Suppressor Cell Activity in a Randomized Trial of Patients Receiving Active Specific Immunotherapy with Melanoma Cell Vaccine and Low Dosages of Cyclophosphamide

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ABSTRACT

Previous studies have shown that melanoma patients develop an immune response to cell surface melanoma-associated antigens. The presence of this antibody response to cell surface antigens has been correlated with a better clinical outcome when melanoma patients are treated with an allogeneic melanoma cell vaccine (MCV) as an active immunotherapy protocol. It was hypothesized that the inability to consistently induce or enhance existing immune responses to melanoma-associated antigens was related to the downregulation by suppressor cells. Patients received treatments of MCV 3 times in a 4-week interval and then every fourth week. The biological response modifier cyclophosphamide (CYP) is an immunomodulator of suppressor T-cell function. In this study we set out to determine whether CYP given prior to MCV could reduce suppressor cell activity during vaccination. In a randomized trial stage II and III melanoma patients (n = 41) were given MCV alone or in conjunction with CYP at dosages of 300, 150, or 75 mg/m². CYP was given 3 days prior to each MCV treatment. Suppressor cell activity in patients was monitored by a concanavalin A suppressor assay using peripheral blood lymphocytes from serial phlebotomies during a 12-week period of treatment. In each trial group there were patients who had major reduction in suppressor cell activity (≥50%). Overall, the greatest reduction in suppressor cell activity occurred in patients receiving 300 mg/m² CYP compared to the other CYP dosages or MCV alone. For the first two treatments at all CYP dosages there was a greater number of patients showing reduced suppressor cell activity compared to later treatments. In a comparison of patients receiving MCV alone to MCV + CYP 300 mg/m² phenotypic analysis of lymphocyte subsets showed significant (*P = 0.03) reduction in the CD8⁺CD11B⁺ (suppressor) cells of the latter group. These studies suggest that CYP can be used at low dosages in conjunction with MCV to reduce suppressor cell activity.

INTRODUCTION

Human melanoma has been demonstrated to be immunogenic in the host. Numerous reports have demonstrated humoral and cellular immune responses that are specific to MAA (1–6). We have demonstrated that anti-melanoma antibodies in melanoma patients can substantiate clinical observations that host immune resistance influences the course of this disease as manifested by disease-free and overall survival (1, 2). Although immunity is apparent in melanoma patients it is usually short term (1) which appears to be related to the downregulation of tumor-specific immune responses (3, 7). Melanoma is very difficult to manage once metastasis has occurred. Patients with tumor-specific immune response(s) (3, 7). Melanoma is very difficult to manage once metastasis has occurred. Patients with tumor-specific immune response(s) (3, 7).

MATERIALS AND METHODS

Patients. Patients for this study were seen in the John Wayne Cancer Clinic of the Jonsson Cancer Center, UCLA. Those entered into the study were high risk stage II and stage III melanoma patients. Most patients had lymphadenectomy(s) and/or other surgery prior to randomization and were entered into the MCV therapy protocol within several weeks of their surgery. The patients were randomized into four treatment groups: MCV alone (10 patients); MCV + 300 mg CYP/m² (11 patients); MCV + 150 mg CYP/m² (10 patients); and MCV + 75 mg CYP/m² (10 patients). Patient eligibility criteria for high risk stage II were: (a) stage II melanoma with regional lymph node metastases (stratified for number of nodes involved for each treatment group); (b) within 2 months of lymphadenectomy surgery and clinically free of disease (NED); (c) no prior chemotherapy, radiation, or immunotherapy; (d) no systemic metastases; and (e) age of 18–75 years. The eligibility criteria for stage III patients were: (a) stage III melanoma with s.c. or visceral metastases; (b) rendered disease-free prior to therapy (NED) or with objectively measurable disease (AWD); and (c) must not have been receiving other prior treatments for a minimum of 30 period (8). It would seem logical that activating host immunity to MAA in such patients may provide control and protection against development of additional metastatic lesions that are not clinically detectable.

