Unexpected Cytokines in Serum of Malignant Melanoma Patients during Sequential Biochemotherapy

Elizabeth A. Grimm, Christine M. Smid, J. Jack Lee, Chi-Hong Tseng, Omar Eton, and Antonio C. Buzaid

Departments of Cancer Biology [E. A. G., C. M. S.], Melanoma and Sarcoma Medical Oncology [O. E.], and Biostatistics [C-H. T., J. J. L.], University of Texas, M. D. Anderson Cancer Center, Houston Texas 77030, and Centro de Oncologia, Hospital Sirio-Libanes, Sao Paulo 01308-050, Brazil [A. C. B.]

ABSTRACT

Biochemotherapy, which combines traditional chemotherapy with immune modulating biologicals, produces an unexpectedly high response rate (>50%) in advanced melanoma patients. We hypothesize that immunological mechanism(s) are responsible for the increased response rate, and particularly that macrophage activation is involved in tumor reduction. Patients were randomized to receive chemotherapy, composed of cisplatin, vinblastine, and dacarbazine (CVD), or biochemotherapy, which is CVD followed by interleukin (IL)-2 and IFN-α2b (CVD-BIO). Laboratory analysis was performed on sera from 41 patients from each arm. Measurements of macrophage activation (neopterin), nitric oxide production (nitrite), and tumor necrosis factor-α (TNF-α), IL-1α, IL-1β, IFN-γ, IL-6, IL-10, and soluble IL-2 receptor (sIL-2R) were performed. Six of the nine biological responses (nitrite, neopterin, IFN-γ, IL-6, soluble IL-2R, and IL-10) significantly (P < 0.0002) increased in the CVD-BIO patients but not in the CVD patients. The increased IL-6 (P = 0.04) and IL-10 (P = 0.05) correlated with patient response, but only when the minor responders were included in the analysis. Evidence of macrophage activation was found in CVD-BIO patients and not in those receiving CVD alone. In addition, an unusual cytokine elaboration composed of IL-6, IFN-γ, IL-10, nitrite, neopterin, and sIL-2R, but not the expected TNF-α and IL-1, was detected. A trend of higher increase in IL-6 and IL-10 in patients having clinical response was found, suggesting an incomplete Th2 pattern of cytokine elaboration. These data show that macrophage activation does not appear to be critical in the response to CVD-BIO, but that IL-10 and IL-6 induced by the BIO component of the CVD-BIO were associated with tumor regression, and that their biology should be pursued further in the analysis of mechanism(s) of response.

INTRODUCTION

Progress toward the treatment of patients with advanced metastatic melanoma has been suggested by the recent results from several biochemotherapy trials in which the response rates in the 50–60% range were reported, after treatment with cisplatin-based chemotherapy combined with IL-2 based immunotherapy (1–3). Previously, immunologists commonly considered standard chemotherapies to be immunosuppressive and believed that their use would inhibit immunotherapeutic attempts. The outdated assumption of chemotherapy and immunotherapy as counteracting treatments is now being replaced by a new paradigm of a combined treatment involving synergistic interactions through as yet unidentified mechanism(s). Various sequences of administration of these combined modalities have been tested previously at our institution, and the administration of chemotherapy prior to immunotherapy or concurrent with immunotherapy appears more effective than when the immunotherapy was given first (4, 5).

Several research groups have examined parameters of biological responses during biochemotherapy. Evidence of T-cell activation, by the detection of high levels of sIL-2R shed into sera, was reported by Mouawad et al. (6). Not only were T cells activated in all patients, but also there was a correlation between the elevated sIL-2R and the clinical response (6). Mouawad’s group reported recently a negative correlation between IL-6 levels in pretherapy serum and response to biochemotherapy (7). Our laboratory reported previously a borderline significance of increased nitrite levels with patient response during a concurrent biochemotherapy trial in 45 stage III patients, suggesting that nitric oxide production may be involved in effective therapy (8, 9). From a separate report on 16 of these patients for whom pretherapy lymphocytes were obtained, an in vitro test for the cisplatin-induced DNA damage was found to provide correlation with biochemotherapy clinical responses (P = 0.0007–0.024, depending on cisplatin dosage), thereby suggesting a potential tool for predicting response to biochemotherapy (10).

