Human Xenograft-Nude Mouse Model of Adoptive Immunotherapy with Human Melanoma-specific Cytotoxic T-Cells

Nancy J. Crowley, Carol E. Vervaert, and Hilliard F. Seigler
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ABSTRACT

We investigated the efficacy of human melanoma-specific cytotoxic T-cells (CTLs) in treating experimental human melanoma metastases in a nude mouse model of adoptive immunotherapy. Hepatic metastases were generated by the intrasplenic injection of $1.5 \times 10^6$ human melanoma cells. Animals were then randomized to receive saline, interleukin-2 only, or CTLs and interleukin-2. CTLs were effective when administered 3 or 7 days after generation of hepatic metastases, with 96 and 88% of animals disease-free, respectively, when examined at one month. Interleukin-2 alone was not effective. In addition, CTLs were effective when as few as $2.5 \times 10^5$ T-cells were adoptively transferred. Only 33% of the animals were tumor-free when CTLs were administered on day 10, and CTLs were not effective when given at day 14. Human CTLs that were not cytotoxic for the tumor line used in vivo, when tested in a $^{51}$Cr assay, were also not effective in the model of immunotherapy. This suggests that the tumor-specific CTLs maintain their specificity in vivo, and eliminates a nonspecific inflammation directed against the human CTLs as a possible cause of the antitumor effect. These studies lay the foundation for clinical trials of CTLs in the adoptive immunotherapy of patients with metastatic melanoma.

INTRODUCTION

The identification and expansion of lymphocyte populations capable of mediating tumor regression upon adoptive transfer to the tumor-bearing host remain areas of intense investigation. Initial investigations were directed towards LAK 3 cells, generated by culturing lymphocytes in high doses of IL-2 (1, 2). Adoptive transfer of both LAK cells and IL-2 has resulted in antitumor effects in both murine (3-5) and human (6, 7) systems. Response rates have been low, however, and are primarily partial responses (2, 6, 7). In addition, significant toxic side effects are associated with the high doses of IL-2 required for a therapeutic response (6, 8). Investigations aimed at developing immunotherapeutic protocols that are more effective than LAK/IL-2 therapy have focused on TILs, isolated from a variety of human solid tumors and expanded in vitro (9-16). Murine studies have suggested that TILs may be 50 to 100 times more potent than LAK cells in the treatment of human solid tumors in vivo (17). Human trials have yielded similar promising results, with TIL therapy producing a greater response rate than therapy with LAK cells, as well as responses in patients who had previously failed LAK cell therapy (18, 19). Attempts to design TILs with improved antitumor potency, by using retroviral gene transduction, are currently underway (20).

CTLs are also under investigation as potential effectors for the adoptive immunotherapy of human cancers. Our laboratory has been investigating melanoma-specific CTLs and has found them to have several advantages over LAK cells and TILs. We have demonstrated that CTLs can be reliably generated by repeated antigenic stimulation of lymph node cells or peripheral blood lymphocytes with autologous tumor, in the presence of low doses of IL-2 (21-24). Unlike LAK cells and many TILs, cytotoxic T-cells are major histocompatibility complex-restricted, and specifically lyse the autologous tumor as well as allogeneic melanomas, which express the restricting HLA-A region antigen (25, 26). We have also demonstrated that HLA-A region matched allogeneic melanomas can substitute for the autologous tumor as the stimulating tumor required to generate specific CTLs (27, 28). The allogeneic-stimulated CTLs are specific for the autologous tumor and demonstrate the same pattern of HLA restriction as the autologous-stimulated T-cell line.

The option of using HLA-matched allogeneic melanoma as a substitute for the autologous tumor is crucial when applying this technology clinically. With our previously described methods, we can generate specific CTLs from readily available precursors. Lymph node cells are often available from surgical specimens; peripheral blood lymphocytes are the most accessible lymphocytes and are a renewable source. The amount of tumor needed for restimulation can easily be obtained using established, HLA-matched allogeneic melanoma lines, should autologous tumor be available in limited quantities only. This will allow the generation of CTLs for all patients, including patients with clinical stage I disease, or patients with stage II disease following lymphadenectomy, who are at high risk for recurrence. This is a distinct advantage when compared to TILs, isolated from metastatic deposits of tumor, which are only available for patients with stage III disease.