In our clinic we have established an active specific immunotherapy protocol designed to enhance or induce immunity to MAA. This involves immunization of melanoma patients after surgery with an allogeneic MCV consisting of melanoma cell lines which were selected on the basis of expression of cell surface MAA to which melanoma patients have been shown to produce antibodies (1, 9). We have previously demonstrated that many patients receiving MCV have only a temporary or no elevation in antibody titers to melanoma-associated antigens. It is believed that this lack of immune response after treatment with MCV may be due to immune suppression or downregulation by the immune system.

In this study we sought to determine whether immunomodulation of the host immune system with the biological response modifier CYP given prior to vaccination could reduce suppressor cell activity, thus enhancing responses to the MCV. CYP is an alkylating agent which is known to modulate immune responses in tumor-bearing animals and humans (10–15). Suppressor T-cells are known to be very sensitive to this drug at concentrations that do not affect other subpopulations of lymphocytes (10, 16–18). The drug can enhance host cellular immune recognition and responses against tumor cells (10, 13, 15). Higher dosages of CYP are known to have side effects on patients and be toxic to various immune cells, particularly B-cells (17, 19). We set up a randomized trial to investigate various low dosages of CYP (300, 150, and 75 mg/m² body weight) given in conjunction with MCV. The immunomodulation of suppressor cell activity in melanoma patients receiving multiple treatments of MCV with various low dosages of CYP is presented in this report.

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1 The abbreviations used are: con A; concanavalin A, CYP; cyclophosphamide, MCV; melanoma cell vaccine, PBL; peripheral blood lymphocyte; TPBL, target PBL; PHA, phytohemagglutinin antigen; NED, no evidence of disease; AWD, alive with disease; Cn, day of treatment; Vn, MCV treatment.
days and no prior immunotherapy; (d) no brain metastases; (e) patients with life expectancies of ≥4 months; and (f) age of 18–75 years.

Treatment Protocol. A polyvalent vaccine consisting of 3 human melanoma cell lines was chosen for immunization on the basis that several melanoma-associated antigens are known to induce antibody responses in patients to autologous melanomas (1, 2). The cell lines were chosen for their expression of antigens GM2 (20), GD2 (21) and M-TAA (22). One of the first two antigens are found to be present in >90% of melanoma biopsy specimens, whereas the latter is in >70% of melanoma biopsy specimens. The 3 melanoma cell lines were established in our laboratory. Cells were grown in RPMI 1640 medium (Flow Laboratories) supplemented with 10% fetal calf serum (heat inactivated) and antibiotics. After several passages cells were harvested by trypsinization, washed 4 times, counted, adjusted to 25 x 10^6 cells in freezing medium (RPMI 1640 + 10% human albumin + 10% dimethyl sulfoxide) and program froze to be stored in liquid nitrogen.

The vials contained equal amounts of each cell line. Before being administered to patients the cells were irradiated at 150 Gy. The vaccine is produced in large batches and carefully analyzed for antigen expression to determine variance from one lot to another. MCV is screened for hepatitis, bacteria, and Mycoplasma contamination by an outside laboratory before being considered for administration to patient. Within several hours of patient arrival for treatment the MCV is thawed, washed 3 times in RPMI 1640 medium, and put into a syringe for intradermal inoculation. In the first 2 treatments the MCV is mixed with one or one-half of a vial of Glaxo strain Bacille Calmette-Guérin (8–25 x 10^6 organisms/vial) (Glaxo, Greenford, England). In Fig. 1 a diagram of the MCV protocol is given. Briefly, MCV is administered by intradermal inoculation into 8 sites in the axilla and groin every 2 weeks on 3 consecutive occasions and then at 4-week intervals for 1 year. CYP (Cytoxan, Mead Johnson and Co., Evansville, IN) is given 3 days prior to each MCV treatment (Fig. 1).