Taken together, existing preliminary data suggest the hypothesis that a heterogeneous set of biological factors, initiated by higher susceptibility to DNA damage from the initial chemotherapy and then involving immune products from subsets of biotherapy-activated macrophages and T cells, is involved with...

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2 To whom requests for reprints should be addressed, at Department of Cancer Biology, Box 79, The University of Texas M. D. Anderson Cancer Center, 1315 Holcombe Boulevard, Houston, TX 77030.

3 The abbreviations used are: IL, interleukin; sIL-2R, soluble IL-2 receptor α chain; CVD, combination chemotherapy, composed of cisplatin, vinblastine, and dacarbazine; CVD-BIO, biochemotherapy composed of CVD followed by IL-2 and IFN-α; ANOVA, repeated measures analysis of variance; TNF, tumor necrosis factor; CR, complete response; PR, partial response; MR, minor response; SD, stable disease.
clinical response. Whether these factors represent secondary events or direct factors in the mechanism of response currently remains unknown. Therefore, to more clearly define biological events unique to the biotherapy (BIO) component of the biochemotherapy, we performed the current study using patient material from a randomized trial that consisted of a larger number of stage IV melanoma patients than used in earlier reports. Patients were recruited from a clinical trial in which randomization was to either chemotherapy alone (CVD) or biochemotherapy, therefore permitting analysis of data concerning the contribution of BIO components in the setting of biochemotherapy. On the basis of our earlier nitrite data, we hypothesized that macrophages were likely to be involved in tumor destruction; therefore, the measurement of markers of macrophage activation was considered a priority. We further asked whether the chemotherapy would inhibit any of the well-known biological responses in response to IL-2 or IFN-\(\alpha\), such as the IL-1s and TNF, and for any systemic biological responses that did occur, whether serum levels correlated with clinical response or survival.

PATIENTS AND METHODS

Patient Treatment/Sample Collection. As part of an institutionally approved clinical trial in accord with assurance filed with and approved by the United States Department of Health and Human Services, stage IV melanoma patients who had received no prior systemic therapy, were randomized to receive either chemotherapy alone (CVD) or sequential biochemotherapy (CVD-BIO). The clinical protocol is currently still accruing the last of the 200 intended patients. Sera collected from the first 82 evaluable patients were used in this research. All patients provided written consent for multiple blood samples. Of these, 41 had been randomized to CVD only and 41 to CVD-BIO. The CVD for both groups consisted of 20 mg/m\(^2\) i.v. cisplatin on days 1–4, 1.5 mg/m\(^2\) i.v. vinblastine on days 1–4, and 800 mg/m\(^2\) i.v. dacarbazine on day 1. The sequential biochemotherapy regime consisted of CVD, followed by 9 \(\times\) 10\(^6\) IU/m\(^2\) continuous infusion IL-2 (Cetus-Chiron) on days 5–9 and 5 \(\times\) 10\(^6\) units/m\(^2\) IFN-\(\alpha\)2b (Roferon-A) by s.c. injection, also on days 5–9 of the first cycle. Both biologicals were repeated on days 16–20. The scheme for the treatment protocol and blood draws is described in Fig. 1. All patients had blood samples drawn prior to initiation of the therapy and then on days 5, 6, and 9. These particular days for sera collection were selected as pretherapy; day 5, which was the last day of chemotherapy; day 6, the first of biological therapy; and day 9, which was the last day of biological therapy. In a previous biochemotherapy trial studied by us, the peak nitrite levels were found on day 5 (8), so that we intended that the most critical biological correlated would occur prior to day 9. Blood was collected into red-top Vacutainer tubes. Within 2 h of collection, the tubes containing the clotted blood were centrifuged, and patient serum was aspirated, aliquoted, and frozen at \(-80^\circ\)C for later analysis.

