In Vivo Effect and Parallel in Vitro Lymphocyte-mediated Tumor Cytolysis after Phase I Xenogeneic Immune RNA Treatment of Patients with Widespread Melanoma or Metastatic Renal Cell Carcinoma

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ABSTRACT

Seven patients with metastatic renal cell carcinoma or widespread melanoma were included in a Phase I clinical trial of xenogeneic immune RNA (I-RNA). I-RNA, extracted from the lymphoid tissues of guinea pigs immunized with the patient's primary tumor, was incubated in vitro with autologous lymphocytes harvested by leukapheresis. Each patient received five separate i.v. injections (every other day) of 3 to 5 x 10^8 autologous lymphocytes immediately after in vitro incubation with I-RNA. Peripheral blood lymphocytes (PBL) were frozen and stored before and after each I-RNA treatment for later serial assay of lymphocyte-mediated tumor cytolysis (LMC). No toxicity was noted during or after I-RNA treatment. One patient with renal cell carcinoma had complete resolution of multiple pulmonary metastases, beginning 3 months and lasting 18 months after I-RNA treatment. Two other patients with visceral metastases from renal cell carcinoma demonstrated >50% regression, two patients showed stabilization of previously growing renal cell carcinoma pulmonary metastases, and one renal cell carcinoma patient and the single patient with widespread recurrent melanoma had no alteration in their rapidly progressive tumor courses. All serial PBL samples from individual patients were tested simultaneously for in vitro LMC against allogeneic renal cell carcinoma and melanoma targets. LMC was boosted in PBL samples after in vitro I-RNA treatment. Progressive increase in LMC was demonstrated in serial PBL samples harvested from patients during I-RNA therapy. Increased LMC was found in PBL samples harvested 3 to 9 months after I-RNA therapy. Statistically significant boosts in the cytolytic effect of PBL samples harvested from treated renal cell carcinoma patients were restricted to renal cell carcinoma targets. Similarly, only PBL samples harvested during I-RNA treatment of the patient with melanoma showed increased LMC on the allogeneic melanoma targets. In vitro LMC activity was clearly boosted in all treated patients regardless of their clinical course after I-RNA therapy. The relationship between the present Phase I human results and previous animal studies of adjuvant I-RNA therapy is discussed. Application of xenogeneic I-RNA to a Phase III human trial is proposed.

INTRODUCTION

Despite considerable controversy concerning the transfer of specific immunological information by xenogeneic I-RNA (5), this laboratory has recently reported the prevention of pulmonary metastases by tumor-specific xenogeneic I-RNA used in an adjuvant setting after excision of B16 melanoma isografts in C57BL/6J mice (11). The in vivo effect of splenocytes harvested from I-RNA-treated animals was demonstrated by serial in vitro tumor cytolysis assays (13), and the increased antigenicity and immunogenicity of B16 cells predestined to metastasize from the heterogeneous B16 isograft was proposed as one possible explanation for successful I-RNA therapy in an adjuvant setting despite a lack of clear-cut therapeutic effect against established tumor isografts (8). On the basis of these animal studies, we have suggested the application of xenogeneic I-RNA to human tumor therapy trials.

Although direct i.v. injection of xenogeneic I-RNA has been reported in the treatment of patients with various cancers (4, 6, 7), all previous human protocols have differed from our therapy model, in which potential effector cells have been incubated with tumor-specific xenogeneic I-RNA in vitro and then infused i.v. into patients. Therefore, before application to any randomized prospective human trial, we have determined in this preliminary study the clinical toxicity and in vitro effects on LMC during and after xenogeneic I-RNA treatment of patients with widespread or metastatic cancers. The results of this Phase I human I-RNA trial are presented.

MATERIALS AND METHODS

Patients. Patients with widespread or metastatic cancers, for whom no effective therapy is known, were included in this Phase I trial. Six such patients with metastatic renal cell carcinoma and a single patient with widespread recurrent melanoma have been studied. The extent of their disease before treatment and their clinical course during and after I-RNA therapy are summarized in Table 1.