Lymphocyte Collection. Blood was collected in heparin-containing tubes from patients immediately prior to treatment. The PBLs were separated from blood by using a standard Ficoll-Hypaque gradient centrifugation, washed twice, counted, aliquoted, and cryopreserved. For cryopreservation lymphocytes were resuspended in RPMI 1640, 10% dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO), plus 40% heat-inactivated agamma human AB serum (Biocell, Carson, CA). The cells were aliquoted in vials and frozen by time-controlled cryopreservation in liquid nitrogen. When cryopreserved cells were used all lymphocytes from serial phlebotomies were thawed at the same time. Cells were thawed rapidly, washed twice with culture medium, and assayed for viability. The PBLs were then resuspended at 10^6/ml in culture medium containing 0.5 μg/ml PHA (Wellcome). One hundred μl of PBLs was added to each well in a 96-well microtiter plate (Costar). Control and MCV-treated PBLs were inactivated by mitomycin C (Sigma) treatment and were washed 3 times in culture medium.

The assay was set up by adding 10^5 mitomycin C-treated control PBLs or con A-treated cells to microwells containing TPBLs. Quadruplicate wells for both the control and con A-treated PBLs of each phlebotomy point were set up. The cultures were incubated at 37°C in a humidified, 7% CO2 atmosphere incubator for 84 h. In the last 18 h the cultures were pulsed with 1 μCi [3H]-thymidine. Cells were then harvested with a PHD harvester (Cambridge, MA) and counted in scintillation fluid using a Beckman LS-330 counter (Beckman Instruments, Inc., Fullerton, CA).

Suppressor cell activity was measured as the percentage of suppression of the PBL response to PHA. Data was calculated in cpm before being expressed as percentage of suppression.

% suppression
\[
= \left( \frac{1 - \frac{\text{cpm of con A PBL + TPBL + PHA cultures}}{\text{cpm of control PBL + TPBL + PHA cultures}}}{100} \right) \times 100
\]

Several specific T-cell subset populations of patients' PBLs were assayed using fluorochrome-conjugated monoclonal antibodies. Cells were stained simultaneously with fluorescein- and rhodamine-conjugated monoclonal antibodies. The CD4 (T4) and TQ1 marker monoclonal antibodies were obtained from Coulter Electronics (Hialeah, FL). CD8 (Leu2a) and CD11B (Leu15) marker monoclonal antibodies were obtained from Becton Dickinson (Mountain View, CA). Specific subset populations were analyzed by dual color immunofluorescence flow cytometry on an EPICS V cell sorter (Coulter). Relative isotype-matched fluorochrome-conjugated antibodies were used as controls. CD4"TQ1+" are a T-suppressor inducer subpopulation (24, 25). CD4"TQ+" are a helper function for B-cell immunoglobulin production (24, 25). CD8"CD11B+" are a subpopulation of suppressor T-cells (26). CD8"CD11B+" are a subpopulation of cytotoxic precursor and effector cells (26). The percentage of various marker combinations were determined using the MDADS computer software program (Coulter).

RESULTS

Clinical Documentation of Patients. In Table 1 clinical documentation of the patients that were studied is given. The clinical
MELANOMA CELL VACCINE AND CYCLOPHOSPHAMIDE

Table 1 Clinical stratification of patients

<table>
<thead>
<tr>
<th>Sex</th>
<th>Male</th>
<th>Female</th>
<th>MCV + CYP (300 mg/m²)</th>
<th>MCV + CYP (150 mg/m²)</th>
<th>MCV + CYP (75 mg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical status</td>
<td>Stage I</td>
<td>Stage II</td>
<td>Stage III</td>
<td>Entry status</td>
<td>NED</td>
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<td>5</td>
<td>3</td>
<td>3</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>4</td>
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<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Number of patients.

Serial Analysis of Suppressor Cell Activity of MCV-treated Patients. Blood samples from patients who received MCV treatment alone were analyzed for suppressor cell activity. There was no significant pattern of suppressor cell activity observed among the 10 patients. Some patients initially had low suppressor cell activity which eventually increased by the 12th week, whereas in other patients the opposite effect was observed. In Fig. 2 examples of longitudinal analysis of suppressor cell activity are given. The mean (n = 10) suppressor cell activity at the first vaccine treatment was 25% suppression and at the forth vaccine treatment the mean was 40.5%. Analysis of the differences of percentage of suppression between Vn and Vn + 1 indicated a trend of lesser inhibition of suppressor cell activity as the number of MCV treatments increased (Table 2).