Evaluation of clinical response was measured in all patients on day 42 and was required to last at least 1 month. Standard response criteria were used: a CR was indicated by no clinical evidence of any residual tumor; a PR was indicated by \(>50\%\) decrease in the sum of the products of the greatest perpendicular diameters of measurable lesions; we also noted MRs for biological analysis purposes, which were indicated by \(<50\%\) tumor shrinkage but \(>25\%\); SD was indicated by \(\leq 25\%\) shrinkage or no change; and PD was indicated by an increase in tumor size. The details of the patient characteristics and clinical results for the entire trial were recently presented and published (11).

ELISA for Cytokines and IL-2R Levels. All ELISA kits were purchased from Endogen (Woburn, MA); the manufacturer’s protocol was followed for determining the IL-1\(\alpha\), IL-1\(\beta\), TNF-\(\alpha\), IL-6, IL-10, IFN-\(\gamma\), and IL-2R levels, and all tests were performed in triplicate. Briefly, standards and samples were plated into 96-well plates precoated with capture antibody. A biotinylated antibody was added to the wells, and the plate was incubated for 2 h at room temperature. After the incubation, the plate was washed three times with wash buffer. Streptavidin-horseradish peroxidase was then added to the wells, and the plates incubated for 30 min at room temperature, followed by three washes. The premixed substrate solution was then added to the wells, and the plate was developed in the dark for 30 min at room temperature. Once the plate controls had
developed, a stop solution was added to the reaction mixture, and the absorbance was read on a DYNEX plate reader at 450 nm with a reference of 550 nm. The mean values at each time point were then used directly for the analysis reported. The normal range values for each cytokine were obtained from Endogen and are indicated in the figure legends. It is important to note that the antibody pairs for the Endogen IL-6 ELISA are known to measure only the M, 30,000 functional form of this molecule, not any of the numerous inactive chaperoned forms of this cytokine.

**Nitrite Measurements by the Greiss Assay.** Serum nitrite, produced via reduction of nitrate, has often been used as a surrogate marker for nitric oxide (NO) production. The total level of the oxidation product nitrite (NO$_2$) in the patient serum was determined at each time point using the Griess reaction (12, 13). The Griess assay measures nitrite only; therefore, all nitrate, considered the more stable form in human sera, was enzymatically converted to nitrite by the addition of nitrate reductase to all samples, as reported previously from this laboratory (8). In brief, the Griess reaction assay used a standard curve consisting of 400, 200, 100, 50, 25, 12.5, and 0 μM NaNO$_2$. Five μl of 30% ZnSO$_4$ in H$_2$O were added to 100 μl of all standards and samples in Eppendorf tubes. The tubes were then centrifuged in an Eppendorf microcentrifuge at 14,000 rpm for 12 min. An aliquot of the supernatant (56 μl) was transferred to a fresh tube, and 62 μl of *Escherichia coli* nitrate reductase was added to the aliquot and mixed and then incubated for 1.5 h at 37°C. Again, the samples were centrifuged for 5 min at 12,000 rpm. Eighty μl of the supernatant were transferred to a 96-well plate, and then 80 μl of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% H$_3$PO$_4$) were added to each well. The plate was incubated for 10 min at room temperature and then read on a DYNEX spectrophotometer at 540 nm. NO$_3$ levels were extrapolated from a standard curve included in each day’s assay.

**Neopterin Determination.** A neopterin RIA kit (IBL, Hamburg, Germany) was used to measure serum neopterin levels, considered unique and indicative of macrophage activation (8). In brief, standards, samples (in triplicate), or controls were mixed with $^{125}$I tracer. The solid-phase reagent was added to all samples, which were then incubated for 30 min at 37°C. After a 10-min wash at room temperature, the samples were centrifuged for 10 min at 3000 × g. The supernatant was decanted, and the radioactivity was counted for 1 min on a gamma counter. Sample values were calculated from the standard curve generated during the assay.