Protocol Design. For the human I-RNA therapy plan, we adhered as closely as possible to our previously successful animal treatment protocol (12, 13). After excision of primary or recurrent tumor, each patient's tumor tissue was used for immunization of guinea pigs as described below. After recovery...
from surgery, patients had Scribner arteriovenous shunts placed in their left forearms. These shunts were used for serial leukapheresis to obtain autologous lymphocytes for in vitro I-RNA incubation and to reinftire the treated autologous cells. Each patient underwent 5 treatments (every other day), and arteriovenous shunts were removed after the last autologous lymphocyte infusion. Aliquots of the patient's PBL were frozen and stored at −70°C in Waymouth's medium plus 10% dimethyl sulfoxide immediately before and after each in vitro I-RNA treatment. Serial PBL specimens from each patient were simultaneously tested for evidence of change in LMC effect. A scheme of the Phase I treatment protocol is outlined in Chart 1.

Preparation of I-RNA. The details of I-RNA preparation have been described elsewhere (14). Briefly, Hartley guinea pigs were immunized by footpad injection of a mixture of fresh tumor cells from the patient's primary tumor and complete Freund's adjuvant. Two weeks later, spleens and lymph nodes of immunized guinea pigs were harvested, and RNA was extracted from these tissues by the hot phenol method. RNA was stored in ethanol at −20°C until use. Only nondegraded RNA, as determined by ultracentrifugation on sucrose density gradients, was used (10). After the complete removal of ethanol, RNA was dissolved in RPMI Medium 1640 and added to a lymphocyte suspension prepared from the patient's PBL obtained by leukapheresis (Haemonetics M30 Pheresis Machine; Haemonetics, Inc., Braintree, Mass.), and further purified by Ficoll-Hypaque gradient centrifugation (1). The concentration of I-RNA added was 750 to 1000 μg for every 5 × 10⁹ PBL in 1 ml of RPMI Medium 1640 and added to a lymphocyte suspension prepared from the patient's PBL obtained by leukapheresis (Haemonetics M30 Pheresis Machine; Haemonetics, Inc., Braintree, Mass.), and further purified by Ficoll-Hypaque gradient centrifugation (1). The concentration of I-RNA added was 750 to 1000 μg for every 5 × 10⁹ PBL in 1 ml of RPMI Medium 1640. After incubation at 37°C in a 10% CO₂:90% air atmosphere for 60 min, PBL were collected by centrifugation and concentrated to 3 to 5 × 10⁹ cells/50 ml of phosphate-buffered saline (1.34 g K₂HPO₄, 0.32 g KH₂PO₄, 8.5 g NaCl, 1 H₂O). Aliquots of 1 × 10⁷ PBL before and after each in vitro I-RNA incubation were frozen and stored at −70°C for subsequent in vitro LMC assays.

Tumor Targets for in Vitro LMC Assays. Reference allogeneic human melanoma and renal cell carcinomas were used as tumor targets for in vitro assays of LMC. Two human renal cell carcinoma lines were kindly supplied by Dr. J. deKernion (University of California, Los Angeles, Calif.). Both of these cultures (Pastor and A498) were explanted from primary human renal cell carcinomas and shown to maintain their histology as heterotransplants in nude mice. S85A and H130M were explants of pulmonary and liver metastases, respectively, from patients with metastatic melanoma. Both of these reference melanoma lines produce melanin and have been shown to have features characteristic of melanoma on electron microscopy. Early generations of each human tumor line were frozen and stored at −70°C in Waymouth's medium plus 10% dimethyl sulfoxide and were periodically defrosted at 2- to 3-week intervals for use as tumor targets in LMC assays. Monolayer cultures of these cells were maintained in Waymouth's medium containing 10 to 20% fetal calf serum.

In Vitro Assays of LMC. Effector cells tested for in vitro LMC consisted of PBL harvested from patient by serial leukapheresis, aliquoted, and frozen to −70°C before and after each in vitro I-RNA incubation. PBL were purified after leukapheresis by Ficoll-Hypaque gradient centrifugation before I-RNA incubation (1). Purified PBL samples consisted of >90 to 95% viable monocytes. All assays on a given patient's serial PBL samples before and after each I-RNA treatment were performed in parallel. Several patients had PBL samples harvested 3 to 9 months after I-RNA treatment. When possible, PBL sets from patients with renal cell carcinoma were tested in parallel with the serial PBL set harvested from the patient with metastatic melanoma. These parallel tests were performed in a criss-cross fashion, using both the reference allogeneic melanoma and renal cell carcinoma tumor targets.