Serial Analysis of Suppressor Cell Activity for Patients Receiving MCV plus CYP.Suppressor cell activity was monitored from serial blood samples obtained from patients receiving MCV plus different dosages of CYP. The activity was monitored on the day of administration of CYP and MCV treatment. In Fig. 3 representative examples of patients receiving 75, 150, and 300 mg CYP/m² treatments are given. Individual patients had variable responses to the different dosages of CYP as observed in patients treated with MCV alone. To analyze suppressor cell activity during treatment percentage of suppression was determined on the day of CYP treatment (Cn) and 3 days later after MCV treatment (Vn). The reduction in percentage of suppression between Cn and Vn was considered a positive effect of CYP treatment, whereas no change in activity or increase in activity was considered as no response. In analysis...
of the couplets (Cn-Vn) of different serial blood samples there was at least 1 patient in each CYP dosage group who had >50% reduction in suppressor cell activity after 3 days.

At the start of treatment for each CYP dose there was variability in individual patients' suppressor cell activities. The mean suppressor cell activity at the initiation of treatment was 32.3, 24.5, and 40% for 75, 150, and 300 mg CYP/m², respectively. The mean suppressor cell activity at the fourth treatment was 28.4, 25.4, and 33.7% for patients treated with 75, 150, and 300 mg CYP/m², respectively. In Table 2 a summary of percentage of suppression differences at the Cn-Vn intervals for each dose of CYP is given. For all CYP dosages the most reduction (mean) in percentage of suppression occurred in the first 2 CYP treatments. The 300 mg CYP/m² dose had the highest overall mean reduction in suppressor cell activity.

In comparing the mean of reduction of percentage of suppression of C1V1 + C2V2 versus C3V3 + C4V4 we found that only the 300 mg CYP/m² group had statistical significance (P < 0.01; Student's t test). In the MCV group there were no significant differences between the couplets.

To evaluate the percentage of patients having reduction of suppression in response to CYP treatment Cn-Vn couplets for individual patients were assessed. The patient is considered as responding to CYP treatment if there is a reduction in suppressor cell activity after 3 days of treatment. For each Cn-Vn couplet the number of patients showing reduction in suppressor cell activity was determined for each CYP dose (Fig. 4). The pattern of reduction of suppressor cell activity was similar for the different CYP dosages. At the first and second treatments the percentage of patients was the highest; thereafter the level declined. Consistently, the 300 mg/m² group had the highest percentage of patients for each couplet (Cn-Vn) group. The 75 and 150 mg/m² treatment groups had similar responses, except at the 75 mg/m² C2-V2 couplet there was a higher percentage of patients. The best response to CYP 300 and 75 mg/m² group was at the C2-V2 couplet, whereas for CYP 150 mg/m² it was the C1-V1 couplet.

Overall Effect of CYP on Suppressor Cell Activity. The overall cumulative effect of CYP on reduction of suppressor cell activity was analyzed by taking the initial treatment value at C1 and subtracting the mean of suppressor cell activity for C2, C3, and C4 in each patient [C1 - (mean of C2 + C3 + C4)]. This derived value represented overall mean reduction, if any, in suppressor cell activity from the initiation of treatment. In Fig. 5 the percentage of patients having reduction in suppressor cell activity for the individual CYP treatments is presented. The percentage of patients having an overall reduction in suppression was significantly greater in the 300 mg CYP group (P < 0.05; analysis of variance) compared to MCV alone or the 75 mg CYP group. There were no significant differences in the reduction of suppressor activity between MCV alone and 150 or 75 mg CYP/m² treatments. When patients were compared with regard to their entry status (AWD, NED) into different treatment groups there were no significant differences.