**Statistical Analysis.** All ELISA and Griess reaction data were initially expressed as mean ± SE of triplicate values. Repeated measures ANOVA was used to compare data for each marker as changes over time and also response over time. F test was then used for determination of significance levels. The test for correlation of dependence between IL-6 and IL-10 were performed using Pearson correlation statistics. All tests reported here were two-sided tests. The Cox regression model was used for comparison of survival with responses in the CVD-BIO group. A two-sided $P \leq 0.05$ was considered statistically significant.

### RESULTS

Of the 41 patients randomized to receive CVD-BIO, 21 (51%) demonstrated an objective clinical response (CR + PR). Of the 41 patients randomized to receive CVD alone, 9 (22%) demonstrated a CR or PR (Table 1). The major response rate in the CVD-BIO group was significantly higher than the response rate in the CVD group ($P = 0.008$, $\chi^2$ test). Because we found no marker of biological response correlating with clinical response using the above standard definitions (response to include only CR and PR), we then included patients with minor clinical responses as biological “responders” based on a 25–50% regression of tumor that could be considered as important biologically, although not as important clinically. Using this modified definition, there were 26 responders to biotherapy (63%), and to chemotherapy there were 13 (32%). Addition of BIO to the CVD for advanced melanoma patients appears to double the response rate.

High response rates are not necessarily indicative of prolonged survival. Using the Cox regression, response was found to significantly correlate with survival ($P = 0.0002$). At the time of this writing, 8 patients who received CVD-BIO were alive compared with only 4 of those who received only CVD. Therefore, it appears that the mechanism of the increased response as measured at 42 days in this study may also be part of a mechanism that leads to long-term survival.

**Biological Responses Observed in Sera from Patients Receiving IL-2 and IFN-α after Chemotherapy but not after Chemotherapy Alone.** Laboratory measurements for nine serum biological markers were performed on available patient sera. The most common IL-2-driven secondary cytokines, TNF-α, IL-1α, and IL-1β, were found not to increase at all in either treatment group. Analysis of the levels of each of those cytokines was stopped after 40, 42, and 37 patients, respectively, to conserve sera. The results of laboratory values were within normal levels at all time points. For IL-1 and TNF, no significant increase or decrease was evident (Fig. 2) from either arm of the study, although CVD-BIO patients with detectable IL-1α values at baseline did have a substantial decrease in these during therapy (12.1–1.9 pg/ml), which did not occur in the CVD alone patients. This absence of Th1 cytokines was very unexpected as TNF-α elaboration into sera has been considered a hallmark of IL-2 infusion (14, 15). The other six biologicals (neopterin, nitrite, IFN-γ, sIL-2R, IL-6, and IL-10) all increased.

<table>
<thead>
<tr>
<th>Response</th>
<th>CVD (%)</th>
<th>CVD-BIO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR$^a$</td>
<td>0 (0)</td>
<td>4 (10)</td>
</tr>
<tr>
<td>PR$^b$</td>
<td>9 (22)</td>
<td>17 (41)</td>
</tr>
<tr>
<td>MR</td>
<td>4 (10)</td>
<td>5 (12)</td>
</tr>
<tr>
<td>SD</td>
<td>12 (29)</td>
<td>5 (12)</td>
</tr>
<tr>
<td>PD$^c$</td>
<td>16 (39)</td>
<td>10 (24)</td>
</tr>
<tr>
<td>41</td>
<td></td>
<td>41</td>
</tr>
</tbody>
</table>

$^a$ The major response rate in the CVD-BIO arm is significantly higher than the response rate in the CVD arm ($P = 0.008$, $\chi^2$ test).