The efficacy of the melanoma-specific CTLs in vitro has been well documented (21-26). To examine the efficacy of the specific CTLs in vivo, we have developed a human xenograft-nude mouse model for the adoptive immunotherapy of metastatic melanoma (29). The murine model discussed is unique in that human tumor-specific CTLs are adoptively transferred to nude mice for the treatment of human melanoma xenografts. We have demonstrated that CTLs are effective for the treatment of micrometastatic and early metastatic disease. We describe here our results for the treatment of human melanoma hepatic metastases in nude mice with human CTLs and exogenous IL-2.

MATERIALS AND METHODS

Tumor Lines. Fresh melanoma cells were obtained from surgically excised tumor-involved nodes, as described previously (28). Melanoma tumor lines were cultured in 75-cm$^2$ flasks (Costar) with Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% FBS (Gibco), 100 units/ml penicillin, 100 $\mu$g/ml streptomycin, 0.25 $\mu$g/ml amphotericin B, and 50 $\mu$g/ml gentamicin. Cell lines were maintained in monolayer and passaged by trypsinization with 0.0625% trypsin plus EDTA as required. Early passages were frozen in FBS plus 10% dimethyl sulfoxide. Each specimen received was assigned a se-
Cultured melanoma cell lines were used for T-cell stimulation and in vitro cytotoxicity assays. For the in vivo assays, a tumor maintained by serial passage in the nude mouse was used.

Establishment of Cytotoxic T-Cell Lines. Lymphocytes were obtained from either peripheral blood or draining lymph nodes, as described previously (28). In the present experiments, DT169 (HLA A1, A2) or DT208 (HLA-A3, A11) peripheral blood lymphocytes were cultured in 24-well plates at an initial density of 1-2 x 10^6 cells/cm^2 of dependent surface area in culture medium consisting of RPMI 1640 (Gibco) supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, and 50 µg/ml gentamicin. Recombinant interleukin-2 was added at a final concentration of 5 units/ml. The T-cells were cocultured with an allogeneic melanoma expressing the restricting HLA-A region antigen: DT169 CTLs were restimulated with the same melanoma cell line every 10 to 14 days. The culture medium containing 5 units/ml recombinant IL-2 was replenished every 3 to 5 days and the cells maintained at a density of 1-2 x 10^6/cm^2 of dependent surface area. T-cell lines are designated DT, while tumor lines are designated DM.

Interleukin-2. The interleukin-2 was generously supplied by DuPont Laboratories, Glenolden, PA. An IL-2 assay with the IL-2-dependent murine cytotoxic T-cell line showed half-maximal proliferation at 0.5 unit (data not shown).

Cytotoxicity Assays. CTLs were tested within 3 days of any in vivo experiment to confirm their specificity. Cell-mediated lysis was determined in vitro using a standard ^51Cr release assay, as described previously (28).

The cytotoxic index was calculated as follows:

\[
\text{Cytotoxic index} = \frac{\text{cpm (experimental)} - \text{cpm (medium)}}{\text{cpm (HCl)} - \text{cpm (medium)}} \times 100\%
\]

Nude Mice. BALB/c strain male nude mice 8 weeks old were obtained from Charles River Laboratories (Raleigh, NC) and maintained under pathogen-free conditions in a laminar air-flow room. Male nude mice were anesthetized with an i.p. injection (0.2 ml) of a mixture of 20 mg/ml ketamine and 2 mg/ml xylazine. The skin was prepped with betadine, and a left subcostal incision made. One ml of the tumor suspension was slowly injected, via a 27-gauge needle, beneath the splenic capsule. After 2 to 3 min, the spleen was excised and the incision closed.

Adoptive Immunotherapy Model. Three days following induction of human melanoma hepatic metastases, the mice were randomized into three groups: (a) controls; (b) IL-2 only; or (c) CTLs and IL-2. Animals treated with specific CTLs received 2 x 10^7 CTLs i.p., followed by i.p. injections of 5000 units IL-2 in 0.5 ml HBSS twice daily for 3 days. Animals treated with IL-2 only received the above dose of IL-2; control animals received twice daily injections of 0.5 ml HBSS. After one month, animals were sacrificed and their livers examined for metastatic melanoma. In subsequent experiments, mice received decreasing doses of CTLs (1 x 10^7, 5 x 10^6, and 2.5 x 10^6) on day 3 following injection. Additional mice received CTLs at later time periods (7, 10, or 14 days) following generation of hepatic metastases.