Relation of Suppressor Cell Activity to Clinical Status. Clinical status of patients at 12 months of therapy was correlated to suppressor cell activity (Table 3). Correlation of overall clinical response to suppressor activity was higher for CYP treatment groups MCV + CYP 75 mg/m² compared to MCV alone (45.5 and 40%, respectively). Statistical analysis of the correlation of clinical status to overall suppressor cell activity in the MCV + CYP treatment groups (all CYP groups) was significant (P < 0.05; χ²). An evaluation of positive clinical status to positive suppressor cell activity showed a trend favoring the MCV + CYP groups, especially for MCV + CYP 300 mg/m² compared to MCV alone (Table 3).

Lymphocyte Subset Analysis of Patients Receiving Therapy. Lymphocyte subsets were analyzed from serial PBL samples.
from 4 patients in the MCV and MCV plus 300 mg CYP/m² groups. A summary of 6 serial lymphocyte samples analyzed by dual color flow cytometry is given in Table 4. PBLs were analyzed at the time of each vaccine (VI–V5) treatment. The cell subset having the highest positive staining was the CD4*TQ1~ (helper cells). The CD4~TQ1+ cells at pre- and MCV treatments were significantly (Student’s t test) lower than the MCV + CYP group (P < 0.02 and = 0.03, respectively). The CD8*CD11B+ (suppressor) cell subset at pre- and MCV treatments was significantly higher than MCV + CYP (P = 0.03 and < 0.05, respectively). All other subset comparisons of either pretreatment or MCV compared to MCV + CYP were not significant. The analysis of individual subsets for each vaccine treatment is given in Table 5. When the two treatment groups were compared, only the CD8*CD11B+ subset had significant (P < 0.05 for V1, V3, and V5) differences for more than one cycle of MCV treatment.

**DISCUSSION**

In this study we set out to determine whether CYP as an immunomodulator could reduce suppressor cell activity in patients receiving MCV. We sought a drug dosage which would not inhibit positive immune effector mechanisms toward melanoma, such as antibody production to tumor-associated antigen. The effect of CYP on enhancement or inhibition of antibody production to melanoma-associated antigen has been previously assessed (1). We reviewed the literature and to the best of our knowledge this is the largest clinical trial in which suppressor cell activity of various low dosages of CYP <300 mg/m² as an immunomodulator was assessed in a significant series of patients with normal performance status and a single histological type of cancer (28–30). Our study is unique compared to other published trials in that the patients: (a) all had the same neoplasm, (b) all had normal performance status, (c) did not have any chemo-, immuno-, or radiotherapy(s) prior to receiving CYP, (d) did not receive any other therapies during treatment, and (e) were randomized to receive low dosages of CYP and MCV or MCV alone. When analyzing immunological parameters these criteria are very important. The effect of therapy on the human immune system may either be immediate or delayed for several months. Therefore, to analyze cellular functions such as suppressor cell activity it is important that patients entering the randomized trial be as homogeneous as possible.

In melanoma patients there is considerable heterogeneity in immunological status, tumor burden, immunogenicity of tumor, and duration of tumor presence. Analysis within a patients’ serial bleed by comparison of pre- to posttreatment is often the most informative to evaluate the effect of the drug. In comparing all 3 dosages of CYP the 300-mg/m² dose consistently gave the most reduction in suppressor cell activity within individual patients as well as the highest number of responding patients. There were, however, patients who had very significant reductions (>50%) in suppressor cell activity with the other dosages of CYP. The assessment of suppressor cell activity at individual couplet points (Cn-Vn) and during a 12-week period indicated that 300 mg CYP/m² was consistently effective. Much to our surprise in patients receiving MCV treatment alone, >40% had an overall reduction in suppressor cell activity during the course of 4 treatments. This may be related to activation of the patients’ immune responses during therapy. These results led us to investigate lymphocyte phenotype subsets to compare patients receiving MCV with or without 300 mg CYP/m².

