. Then for a given parameter, we determined whether the mean 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

Major T-Lymphocyte Subsets. As shown in Fig. 2, at no time after the initiation of CY plus vaccine were there any significant changes in the percentages of total T-cells (CD3+), helper-inducer T-cells (CD4+), or suppressor-cytotoxic T-cells (CD8+).

CD4+ Suppressor-Inducer T-Cells. As illustrated in Fig. 3, C and D, cryopreserved PBL exhibited diminished fluorescence with FITC-anti-Leu-8 compared with freshly separated PBL. This reduced expression of Leu 8 introduced a technical variable that precluded a reliable comparison of pre- and posttreatment samples. However, none of the other leukocyte antigens evaluated in this study was affected by cryopreservation, as illustrated by the two-dimensional histograms of FITC-anti-CD4 and PE-anti-2H4 in Fig. 3, A and B.

Prior to treatment with CY plus vaccine, 24.3 ± 2.2% of PBL were CD4+, 2H4+, and 19.7 ± 1.0% were CD4+, 4B4+. PBL obtained from a group of 11 age-matched patients who had been surgically cured of superficial primary melanoma were of similar composition: CD4+, 24.4 ± 2.9%; CD4+, 4B4+, 19.5 ± 2.2%. Thus the relative proportions of these subsets did not appear to be altered by the development of metastases.

Table 1 Clinical characteristics of the patients

<table>
<thead>
<tr>
<th>Sex</th>
<th>Men</th>
<th>Women</th>
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<tr>
<td>Age</td>
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<td>Karnovsky status</td>
<td>90 (50–100) *</td>
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<td></td>
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<tr>
<td>Visceral</td>
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<td>Skin or nodal only</td>
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<td></td>
</tr>
<tr>
<td>Resected</td>
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<td></td>
</tr>
<tr>
<td>Prior chemotherapies</td>
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<td>10</td>
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<tr>
<td>Time to death</td>
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</tr>
<tr>
<td>No. of alive</td>
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<td></td>
</tr>
<tr>
<td>Time alive</td>
<td>12.5 mos (5.9–58.5) *</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>11.0 mos (2.3–58.5) +</td>
<td></td>
</tr>
</tbody>
</table>

* Median (range).

Fig. 1. Schema indicating points at which PBL were obtained. CY, 300 mg/m² i.v. V, melanoma vaccine (autologous, irradiated melanoma cells plus Bacillus Calmette-Guérin) injected intra-dermally. LY, blood collected for separation of PBL.

Fig. 2. Stability of major T-cell subsets after CY plus vaccine. Points, mean percentage of CD3+, CD4+, or CD8+ PBL (±SE) for the group of patients before and after initiation of CY plus vaccine. For each point, the change compared with day 0 was analyzed by a t test for nonindependent samples: for all points, P not significant.

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Treatment of melanoma patients with CY plus vaccine resulted in a gradual fall in the proportion of CD4+ T-cells expressing the 2H4 antigen (Fig. 4). It is noteworthy that the reduction of CD4+, 2H4+ T-cells did not become apparent until day 28 after the first dose of CY and reached statistical significance only on days 49 (21 days after the second dose) and 105 (21 days after the fourth dose).

In contrast, the proportion of CD4+ T-cells expressing the antigen 4B4 increased slightly following the institution of CY plus vaccine (Fig. 5). However, the spread of values at each time point was wide, and, therefore, the observed increases in CD4+, 4B4+ T-cells were not statistically significant.

Similar results were obtained when absolute numbers of circulating cells, rather than percentages, were analyzed (Fig. 6). Thus, there was a progressive fall in the absolute number of CD4+, 2H4+ T-cells, which was statistically significant on days 49 and 105, while the absolute number of CD4+, 4B4+ cells remained unchanged. As expected, the absolute number of all CD4+ T-cells also fell from 730 ± 52 on day 0 to 598 ± 50 on day 105, although the change did not reach statistical significance.

We considered the possibility that these changes could be attributed solely to progression of metastatic disease. However, a comparison of patients with progressive metastases with patients who remained disease free showed no significant differences in the reduction of the percentage of CD4+ T-cells expressing 2H4+. The mean ± SE changes were as follows. Day 49: metastases (n = 16), −5.8 ± 1.8%; disease free (n = 13), −5.0 ± 2.2%, P not significant. Day 105: metastases (n = 12), −11.0 ± 1.9%; disease free (n = 9), −6.5 ± 4.2%, P not significant.