The in vitro assay used to demonstrate LMC is a modification of the "long-term" ⁵¹Cr technique described in detail earlier (9). Tumor targets (1 × 10⁶/0.2 ml Waymouth's medium, containing 10% heat-inactivated fetal calf serum) were incubated in flat-bottom sterile 2-ml glass ampuls (Flow Laboratories, Inc., Rockville, Md.) for 2 to 4 hr at 37°C in a 5% CO₂:95% air atmosphere. After target cell attachment, medium was aspirated and replaced with 0.2-ml volumes of sodium chromate (⁵¹Cr: specific activity, 56 mCi/mg). Target cells were labeled in a concentration of 0.2 mCi/ml medium. After 2 hr of incubation, ⁵¹Cr solution was aspirated, and each ampul was gently washed 5 times with 0.7-ml volumes of Waymouth's medium containing 5% inactivated fetal calf serum. Human PBL were added in 0.2-ml volumes of Waymouth's medium at effector cell:target cell ratios of 100:1, 30:1, and 10:1. After 1 hr, 1.8 ml of Waymouth's medium containing 5% inactivated fetal calf serum were added to each ampul. After 20 to 24 hr of incubation at 37°C in a 5% CO₂:95% air atmosphere, each ampul was measured for total radioactivity in a γ-counter. Ampuls were then centrifuged at 600 × g for 45 min, and 0.2 ml of supernatant was removed for measurement of released radio-

activity. Cytolytic effect was calculated as follows:

\[
\text{% of cytolysis} = \frac{\text{Radioactivity released/0.2 ml} \times 10}{\text{Total radioactivity} \times 100}
\]

Each PBL population was tested in triplicate at the 3 effector cell:target cell ratios, and mean percentage of cytosis ± S.E. was calculated.

For several patients' PBL samples, LMC was paralleled by in vitro assay of reference allogeneic tumor-target adherence. The assay used is a modification of the technique described by Cohen et al. (2). Reference tumor target cells (10^5) were labeled with 0.25 μCi of [125I]iododeoxyuridine (specific activity, 2000 Ci/mmol; New England Nuclear, Boston, Mass.) in each well of a Falcon Microtest II culture plate at 37° in a 10% CO2: 90% water-saturated air atmosphere for 24 hr. Excess [125I]iododeoxyuridine was then aspirated, and each cell was washed once with RPMI Medium 1640. PBL were suspended in complete RPMI Medium 1640 containing 10% heat-inactivated fetal calf serum, 2 mm L-glutamine, 100 units of penicillin per ml, and 100 μg streptomycin per ml and added to the labeled reference tumor target cells at lymphocyte:target cell ratios of 250:1 and 125:1. After 48 hr of incubation, splenocytes and detached target cells were removed by washing the wells with RPMI Medium 1640. Remaining adherent tumor target cells were fixed with a thin film of plastic spray, and individual wells were cut out with a band saw. Residual radioactivity, reflecting remaining tumor cells in each well, was counted in a Packard Model 5110 γ counter. The mean cpm were obtained from 8 wells. Cytotoxicity index (CI) was calculated as follows:

\[
\text{CI} = \frac{\text{cpm of tumor cells exposed to PBL after I-RNA treatment} - \text{cpm of tumor cells exposed to PBL before I-RNA treatment}}{\text{cpm of tumor cells exposed to PBL before I-RNA treatment}} \times 100
\]

Data were analyzed for statistical significance by Student's t test.

RESULTS

In Vivo Effect of I-RNA Therapy. No toxicity was identified during or after xenogeneic I-RNA therapy in any of the patients studied. Six of 7 patients completed the full course of Phase I therapy. The one patient (Patient 6) excluded after a single I-RNA treatment experienced progressive aphasia from known renal cell carcinoma brain metastases. The last 3 patients in this trial were leukapheresed and reinfused with autologous I-RNA-treated lymphocytes as outpatients.