The data from the subset analysis supported the functional suppressor data from the con A assay. There was significant reduction in the suppressor cell subset CD8*CD11B+ in patients receiving 300 mg CYP/m² + MCV versus MCV alone or pretreatment. At 4 of 5 individual Vn points, a significant reduction in CD8*CD11B+ cells occurred. CYP treatment did not significantly affect the number of CD4~TQ1~ (suppressor inducer) cells. There was a significant increase in CD4*TQ1+ cells in the CYP treatment group compared to MCV alone or pretreatment. Although this subset has not been clearly defined, it is likely to be part of the CD8+ cell subset (30, 31). The TQ1 antigen is found on about 50% of PBLs, with distribution of 70–85% on CD4 cells and 30–60% on CD8 cells (24, 25, 32). CYP did not significantly alter the cytotoxic T-cell subset CD8~CD11B~. Our studies indicate that the percentage of CD4+ and CD8+ cells overall are not altered after treatment with 300 mg CYP/m², although subsets expressing these major markers may vary in numbers.

Studies by Bast et al. (28) have shown that treatment with CYP dosages of 200–600 mg/m² gave selective reduction of circulating CD8+ cells in patients with melanoma. Our studies indicated no overall reduction of CD8+ cells after treatment but reduction in a specific subset. Berd and Mastrangelo (29) have reported that there were no significant changes in the CD8+CD11B+ cell subset in advanced stage melanoma patients receiving vaccine plus 300 mg CYP/m². One of the major

### Table 3
**Relation of suppressor cell activity to clinical status**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Overall correlation of clinical response to suppressor cell activity</th>
<th>Correlation of (+) suppressor cell activity</th>
<th>( + ) suppressor cell activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCV</td>
<td>4/10 (40%)</td>
<td>2/4 (50%)</td>
<td></td>
</tr>
<tr>
<td>MCV + CYP</td>
<td>5/11 (45.5%)</td>
<td>2/4 (50%)</td>
<td></td>
</tr>
<tr>
<td>MCV + CYP (150 mg)</td>
<td>8/10 (80%)</td>
<td>3/5 (60%)</td>
<td></td>
</tr>
<tr>
<td>MCV + CYP (300 mg)</td>
<td>8/10 (80%)</td>
<td>4/6 (66.7%)</td>
<td></td>
</tr>
</tbody>
</table>

*Correlation of patients suppressor cell activity to clinical status at 12 months of therapy. Suppressor cell activity was then categorized as positive or negative: a positive was patients with low overall suppression during treatment (mean <30%) or cumulative reduction in suppression as described in Fig. 5; a negative was high overall suppression during treatment (>30%) or no cumulative reduction in % suppression after CYP. Clinical status of patients at start of treatment and 12 months after treatment was evaluated as follows: a positive response was considered to be when patients had no change in clinical status (NED to NED, AWD to AWD); a negative response was considered to be when patients had progression of disease (NED to recurrence of death, AWD to death). Correlation refers to congruence in clinical course and suppressor activity, i.e., if suppressor cell activity was not reduced, the clinical course was adverse or, if clinical course was favorable, the suppressor cell activity was reduced.

### Table 4
**Phenotypic analysis of lymphocyte subsets**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Positive staining lymphocytes</th>
<th>CD4<del>TQ1</del></th>
<th>CD4~TQ1+</th>
<th>CD8*CD11B+</th>
<th>CD8~CD11B+</th>
<th>CD8~CD11B-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment</td>
<td>19.0 ± 3.8 &amp; 35.7 ± 4.0</td>
<td>10.8 ± 2.1</td>
<td>9.1 ± 1.2</td>
<td>15.5 ± 1.9</td>
<td>9.4 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>MCV</td>
<td>19.2 ± 1.6</td>
<td>18.6 ± 1.8</td>
<td>6.4 ± 0.5</td>
<td>16.9 ± 2</td>
<td>11.1 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>MCV + 300 mg CYP/m²</td>
<td>19.7 ± 2.4</td>
<td>31.1 ± 1.5</td>
<td>15.6 ± 1.7</td>
<td>12.9 ± 0.8</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In each treatment group 4 patients were analyzed. Pretreatment represents the mean values of 8 patients prior to treatment (VO). The values in the treatment groups represent the mean of 4 patients; for each patient the mean of 5 points (V1–5) was taken.