In addition, we were able to study lymphocytes from 6 patients from a previous study (5) who had received vaccine on the same schedule, but without CY pretreatment; all of these patients had progressive metastases. In 5 of these patients, the percentage of CD4+ T-cells expressing 2H4 on day 49 actually
increased: +4.5%, +19.9%, +9.7%, +8.3%, and +0.9%. Day 49 PBL obtained from the other patient exhibited a substantial decrease in CD4+, 2H4+ cells (−58.0%). However, that sample was unusual in that 75% of the lymphocytes were negative for CD3, CD4, and CD8, and so the apparent depletion of 2H4+ cells was nonspecific.

**CD8+, Leu-15+ Suppressor T-Cells.** CD8+ T-cells can be functionally subdivided on the basis of the expression of the Leu-15 antigen (18): while CD8+, Leu-15− cells are cytotoxic effectors, CD8+, Leu-15+ cells suppress *in vitro* immune responses. In our patient population, 38.6 ± 2.5% of CD8+ T-cells expressed Leu-15 prior to treatment. CY plus vaccine administration had no selective effect on this subpopulation (percentage of CD8+ cells that were Leu-15+: day 28: 37.0 ± 5.8%; day 49: 36.6 ± 3.6%; day 105: 38.1 ± 6.4%).

**T-Cell Activation Antigens.** We hypothesized that CY immunopotentiation could result from depletion of a minor population of suppressor or suppressor-inducer cells that had been activated by *in vivo* exposure to tumor antigens. Therefore, we used two-color flow cytometric analysis to detect PBL that coexpressed CD4 or CDS, and either the interleukin 2 receptor, tac, or the recently described activation antigen, Tal (20).

Expression of tac was limited to a small percentage of CD4+ T-cells, as reported in a previous investigation (22). The percentage of CD4+, tac+ T-cells was somewhat greater in patients with metastatic melanoma than in patients who were disease free; metastases: 3.1 ± 0.4%; disease free: 1.9 ± 0.4%; *P* = .050, 2-tailed *t* test. However, CY plus vaccine treatment caused no significant changes in the percentage of CD4+, tac+ lymphocytes at any time point (data not shown).

Since the Tal antigen was previously reported to be present on only a small fraction of resting peripheral blood T-cells (20), we were surprised to find that a majority (57.0 ± 3.7%, *n* = 18) of peripheral blood T-cells (CD3+) from our study patients with metastatic melanoma expressed that antigen prior to treatment. This finding was not a consequence of advanced cancer, because Tal was expressed by a high proportion of both CD4+ and CD8+ T-cells obtained from age-matched patients (mean age, 55) who had been surgically cured of melanoma, and from a group of 9 normal volunteers (mean age, 29). For example, the percentage of CD4+ T-cells that was Tal+ was as follows: patients with metastases, 70.1 ± 2.7%; patients cured, 57.6 ± 2.9%; normal volunteers, 54.1 ± 2.1%. This unexpectedly high expression of Tal was not due to a cryopreservation artifact, since testing freshly separated PBL gave similar results. Treatment with CY plus vaccine did not cause any reduction in Tal+ cells.

**DISCUSSION**

Immunopotentiation by CY is probably mediated by selective inhibition of suppressor T-cell function (7–10, 23). However, in neither experimental nor human systems has a phenotypically defined, CY-sensitive cell been identified. The characterization and isolation of such a cell would have practical as well as theoretical importance, because it would provide a simple way of immunologically monitoring CY-treated patients.

Bast *et al.* (11) reported that low doses of CY (200–400 mg/m²) produced selective depletion of circulating CD8+ T-lymphocytes in patients with metastatic melanoma. However, their conclusion was based on the study of only 4 patients, 3 of whom exhibited a significant change. In two larger studies, we did not detect a selective depletion of CD8+ T-cells in metastatic melanoma patients treated with a single dose of CY, either 1000 or 300 mg/m² (9, 12). As shown in the present study, the repeated administration of CY did not cause reduction in the percentage or number of CD8+ cells, or in the suppressor subpopulation as defined by expression of Leu-15.