The clinical course of each patient is summarized in Table 1. The first patient showed a 50% decrease in multiple pulmonary metastases at 3 months after I-RNA therapy with complete resolution of all pulmonary metastases by 6 months. She remained without evidence of disease until 18 months after therapy. Two other patients with metastatic renal cell carcinoma demonstrated more than a 50% decrease in tumor metastasis size after therapy. An additional 2 patients showed only transient stabilization in the growth of their renal cell carcinoma pulmonary metastases after I-RNA therapy. The one patient with brain metastases from renal cell carcinoma and the single patient with widespread recurrent melanoma showed no discernible change in tumor growth during or after I-RNA treatment. Since this Phase I clinical protocol was purposely nonrandomized, no direct relationship between I-RNA therapy and subsequent clinical course in any of these patients can be established.

Serial in Vitro LMC. Regardless of their varied clinical courses after I-RNA treatment, all patients showed progressive boosting in their LMC effect on reference allogeneic tumor targets. Increased in vitro LMC could be demonstrated in...
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After 5th I-RNA released after exposure to PBL harvested before the first I-RNA treatment.

% of $^{51}$Cr released after exposure to l-RNA-treated PBL minus mean percentage of $^{51}$Cr released after exposure to PBL harvested before the first I-RNA treatment.

Table 2

<table>
<thead>
<tr>
<th>Time of PBL harvest</th>
<th>Effector: target ratio</th>
<th>% of $^{51}$Cr released on renal cancer targets (Pastor)</th>
<th>Cytotoxic effecta</th>
<th>% of $^{51}$Cr released on melanoma targets (SS5A)</th>
<th>Cytotoxic effecta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before 1st I-RNA</td>
<td>100:1</td>
<td>37 ± 1b</td>
<td>50 ± 2</td>
<td>50 ± 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>39 ± 1c</td>
<td>50 ± 3</td>
<td>50 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>47 ± 2</td>
<td>50 ± 2</td>
<td>50 ± 2</td>
<td></td>
</tr>
<tr>
<td>After 1st I-RNA</td>
<td>100:1</td>
<td>53 ± 1c</td>
<td>47 ± 4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>57 ± 3d</td>
<td>50 ± 2</td>
<td>50 ± 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>61 ± 14c</td>
<td>50 ± 1</td>
<td>0</td>
<td></td>
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<tr>
<td>Before 2nd I-RNA</td>
<td>100:1</td>
<td>54 ± 4c</td>
<td>49 ± 1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>52 ± 2c</td>
<td>47 ± 3</td>
<td>3e</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>54 ± 2c</td>
<td>53 ± 8</td>
<td>3a</td>
<td></td>
</tr>
<tr>
<td>After 2nd I-RNA</td>
<td>100:1</td>
<td>52 ± 3c</td>
<td>43 ± 4</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>30:1</td>
<td>62 ± 5c</td>
<td>49 ± 1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10:1</td>
<td>62 ± 6c</td>
<td>55 ± 4</td>
<td>5a</td>
<td></td>
</tr>
</tbody>
</table>

a Cytotoxic effect is calculated as the difference between mean percentage of $^{51}$Cr released after exposure to I-RNA-treated PBL minus mean percentage of $^{51}$Cr released after exposure to PBL harvested before the first I-RNA treatment.
b Mean ± S.E.
c Student's t test, p < 0.001.
d Student's t test, p < 0.01.
e Student's t test, not significant.
f Student's t test, p < 0.05.

Table 3

Cytotoxicity of lymphocytes harvested from Patient 5 (renal cell carcinoma) before and after I-RNA

$[^{125}]$Iododeoxyuridine assay was performed using PBL obtained before and after the fifth I-RNA treatment.

<table>
<thead>
<tr>
<th>PBL harvested</th>
<th>Effector: target ratio</th>
<th>Cytotoxicity index</th>
<th>Cytotoxicity index</th>
<th>Cytotoxicity index</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBL harvested</td>
<td>Effector: target ratio</td>
<td>Cpm on remaining renal cancer targets</td>
<td>Cpm on remaining melanoma targets</td>
<td>Cpm on remaining melanoma targets</td>
</tr>
<tr>
<td>Before 5th I-RNA</td>
<td>250:1</td>
<td>914.7 ± 93.9d</td>
<td>122 ± 14.2</td>
<td>122 ± 14.2</td>
</tr>
<tr>
<td>After 5th I-RNA</td>
<td>250:1</td>
<td>686.5 ± 286d</td>
<td>25d</td>
<td>25d</td>
</tr>
<tr>
<td>Before 5th I-RNA</td>
<td>125:1</td>
<td>1690 ± 119.9d</td>
<td>136 ± 10.5</td>
<td>136 ± 10.5</td>
</tr>
<tr>
<td>After 5th I-RNA</td>
<td>125:1</td>
<td>993 ± 330d</td>
<td>132 ± 25.0</td>
<td>132 ± 25.0</td>
</tr>
</tbody>
</table>