*Mean ± SEM.
There is a variation in patient responses. The results that have been observed from tumor-bearing animals receiving CYP are often more uniform since the animals used in those studies are inbred.

In this study, and others, overall suppressor cell activity has been measured by the Con A assay and flow cytometry of individual T-cell subsets which can be either nonspecific or specific. The immune system is constantly regulating multiple antigen stimulations via antigen-specific and nonspecific suppressor cells. Analysis of the effect of immunomodulating drugs such as CYP may be much more informative if antigen-specific (related to the disease) T-cell subsets could be assessed. However, this is a difficult task, since only recently melanoma-associated antigens that induce autologous serological responses have been characterized (1). The question of which is more important, antigen-specific or nonspecific suppressor cells, will remain unsolved until more antigen-specific cellular responses toward tumors are well characterized.

In previous studies we found that 300 mg CYP/m² enhanced tumor necrosis factor-like production from monocytes, whereas at the lower dosages of 75 and 150 mg/m² production of this factor was unaltered or diminished (34). Our studies indicate that CYP at 300 mg/m² has multiple effects on the cellular immune functions which control eradication of tumor burden. Cytokines released from monocytes can effect T-cell subset functions (35). The effect of CYP on T-suppressor cell activity may be indirect through modulation of monocyte activity.

One of the objectives of using CYP was to downregulate suppressor cell activity prior to MCV treatment. The study suggests that low dose CYP can be used for this purpose in conjunction with MCV. Analysis of the clinical response (12 months) with overall suppressor cell activity reduction indicated a significant correlation for the MCV + CYP groups. The patients receiving the 300 mg CYP/m² had the most favorable clinical response in 12 months as well as the most overall reduction in suppressor cell activity. Although 300 mg/m² was consistently more effective, lower dosages were effective for both clinical and overall suppression in some patients. Since even 300 mg/m² of the drug can have toxic effects on some patients a method is needed for determining the lowest effective dose for a given patient. Designing alternative treatment schedules may maximize the effect of CYP. From our study it is suggested that the initial two treatments have the major effect and that subsequent treatments may be delayed until suppressor cell activity rises. Melanoma patients have varying levels of suppressor cell activity including some that have low activity. Suppressor cell activity was not significantly affected by CYP in these particular patients. This strongly suggests that pretreatment analysis before CYP administration may be very impor-

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Table 5 Phenotypic analysis of lymphocyte subsets at each MCV treatment

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Vaccination</th>
<th>CD4<em>TQ1</em></th>
<th>CD4*TQ1+</th>
<th>CD8<em>CD11B</em></th>
<th>CD8*CD11B+</th>
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<tbody>
<tr>
<td>MCV</td>
<td>V1</td>
<td>16.9 ± 5.9</td>
<td>38.3 ± 6.7</td>
<td>9.8 ± 1.6</td>
<td>12.7 ± 5.8</td>
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<tr>
<td></td>
<td>V2</td>
<td>19.2 ± 1.3</td>
<td>32.6 ± 3.1</td>
<td>8.8 ± 3.4</td>
<td>16.3 ± 3.8</td>
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<td>V3</td>
<td>20.5 ± 4.6</td>
<td>27.4 ± 1.6</td>
<td>8.9 ± 0.9</td>
<td>15.3 ± 3.9</td>
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<td></td>
<td>V4</td>
<td>18.2 ± 4.1</td>
<td>31.6 ± 4</td>
<td>12.5 ± 6.1</td>
<td>16.8 ± 6.2</td>
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<td></td>
<td>V5</td>
<td>18.5 ± 5.6</td>
<td>31.9 ± 0.9</td>
<td>18.5 ± 6.6</td>
<td>22.2 ± 11.3</td>
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<tr>
<td>MCV + 300 mg CYP/m²</td>
<td>V1</td>
<td>25.6 ± 12.6</td>
<td>36.9 ± 5.3</td>
<td>4.8 ± 1.2</td>
<td>11.0 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>V2</td>
<td>19.3 ± 4.8</td>
<td>31.1 ± 1</td>
<td>5.0 ± 0.9</td>
<td>12.9 ± 3.2</td>
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<tr>
<td></td>
<td>V3</td>
<td>17.6 ± 3.4</td>
<td>37.8 ± 5.5</td>
<td>6.0 ± 1.6</td>
<td>12.6 ± 6.2</td>
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<td></td>
<td>V4</td>
<td>22.6 ± 8.8</td>
<td>22.7 ± 6.8</td>
<td>11.4 ± 5.4</td>
<td>23.2 ± 6.9</td>
</tr>
<tr>
<td></td>
<td>V5</td>
<td>17.8 ± 9.8</td>
<td>30.0 ± 0.1</td>
<td>5.2 ± 0.3</td>
<td>13.4 ± 8.8</td>
</tr>
</tbody>
</table>