$^b$ PD, partial disease.
Fig. 2 Cytokine and other tumor marker levels from all patients on all days. A, TNF-α levels measured by ELISA. B, IL-1α. C, IL-1β, also by ELISA. D, IL-6. E, IL-10. F, IFN-γ levels measured by ELISA. G, sIL-2R. H, neopterin levels measured by RIA. I, nitrite levels measured by Griess reaction. Left column, levels from the CVD-BIO patients; right column, levels from the CVD patients.
significantly over time in the CVD-BIO patient sera ($P < 0.0002$), as compared with that in the CVD-alone group (Fig. 2 and Table 2). Not all tests were performed on all patients at all time points because of a lack of serum in some cases and unavailability of neopterin measurement kits in other instances. Sporadic elevation of IFN-γ was noted in a few patients in the CVD-alone group (Fig. 2F), but these values had no statistical significance or correlation with response. Overall, each of these six biological markers were still increasing in value on the last day (day 9) of serum collection, and it was unfortunate that samples were not planned for later times.

**Increased IL-6 and IL-10 Levels in Serum of Patients in the CVD-BIO Group Tend to Correlate with Clinical Responses.** Using a repeated measures ANOVA model, we asked whether any of the six biologicals that increased in sera of patients receiving CVD-BIO, but not in the CVD-alone patients, correlated to patient clinical response. Using the standard definition of CR + PR patients as “responders,” no significant
correlation of any marker with clinical response was detected. However, when we added the data from the 4 CVD and 5 CVD-BIO patients in the MR category to our analysis, weak correlation of clinical response with the increased values of IL-6 (P = 0.04), and a trend of increased IL-10 on days 5 (P = 0.05) and 6 (P = 0.07), was observed (Table 3). The day-9 values for all markers increased in all of the CVD-BIO patients, and the statistically significant association with clinical response at this later time was lost, because of higher variability of the data obtained.

**Table 3** Test for correlation of increased biological markers with the biochemotherapy patient clinical response

<table>
<thead>
<tr>
<th>Serum marker</th>
<th>Day 0</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>sIL-2R</td>
<td>0.81</td>
<td>0.40</td>
<td>0.33</td>
<td>0.87</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.27</td>
<td>0.33</td>
<td>0.04</td>
<td>0.92</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.18</td>
<td>0.05</td>
<td>0.07</td>
<td>0.22</td>
</tr>
<tr>
<td>Neopterin</td>
<td>0.97</td>
<td>0.42</td>
<td>0.19</td>
<td>0.68</td>
</tr>
<tr>
<td>Nitrite</td>
<td>0.48</td>
<td>0.19</td>
<td>0.29</td>
<td>0.91</td>
</tr>
<tr>
<td>INF-γ</td>
<td>0.58</td>
<td>0.49</td>
<td>0.15</td>
<td>0.90</td>
</tr>
</tbody>
</table>

**P** for correlation of cytokine level with response

For correlation of cytokine level with response using ANOVA and the F test table.

**Table 2** Significant increases of biological responses in the CVD-BIO arm versus CVD-only arm

Data above are given as the mean of each marker measured ± SE for day 0 (pretherapy) and day 9 (last day of biologic). All values are in pg/ml except for neopterin, which is ng/ml, and nitrite, which is in μM. The two-sided P is calculated using data from all days using ANOVA.

**DISCUSSION**

In general, melanoma is considered an immunologically responsive tumor, with ~15% of all patients reported to respond to either IFN or IL-2 alone therapy (19). Durable CRs are observed in ~5% of the patients treated with moderate to high doses of IL-2 alone (18, 19). Although chemotherapy alone produces higher overall response rates compared with IL-2 alone, durable CRs are rare (1.5%). The recent suggestions of successful combination of these two treatment modalities, referred to as biochemotherapy or chemoimmunotherapy, has provided a most intriguing possibility for improving melanoma therapy, as well as to reveal more generalized mechanisms of antitumor host responses.