Statistical Methods. The significance of differences between groups was determined by the two-sided Fisher's exact test, with adjustments for multiple comparisons. A P value <0.05 was considered significant.

RESULTS

In Vivo Cytotoxicity. The in vitro cytotoxicity of 169 CTLs, as well as other HLA-A2 restricted CTL lines, has been described previously (25, 28). CTLs were assayed within 3 days of the in vivo experiments to confirm their pattern of cytotoxicity (Table 1). 169 CTLs were specifically cytotoxic for all HLA-A2 melanomas tested, including DM6, the melanoma cell line used for the in vivo experiments; 208 CTLs were used as a specificity control for in vivo experiments, and their pattern of cytotoxicity is also shown (Table 1). These CTLs appear to be restricted by HLA-A11, and specifically lyse the autologous tumor as well as one other HLA-A11 allogeneic melanoma. Although DM14 and DM208 also share a B-region antigen (B8), there was no significant lysis of other allogeneic melanomas that also express B8 (DM93 and DM175), suggesting that the CTLs were not restricted by the B-region antigen. There was no cytotoxic activity against K562 or allogeneic melanomas of different HLA-A types. Most importantly, there was no cytotoxic activity against DM6 (HLA-A2) when assayed in vivo.

<table>
<thead>
<tr>
<th>Table 1 In vitro cytotoxicity of 169 CTLs and 208 CTLs</th>
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* Natural killer target; no HLA antigens expressed.
* E:T, effector: target.

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lished 3-day hepatic metastases were treated with: (a) HBSS; (b) IL-2 only; or (c) $2 \times 10^5$ specific CTLs and IL-2. Each individual experiment was done with 12 to 21 mice, with each treatment group consisting of 4 to 7 animals, depending on the availability of tumor, CTLs, and mice at the time of the experiment. The results for all experiments were then pooled and are shown in Table 2. Twenty-four of 25 control animals had tumor present within the liver, as did 11 of 13 animals receiving IL-2 only. In striking contrast, 17 of 18 animals receiving IL-2 and CTLs were tumor-free (Fig. 1B). The one animal that failed therapy had a single 2-mm nodule of tumor. The treated groups with CTLs and IL-2 demonstrated a statistically significant decrease in the number of animals with liver metastases when compared to control animals ($P < 0.001$) or animals receiving IL-2 only ($P < 0.001$).

Adoptive Immunotherapy of 3-day Hepatic Metastases with Decreasing Numbers of CTLs. To determine the efficacy of the human CTLs, a dose titration curve was performed. Animals were given decreasing numbers of CTLs on day 3 ($2 \times 10^5$, $1 \times 10^5$, $5 \times 10^4$, or $2.5 \times 10^4$), followed by 5000 units IL-2 twice daily for 3 days. Again, each experiment consisted of 4 to 7 animals per treatment group, and the results of all experiments were pooled (Table 3). The CTLs were effective at all doses tested. All treated groups had a significant reduction in the number of mice with hepatic metastases, when compared to control animals. In addition, although some of the animals failed therapy, they often had only one or two small deposits of tumor, which would easily be amenable to surgical resection.

### Table 2 Adoptive immunotherapy of metastatic human melanoma in nude mice with human tumor-specific cytotoxic T-cells

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* 3-day human melanoma hepatic metastases were treated on days 3–5 with 0.5 ml HBSS i.p. daily or 5000 units IL-2 in 0.5 ml HBSS twice daily.

### Table 3 Adoptive immunotherapy of metastatic human melanoma in nude mice with decreasing numbers of human tumor-specific cytotoxic T-cells

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* 3-day human melanoma hepatic metastases were treated on days 3–5 with 0.5 ml HBSS i.p. twice daily or 5000 units IL-2 in 0.5 ml HBSS twice daily.

# Decreasing numbers of 169 CTLs, followed by 5000 units IL-2 in 0.5 ml HBSS twice daily on days 3–5.

$P < 0.001$ versus animals receiving HBSS only or IL-2 only.