There are several immunoregulatory models that are consistent with the observation that CY reduces T-suppressor function in tumor-bearing patients without depleting CD8+ suppressors. Ozer *et al.* (10) showed that *in vitro* exposure of normal human lymphocytes to 4-hydroperoxy-CY prevented the generation of suppressor T-cells by concanavalin A, but did not inhibit the suppressor cells after they had been induced. They postulated the existence of a minor, extremely CY-sensitive, population of “presuppressor” cells. Damle *et al.* (13) studied a T-cell with the phenotype CD4+, Leu 8+, which is not a suppressor, but is necessary for the induction of CD8+ suppressors. Morimoto *et al.* (14) described the surface marker 2H4 (CD45), which also identifies CD4+ cells that are inducers of suppression, but which is apparently distinct from Leu-8. Moreover, they showed that CD4+ T-cells that were 2H4− usually expressed another antigen, 4B4 (CDw29); this subpopulation did not induce suppressor cells but did provide help for B-cells (15).

We hypothesized that augmentation of immune responses after administration of CY might be associated with depletion of CD4+ T-cells expressing Leu-8 or 2H4. Although Leu-8 could not be adequately studied because of its sensitivity to cryopreservation, we did indeed find that patients treated repeatedly with CY plus autologous melanoma cell vaccine exhibited a progressive fall in circulating CD4+, 2H4+ lymphocytes. The statistically significant changes observed on days 49 and 105 are particularly important because they were associated with augmented levels of DTH to autologous melanoma cells measured at those time points (6).

However, the data do not justify the conclusion that the CD4+, 2H4+ suppressor-inducer T-cell is the CY-sensitive cell. Although the depletion of CD4+ cells expressing 2H4 was statistically significant, it was relative modest in degree, about 10, 22, and 35% reduction in absolute numbers on days 28, 49, and 105, respectively. Moreover, statistically significant depletion was not observed until day 49, when two courses of CY plus vaccine had been administered. In contrast, potentiation of DTH is achieved when antigen is given as early as 3 days after CY, by which time PBL exhibit reduction of concanavalin A-inducible T-suppressor activity (3, 4, 9, 12). This discordance may be explained by postulating that the major effect of CY on suppressor-inducer cells is inhibitory, rather than lytic. Thus, functional impairment of this subpopulation may become apparent by 3 days, but depletion of these cells by CY could take much longer and require repeated administration.

At least two other explanations of the data are plausible: (a) the effects of CY and tumor vaccine on 2H4+ cells could be antagonistic, *i.e.*, inhibition by CY and simultaneous stimulation by tumor antigen; (b) CY immunopotentiation could be caused by inhibition of other types of suppressor mechanisms, *e.g.*, suppressor monocyte-macrophages (24).

We were unable to demonstrate that CY plus vaccine caused depletion of activated T-cells, as defined by expression of tac, or of the newly defined activation marker, Tal (20). Although Zoumbos *et al.* (25) observed tac+ CD8+ suppressor cells in PBL of patients with aplastic anemia, in our population of melanoma patients tac expression was limited to CD4+ T-cells; similar results have been reported by Lotze *et al.* (22). The increased expression of tac on CD4+ T-lymphocytes from our patients with metastatic, as compared to surgically cured, mel-
anoma could reflect stimulation of these cells by circulating melanoma-associated antigen.

Our results with the Ta1 antigen were different from the original report of Fox et al. (20), who observed that Ta1 was infrequently expressed on freshly isolated T-cells from normal subjects. In contrast, we found that the majority of T-cells from patients with melanoma, either metastatic or cured, and from a small group of normal volunteers, expressed Ta1. This discrepancy could be due to variations in antibody preparations, or it could have a biological basis, e.g., demographic differences in the study populations.

Finally, this study illustrates both the usefulness and limitations of PBL subpopulation analysis in determining the mode of action of an immunomodulating agent. While studies of phenotype lack the sensitivity of functional assays, they can provide interesting insights into structure-function relationships and indicate directions for future research.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance of their coworkers in the completion of this work: Ellen Hart, who administered the immunotherapy and collected blood samples; Carmella Clark, who processed and analyzed blood specimens and prepared vaccines; and Marsha Hahn Golden, who performed the flow cytometry.

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Effect of Low Dose Cyclophosphamide on the Immune System of Cancer Patients: Depletion of CD4+, 2H4+ Suppressor-inducer T-Cells

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