Prior to the laboratory analysis of patients on this randomized protocol, our early study of mechanisms of response to
biochemotherapy mechanisms suggested that macrophages might be activated via a Th1 cytokine network to regulate tumor growth. On the basis of the hypothesis that Th1 cytokines are likely to lead to productive antitumor immune responses, we asked whether such responses could be detected systemically. Therefore, our analytical studies were designed to critically evaluate the activation of Th1 markers TNF-α, IL-1α, IL-1β, and IFN-γ, all known to activate macrophages. Macrophage activation was measured by several means, the most specific by quantifying neopterin in the sera (8). Macrophage activation is also associated with products of reactive nitrogen species, NO, which in the human is more often considered a product of endothelial cells or even tumor cells themselves (8). A macrophage cytokine product, IL-6, was also included in the analysis. T-cell activation was evaluated by measuring soluble IL-2R shedding. Although our new data continue to support macrophage involvement as increased in nitrite and neopterin, we observed no direct evidence that clinical response was related to higher levels of this known group of macrophage-specific products, as suggested in earlier work (8). In addition, the primary macrophage-activating cytokines (IL-1 and TNF) were not increased; in fact, neither of these expected cytokines (14, 15) was found above background. Therefore, a role of Th1 cytokine elaboration and macrophage activation is no longer considered by us to be a likely primary mechanism regulating antitumor responses during CVD-BIO.

Evidence of systemic activation of T cells is based on the six other markers and cytokines that were up-regulated during CVD-BIO but not during CVD alone, with increased IL-6 and IL-10 being the only ones to correlate significantly with clinical response. Although these two cytokines can be produced by T cells as well as many other cell types, we have noted that both of these cytokines can be present in the cytoplasm of biopsied samples of melanoma cells, along with IL-1α or IL-1β (20, 21). IL-10, and under many circumstances IL-6, can be constitutively secreted from melanoma tumors (20, 21), suggesting the possibility that these two cytokines could be released into sera merely as a result of tumor destruction. Additional studies, which include analysis of tumor biopsies during therapy, are needed to fully understand the extent of these cytokines and their role in tumor destruction.

IL-10, originally named cytokine synthesis inhibitory factor, was recognized for inhibiting production of IL-1, IL-2, TNF, IFN-γ, and other Th1 cytokines and is known to divert immune responses to Th2-mediated ones (22–25). IL-10 protein has been found to constitutively be expressed at greater than normal levels in the serum of many cancer patients, including those with melanoma (25–28). IL-10 has been appreciated as an immunosuppressive factor produced by numerous tumor cell types, but conflicting evidence of IL-10 producing antitumor effects is also noted (28–32). It was demonstrated through the use of IL-10 KO mice that tumor-induced IL-10 can block the generation of Th1-dependent, antigen-specific response (33), which is consistent with our observations of the absence of Th1 cytokine expression. In addition, IL-10 has also been proposed as an autocrine growth factor for melanoma (33). Therefore, we were not surprised that many of our melanoma patients presented with above normal IL-10 levels in their sera (normal range, up to 14 pg/ml in Endogen ELISA). We were, however, extremely surprised by the 100-fold higher levels of IL-10 in

Table 4  Baseline values of cytokines of CVD-BIO patients: correlation with clinical responsea

<table>
<thead>
<tr>
<th></th>
<th>Responder</th>
<th>Nonresponder</th>
<th>Nonresponder</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Above</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>7</td>
<td>6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>IL-6</td>
<td>23</td>
<td>1</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>IL-10</td>
<td>4</td>
<td>20</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>13</td>
<td>10</td>
<td>8</td>
<td>5</td>
</tr>
<tr>
<td>sIL-2R</td>
<td>16</td>
<td>5</td>
<td>7</td>
<td>3</td>
</tr>
</tbody>
</table>

* Statistical analysis was performed using the two-tailed Fisher’s exact test. Normal ranges of serum values for each cytokine were provided by the manufacturer of the ELISA kits as follows: IL-1α, 0–5.4 pg/ml; IL-6, 0–149 pg/ml; IL-10, 0–14.1 pg/ml; IFN-γ 0–1.5 pg/ml; sIL-2R, 2380–9870 pg/ml.