* In each treatment group there are 4 patients. The value given for each vaccination treatment represents the mean of 4 patients. The pretreatment analysis of phenotypic subsets is given in Table 4.

* Mean ± SEM.

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Differences in our protocol is that by day 56 (8 weeks) the patients have received 4 CYP treatments, whereas in their trial only 3 CYP treatments have been given. The reduction in the CD8*CD11B* cell subset were consistent at multiple vaccine (V) points therefore, supporting the validity of the overall evaluation. Also in the study by Berd and Mastrangelo (29) a modest reduction in the CD4*2H4* (suppressor inducer) cells occurred after 7 weeks of CYP and vaccine treatment. The suppressor inducer subset CD4*TQ1* we analyzed showed no significant changes. The CD4*TQ1* and CD4*2H4* are not the same but are believed to overlap. Comparison of the trials may not be appropriate since the extent of disease was not similar, different melanoma vaccines were administered, the number of CYP treatments were not the same, and the scheduling of treatments differed.

Patients consistently gave a similar pattern of response to CYP, regardless of dose, for the first 2 treatments. This type of response may be predicted, since suppressor cells sensitive to CYP will be removed or inhibited early in treatment. This does not imply that suppressor cell activity would be abrogated completely, since there are suppressor cells that are not sensitive to CYP. The efficiency of CYP as a suppressor cell immunomodulator may be limited to the number of CYP-sensitive suppressor cells. There is question as to what phenotype(s) the CYP-sensitive population might be. The CD8*CD11B+ may possibly be included as a CYP-sensitive cell population since there was a reduction during 300 mg CYP/m² treatment. An important question that needs to be addressed is to what extent melanoma tumor burden induces CYP-sensitive suppressor cells in patients? It may be that only patients with melanomas expressing certain tumor and/or particular tumor burden have high levels of these cell types. Also CYP treatment effect is related to the extent of immune suppression the patient has at the start of the study. Due to the limitation of CYP effect on certain subsets of suppressor cells other drugs which modulate suppressor cell activity should be investigated for possible greater efficacy. One such drug, cimetidine, is currently being investigated. Preliminary results suggest that this drug can also modulate suppressor cell activity and immunological responses in melanoma patients (1).

The variation in responses of patients to the various low dosages may be related to in vivo activation of CYP. CYP requires activation to a metabolic form by enzymes from the liver (33). Since the patient population is very heterogeneous the concentration of the final activated form to which the immune cells are exposed would vary from patient to patient receiving the same CYP dose. This may in part explain why
tant. Future studies should be focused in this direction in order to predict which patients are likely to respond so that unnecessary treatment of nonresponders can be avoided.

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REFERENCES


Suppressor Cell Activity in a Randomized Trial of Patients Receiving Active Specific Immunotherapy with Melanoma Cell Vaccine and Low Dosages of Cyclophosphamide