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**Adoptive Immunotherapy of 3-day Hepatic Metastases.** We have previously reported on our method for the generation of human melanoma hepatic metastases in nude mice (29). The growth of human melanoma within the liver was variable, but tended to grow in 3 patterns (Fig. 1A). Control animals had either: (a) livers almost totally replaced with tumor; (b) tumor nodules scattered throughout the liver; or (c) tumor nodules present at the periphery of the liver. Enumeration of individual tumor nodules was not possible because of the variability in growth patterns, and because the tumor tended to grow in large confluent areas. Animals were therefore classified as having large, moderate, or small tumor burdens. The presence of melanoma within the liver was confirmed by histological examination (29). Because of the difficulty in enumerating hepatic metastases, it was elected to define a treatment success as the complete absence of metastatic melanoma. If any tumor was found in the animals treated with CTLs and/or IL-2, the animal was considered a treatment failure. Again, the livers were examined histologically for the presence of micrometastatic melanoma (29).

**Generation of Hepatic Metastases.** We have previously reported that hepatic metastases were inoculated on day 3 (29). Melanoma within the liver was variable, but tended to grow in large confluent areas. Animals were therefore classified as having large, moderate, or small tumor burdens. The presence of melanoma within the liver was confirmed by histological examination (29). Because of the difficulty in enumerating hepatic metastases, it was elected to define a treatment success as the complete absence of metastatic melanoma. If any tumor was found in the animals treated with CTLs and/or IL-2, the animal was considered a treatment failure. Again, the livers were examined histologically for the presence of micrometastatic melanoma (29).

**Adoptive Immunotherapy of 3-day Hepatic Metastases.** Established 3-day hepatic metastases were treated with: (a) HBSS; (b) IL-2 only; or (c) $2 \times 10^5$ specific CTLs and IL-2. Each individual experiment was done with 12 to 21 mice, with each treatment group consisting of 4 to 7 animals, depending on the availability of tumor, CTLs, and mice at the time of the experiment. The results for all experiments were then pooled and are shown in Table 2. Twenty-four of 25 control animals had tumor present within the liver, as did 11 of 13 animals receiving IL-2 only. In striking contrast, 17 of 18 animals receiving IL-2 and CTLs were tumor-free (Fig. 1B). The one animal that failed therapy had a single 2-mm nodule of tumor. The treated groups with CTLs and IL-2 demonstrated a statistically significant decrease in the number of animals with liver metastases when compared to control animals ($P < 0.001$) or animals receiving IL-2 only ($P < 0.001$).

**Adoptive Immunotherapy of 3-day Hepatic Metastases with Decreasing Numbers of CTLs.** To determine the efficacy of the human CTLs, a dose titration curve was performed. Animals were given decreasing numbers of CTLs on day 3 ($2 \times 10^5$, $1 \times 10^5$, $5 \times 10^4$, or $2.5 \times 10^4$), followed by 5000 units IL-2 twice daily for 3 days. Again, each experiment consisted of 4 to 7 animals per treatment group, and the results of all experiments were pooled (Table 3). The CTLs were effective at all doses tested. All treated groups had a significant reduction in the number of mice with hepatic metastases, when compared to control animals. In addition, although some of the animals failed therapy, they often had only one or two small deposits of tumor, which would easily be amenable to surgical resection.

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* 3-day human melanoma hepatic metastases were treated on days 3–5 with 0.5 ml HBSS i.p. daily or 5000 units IL-2 in 0.5 ml HBSS twice daily.

# Decreasing numbers of 169 CTLs, followed by 5000 units IL-2 in 0.5 ml HBSS twice daily on days 3–5.

$P < 0.001$ versus animals receiving HBSS only or IL-2 only.

**Table 3 Adoptive immunotherapy of metastatic human melanoma in nude mice with decreasing numbers of human tumor-specific cytotoxic T-cells**

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* 3-day human melanoma hepatic metastases were treated on days 3–5 with 0.5 ml HBSS i.p. twice daily or 5000 units IL-2 in 0.5 ml HBSS twice daily.