Fig. 3  Correlation of IL-6 with IL-10. Correlation was found to be highly significant on day 9. ELISA data from all CVD-BIO patients were used to perform a test for dependency of these two parameters. The Pearson correlation method indicated that IL-6 and IL-10 do correlate with each other on the days shown.
responding patients’ sera during the actual biochemotherapy treatment. In contrast to the well-accepted immunosuppressive role of IL-10, experimental tumor model literature indicates that IL-10 provides substantial antitumor effects (28–31, 34–37). Recent murine studies implicate the induction of IFN-γ in the mechanism of effective antitumor therapy (38). We did find up-regulation of human IFN-γ in all CVD-BIO patients. Another murine study reported that systemic administration of IL-10 not only resulted in rejection of established melanoma tumors, but the cured mice were resistant to lethal challenge (39). Supported by these mouse models, we now propose the hypothesis that IL-10, under selected circumstances, is involved in successful human melanoma rejection induced by biochemotherapy, possibly via suppressing a Th1 cytokine cascade.

The addition of the 4 (CVD alone group) and 5 (CVD-BIO group) MR patient values as part of the responders for statistical analysis led to both IL-6 and IL-10 levels, achieving borderline significance in correlation with clinical response. Apparently, the addition of more patient numbers added more power to the test; whereas such a grouping is not appropriate for clinical analysis, it is possible that these patients were quite similar biologically to those with greater tumor reduction. However, examination of more patients in each group and separate group analysis will be needed to resolve this issue formally. Studies of regulation of expression of these cytokines is the topic of much current research, and heterogeneity of IL-10 cytokine expression attributable to genetic polymorphisms is known to exist (16, 17). For IL-10 in particular, IL-10 allelic differences may need to be considered as genetic control of antitumor response mechanisms.

A scenario involving the mechanism of the combination therapy we proposed was that the CVD would initiate the DNA damage of tumor cells, and the immunotherapy would then be potentiated. We further proposed that in response to the IL-2 and IFN-α, proinflammatory Th1 cytokines would first be elicited, followed by markers of macrophage activation and finally a decrease of the tumor markers such as IL-6 and IL-10. Our results clearly indicate that this pathway did not occur. The expected Th1 cytokines were apparently inhibited by the CVD or the IL-10 elaboration or both, demonstrated by the fact that neither TNF-α nor IL-1 was increased during the biochemotherapy. This absence of TNF-α induction was unexpected because it was thought to represent the major product in response to IL-2, leading to production of NO, which is known to be responsible for hypotension and vascular leak (40). The CVD-BIO patients do demonstrate hypotension, although manageable, suggesting that alternative pathways of nitric oxide production may be operable (41).

The rapid production of the soluble IL-2R in the CVD-BIO patients strongly indicates that T cells were activated during CVD-BIO, but because IL-6 and IL-10 levels were high, these activated T cells may have been of the Th2 subset. The increased expression of IL-6 and of IL-10, together with their correlation with response, was most unexpected. Presently, it is unknown whether their levels represent Th2 activation, death of melanoma cells releasing their intracellular stores of these markers, secretion products of macrophages, or a combination of these and other biological effects. It is important to determine the source of these cytokines as well as whether any genetic polymorphism exists in the mechanisms related to their expression. Acquisition of sera from later time points is now justified based on the data presented and will be necessary to determine total cytokine production for each of these markers.

Human melanomas are heterogeneous not only from patient to patient but also possibly within individual patients. We hypothesize that each tumor nodule contains a variety of tumor cells with different biological characteristics, and that the endogenous factors supporting tumor growth and invasion are the sum of numerous characteristics, some of them mutable. The factors, which can be changed by intervention, are likely to be complex and interrelated. Because it is known that some melanoma patients with very large tumor burdens can achieve dramatic and long-lasting responses to biological maneuvers, there is no doubt that a subset of patients, yet to be identified by current prognostic indicators, are biologically responsive. Although the mechanism of tumor growth control in response to CVD-BIO is likely to be extremely complex, the high response rate and correlation with IL-10 and IL-6 increased values during therapy suggests possible role(s) for these cytokines.

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