a Cytotoxicity index $[^{125}]$Iododeoxyuridine with PBL before 5th I-RNA - $[^{125}]$Iododeoxyuridine with PBL after 5th I-RNA $x$ 100
b Mean ± S.E.
c Student's t test, p < 0.001.
d Student's t test, p < 0.05.
e Student's t test, not significant.
f Student's t test, p < 0.05.

d NT, not tested.

g Student's t test, not significant.


individual PBL samples after in vitro I-RNA incubation. LMC remained elevated in serial PBL samples obtained before subsequent I-RNA treatment (Table 2). In selected patients, tumor-target adherence decreased after incubation with RNA-treated PBL, compared to PBL before RNA exposure (Table 3). Changes in LMC after I-RNA therapy were restricted to "tissuespecific" allogeneic reference tumor targets. PBL from renal cell carcinoma patients demonstrated increased cytolysis of renal cell carcinoma targets but not melanoma targets (Table 2). Similarly, PBL from the patient with metastatic melanoma demonstrated a boost in LMC during I-RNA treatment only on reference allogeneic melanoma targets (Table 4).

A progressive in vivo effect on LMC was demonstrated by simultaneous in vitro assay of serial PBL samples harvested and stored prior to each in vitro I-RNA exposure (Chart 2). In several patients, the increase in LMC could be demonstrated...
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in PBL samples harvested as long as 9 months after I-RNA treatment.

No correlation between in vivo tumor course and in vitro LMC effect was apparent. Progressive boosting in serial LMC against tissue-type-specific allogeneic tumor targets during and after I-RNA treatment was evident in patients who had clinical evidence of tumor remission (Chart 3) and in patients who had no change in their rapidly progressive tumor course (Chart 4).

DISCUSSION

These results demonstrated that a xenogeneic I-RNA therapy protocol effective in an animal tumor model can be applied to humans without toxicity. Placement of arteriovenous shunts for serial leukaphereses and reinfusion of I-RNA-incubated autologous lymphocytes allowed the last few patients in this study to be treated without continuous hospitalization. Although no formal relationship can be established between therapy and subsequent clinical course in this nonrandomized Phase I trial, we believe that the present results show no evidence of tumor enhancement by I-RNA therapy. Despite the far-advanced tumors in our patients, their clinical courses after I-RNA treatment were at least as promising as results reported in earlier nonrandomized human trials, using xenogeneic I-RNA injected i.v., despite probable deactivation by endogenous RNases (4, 6, 7). Our previous animal studies and this human trial suggest that in vitro I-RNA exposure of autologous lymphocytes and reinfusion of treated lymphocytes is a more effective method of influencing host antitumor immune response and achieving therapeutic benefit (11, 13).

In contrast to our previous animal model data (13), we have found no correlation between clinical courses in the treated patients and serial in vitro LMC effects. However, the I-RNA treatment of C57BL/6J mice after B16 isograft excision was designed as an adjuvant immunotherapy model. In such a minimal residual disease setting, the effects on host LMC demonstrated by in vitro assay might have had a greater in vivo influence on tumor course. By contrast, all of the patients treated in this Phase I trial were chosen for their far-advanced disease state. Despite uniform success in manipulating a single immune parameter (in vitro LMC) in such patients with large tumor volumes, the likelihood of altering overall tumor course in vivo might be much less. This is consistent with previous reports demonstrating no clear-cut therapeutic benefit by I-RNA treatment of established tumor isografts despite evidence of increased in vitro LMC in treated animals (3).

Our Phase I human study of xenogeneic I-RNA treatment has been performed as a preliminary trial of toxicity and as an attempt to demonstrate effects on host LMC. We believe that both the in vivo results and the in vitro immune correlates justify the application of this form of immunotherapy to a randomized prospective human trial. Ideally, patients chosen for such a Phase III study should be treated in a minimal residual disease setting, when any long-lasting therapeutic benefit of xenogeneic I-RNA would be most probable.

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REFERENCES

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