# Decreasing numbers of 169 CTLs, followed by 5000 units IL-2 in 0.5 ml HBSS twice daily on days 3–5. All treatment groups were compared with control animals using an adjusted Fisher’s 2-sided exact t test, and were significantly different. $P$ values were <0.0001, 0.0018, <0.001, and 0.002, respectively.
Adoptive Immunotherapy of 7-day, 10-day, and 14-day Hepatic Metastases. Animals with large tumor burdens often have gross disease, and by day 14 control animals have evidence of gross disease, and by day 14 control animals often have large tumor burdens.

CTLs were extremely effective for the treatment of 7-day hepatic metastases, with nearly all animals tumor-free when examined at 30 days (Table 4). Only a single animal failed therapy, with a small tumor burden. The efficacy decreased with more advanced disease, however. Just over one-third of the mice treated at day 10 were disease free; the remaining animals failed therapy. CTLs were totally ineffective in the treatment of 14-day metastases, and all animals failed therapy, often with large tumor burdens.

**In Vivo Specificity of Tumor-specific CTLs.** CTLs have been clearly shown to be specific for the autologous tumor, and HLA-A region matched allogeneic tumor, when examined in vitro (21-28). In addition, their specificity has been examined in vivo in a Winn assay where, again, they were demonstrated to be specific for the HLA-matched allogeneic melanoma (30). To determine their specificity in vivo in the adoptive immunotherapy model, a second CTL line with no cytotoxic activity towards DM6 in vitro was selected for the specificity control. DT208 CTLs appear restricted by HLA All and do not lyse DM6, the melanoma used in the adoptive immunotherapy experiments, when assayed in vitro. DT208 CTLs were used in vivo to: (a) determine whether CTLs maintained their specificity in vivo; and (b) to rule out any nonspecific inflammatory response directed against the human CTLs that might account for the destruction of the human tumor cells as well. Animals received either 2 x 10⁷ 169 CTLs or 2 x 10⁷ 208 CTLs on day 7, followed by 5000 units of IL-2 twice daily for 3 days. DT208 CTLs were not effective for adoptive immunotherapy (Table 5), suggesting that the CTLs maintain their specificity in vivo, and that nonspecific inflammation directed towards the human CTLs is not responsible for the antitumor effect.

**DISCUSSION**

We have previously demonstrated the efficacy of human melanoma-specific CTLs in vitro and have defined a method for generating specific CTLs from readily available precursors (21-28). In the current study, we have extended those observations and demonstrated that these human tumor-specific CTLs are effective in vivo for the treatment of human melanoma hepatic metastases, in a nude mouse model of adoptive immunotherapy. Although previous investigators have used murine models to examine a variety of effectors for adoptive immunotherapy, including LAK cells (3, 5) and TILs (31, 32), these models have used murine tumors and effectors derived from normal or immunized spleen cells (3-5, 33-37) or from infiltrating murine lymphocytes (31, 32). The murine model of adoptive immunotherapy discussed here is unique in that human CTLs are used for the treatment of metastatic human melanoma.

The athymic mouse-human xenograft model has been used extensively to develop treatment modalities for human malignant neoplasms (38, 39). Initial investigations studied chemo- and immunotherapeutic agents effective against human tumors, with an excellent correlation between response rates in the nude mouse-human xenograft model and response rates seen clinically. The nude mouse-human xenograft model has also been used to study the response rates of various tumors to irradiation, hormonal manipulation, and monoclonal antibody-directed therapy, again with close correlation to response rates seen clinically. The current study extends the use of the athymic mouse-human xenograft model to include a model of adoptive immunotherapy of human cancers. This model lends itself readily to further investigations aimed at optimizing the efficacy of human CTLs, including examining the minimum number of CTLs required for a therapeutic effect, determining the dependence of CTLs on exogenous IL-2, and investigating ways to augment the effectiveness of the specific CTLs by the administration of other cytokines or antimelanoma antibodies. The results of such experiments should provide information that is directly applicable to the treatment of metastatic melanoma in humans with tumor-specific CTLs.

The number of CTLs adoptively transferred in this model is comparable to the number of effectors used in other murine models of adoptive immunotherapy. Murine studies with LAK cells have required 10⁸ for therapeutic efficacy (3, 5, 17), while murine studies with TILs or in vitro sensitized spleen cells have suggested that 10⁷ cells are effective in treating micrometastases (31, 35-35). In the current study, CTLs were effective when as few as 2.5 x 10⁷ lymphocytes were given, indicating that the efficacy of CTLs is comparable with that demonstrated by TILs on a per cell basis. In addition, we have previously demonstrated that the murine-passaged tumor used in these experiments has a decreased level of HLA expression and a decreased susceptibility to lysis by specific CTLs, relative to early passages from tissue culture (29). This would tend to decrease the effectiveness of the CTLs in this nude mouse model. This suggests that the CTLs may be even more effective in the treatment of fresh tumor specimens and, therefore, more effective in the treatment of human metastatic melanoma.

**Therapeutic responses in humans to either LAK cells or TILs**

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<td>7/7</td>
</tr>
<tr>
<td>2 x 10⁷ 169 CTLs + IL-2, day 7</td>
<td>1/8</td>
</tr>
<tr>
<td>2 x 10⁷ 208 CTLs + IL-2, day 7</td>
<td>5/6</td>
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* Human melanoma hepatic metastases were treated on day 7 with 2 x 10⁷ 169 CTLs or 2 x 10⁷ 208 CTLs, followed by 5000 units IL-2 in 0.5 ml HBSS twice daily for 3 days.

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have required high doses of exogenous IL-2, with IL-2-associated toxicity. The morbidity and mortality associated with the administration of high doses of exogenous IL-2 has been well documented in the literature (6–8). Most murine studies with TILs have used 5000 to 7500 units 3 times per day, usually for a total of 5 days. The amount of exogenous IL-2 administered in the current studies (5000 units twice per day for 3 days) was lower, and the duration of treatment shorter, yet the CTLs remained highly effective. If the effectiveness of CTLs is less dependent on exogenous IL-2, it may be possible to use lower doses of IL-2 in humans, with lower associated morbidity and mortality, and still achieve a therapeutic response. Additional animal studies are currently underway to evaluate the efficacy of CTLs in vivo without the administration of exogenous IL-2.

CTLs were clearly demonstrated to be most efficacious in the treatment of micrometastatic (day 3) or early metastatic (day 7) disease. Treatment of more advanced disease (day 10) resulted in a 33% response rate only, and treatment of advanced metastatic disease (day 14) was ineffective. This response parallels that seen in other murine models of immunotherapy. Tumor-specific CTLs have several advantages in this regard. We have demonstrated that CTLs can be generated from readily available precursors: lymph node cells or peripheral blood lymphocytes and autologous tumor or cultured, HLA-matched allogeneic melanomas. Using this method, CTLs can be generated for patients with clinical stage I disease, or for patients with stage II disease following lymphadenectomy. This is in sharp contrast to TILs that are isolated from metastatic deposits of tumor (17) and, therefore, can be obtained only from patients with stage III disease. TILs are only available from patients with advanced disease, where adoptive immunotherapy has been demonstrated to be least efficacious. The ability to generate CTLs for patients with stage I or stage II disease allows the CTLs to be used in patients with micrometastatic disease, where the efficacy of the CTLs has been demonstrated to be the highest. There are a significant number of patients who are clinically disease-free, but who have a high likelihood of harboring micrometastatic disease and sustaining a relapse. This would include, for example, patients with thick primary lesions (>4.0 mm) or patients with multiple positive nodes. This is a group of patients with high potential benefit from adoptive immunotherapy.

Murine studies with TILs suggest that approximately 10^10 effectors would be required for a therapeutic response in humans (17). In human trials, the number of TILs infused has ranged from 8 x 10^9 to 7.5 x 10^10 (11, 18, 19). Using the methods described previously, one could generate comparable numbers of specific CTLs. A single leukapheresis would yield 10^10 lymphocytes, and with a 10- to 100-fold increase in culture, all patients should have adequate numbers of effectors for immunotherapy trials. Because we can generate CTLs for patients with clinical stage I or stage II disease, these CTLs can also be administered in an adjuvant setting to patients with high risk of recurrent disease. This would allow CTLs to be used in vivo for the treatment of humans with micrometastatic disease, where they have been shown to be most efficacious. The above studies lay the foundation for clinical trials of CTLs in the adoptive immunotherapy of patients with metastatic melanoma.

**REFERENCES**

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