Clonal Analysis of Lymphocytes from Tumor, Peripheral Blood, and Nontumorous Kidney in Primary Renal Cell Carcinoma

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ABSTRACT

The immunological properties of lymphocytes from tumor, peripheral blood (PBL), and nontumorous kidney from 16 patients with renal cell carcinoma were characterized at the clonal level with respect to their clonogenic efficiency, phenotypic expression, and cytotoxicity against autologous and allogeneic tumor cells. The objectives were to delineate: (a) the quantitative differences in the immunological properties of tumor-infiltrating lymphocytes (TIL) from patient to patient; and (b) the qualitative differences in immunological properties between TIL and lymphocytes from peripheral blood or nontumorous kidney from a single patient.

A total of 926 clones were characterized for phenotype expression, and 465 clones were characterized for cytotoxicity. The clonogenic efficiency of TIL varied with individuals: high in one patient; relatively high to moderate in seven patients; low in seven patients, and extremely low in the remaining one patient. The levels of autologous tumor cell lysis by TIL clones also varied with individuals. More than one-third of the TIL clones established in 4 of 13 patients displayed significant (>10%) lysis against autologous tumor cells, and in each of the four patients the average percentage of lysis in the total TIL clones was higher than 10%.

In two patients, 5 of 26 or 3 of 13 TIL clones were cytotoxic, but averages of percentage of lysis in the total clones were <10%. One or 2 TIL clones of 10-27 total clones were cytotoxic in each of 4 patients, while no cytotoxic TIL clones were found in the remaining 3 patients. Clonogenic efficiency did not correlate with the level of cytotoxicity, and TIL from no tumors displayed both high proliferation and high cytotoxicity at the clonal level. In a majority of patients (12 of 13), most cytotoxic TIL clones against autologous tumor cells also lysed allogeneic tumor cells. In contrast, TIL clones lysed only autologous tumor cells in the remaining one patient (patient 2). The clonogenic efficiency of TIL was lower than that of PBL in 6 of 12 patients, while the opposite was true in the remaining 6 patients. The level of cytotoxicity in the PBL clones of these 12 patients primarily correlated with that of the TIL clones. With one exception (patient 2), most cytotoxic PBL clones against autologous tumor cells also lysed allogeneic targets in a majority of patients. CD4+CD8+ T-cell clones (70-85%) predominated in all patients regardless of the different lymphocyte sources. There was a low but substantial proportion of CD4+CD8- T-cell clones (15-25%), and the percentages of CD4-CD8+ clones were very low (<10%). Phenotypic expression of lymphocyte clones, including CD3, CD4, CD8, CD56 (Leu-19), or Leu-7 antigen, did not correlate with their cytotoxic activity. These results suggest that there are: (a) quantitative differences in the immunological properties of the TIL studied here among renal cell carcinoma patients; but (b) no fundamental qualitative differences in immunological properties between TIL and PBL or lymphocytes from nontumorous kidney at the T-cell level with respect to clonogenic efficiency, phenotypic expression, and cytotoxicity.

INTRODUCTION

Over the past several years, biological therapies for the treatment of malignancy, utilizing IL-24 with or without LAK cells, have been introduced (1-4). Various investigators using a variety of doses and schedules of IL-2 have obtained reproducible objective responses in patients with metastatic melanoma and RCC. However, even for these two responding cancers, only a minority of patients (20%) have responded to the biotherapy, suggesting possible different patterns of host-tumor interactions among individual patients. To date, no clinical or laboratory parameters have been established for understanding the difference in host-tumor interactions between responders and nonresponders. Infusion of LAK cells has little effect on the response rates of RCC or melanoma, suggesting that ex-vivo-activated LAK cells offer little increased antitumor activity over that observed with IL-2 alone (1-4). Furthermore, in an extensive and detailed correlative study performed by the National Cancer Institute extramural IL-2/LAK working group, no correlations were found between tumor reduction or clinical toxicity and nine separate laboratory parameters on PBL (4). This absence of correlations suggests that PBL are not directly involved in biotherapy-induced antitumor immunity.

It is more likely that lymphocytes present within tumors (TIL) are involved in the mechanisms of biotherapy-induced tumor regression. In the initial National Cancer Institute trials infusing activated TIL with IL-2, more than one-half of the metastatic melanoma patients responded (5). IL-2-activated melanoma TIL displayed autologous tumor-specific cytotoxicity, while IL-2-activated PBL showed MHC-nonrestricted cytotoxicity, as reported by us (6, 7) and others (8-10). These results suggest that the immunological properties of melanoma TIL are qualitatively distinct from those of PBL, and melanoma TIL play an important role in tumor regression. In contrast to melanoma TIL, RCC-TIL consisted of CD3+CD16+ T-cells and CD3+CD16+ NK cells (6, 11). Both T- and NK cells from TIL and PBL displayed MHC-nonrestricted cytotoxicity following in vitro activation with IL-2 (6, 11-18), demonstrating no fundamental qualitative differences between TIL and PBL in RCC patients at the bulk culture level.

In this study, we investigated: (a) whether the immunological properties of RCC-TIL differ among individual patients; and (b) whether they are different from those of PBL or lymphocytes found in nontumorous kidney. We used LDA with lymphocytes from the tumor, peripheral blood, and nontumorous kidney of each patient to establish lymphocyte clones and studied their

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4 The abbreviations used are: TIL, tumor-infiltrating lymphocytes; PBL, peripheral blood lymphocytes; LAK, lymphokine-activated killer; LDA, limiting dilution analysis; RCC, renal cell carcinoma; IL-2, interleukin 2; MHC, major histocompatibility complex; NK, natural killer; rIL-2, recombinant human IL-2; mAh, monoclonal antibodies; FCS, fetal calf serum; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; FITC, fluorescein isothiocyanate.
immunological properties at the clonal level for the following reasons: (a) significant differences in immunological properties between RCC-TIL and PBL from the same patients were not observed at the bulk culture level with 100–1000 units/ml rIFN-2 (6, 11–18); (b) bulk culture of mixed populations of lymphocytes is not always a better method for identifying possible differences in immunological properties among different lymphocyte sources; (c) it is possible that bulk culture preferentially activates certain types of lymphocytes, resulting in disappearance of another type of lymphocyte; (d) differences at the clonal level, if any, might be more discernible than those at the bulk culture level. Three parameters (clonogenic efficiency, phenotypic expression, and cytotoxicity) were chosen to measure the immunological properties of lymphocyte clones because proliferation and cytotoxicity were important for the in vitro assessment of antitumor response, and phenotypic analysis was necessary for understanding the T-cell subsets responsible for these functions. We found that anti-autologous tumor response mediated by TIL clones, as measured by clonogenic efficiency and cytotoxicity against autologous tumor cells, varied widely among individual tumors. There were no significant qualitative differences in the immunological properties between TIL and PBL or lymphocytes from nontumorous kidney with respect to clonogenic efficiency, phenotypic expression, and cytotoxicity.

MATERIALS AND METHODS

Cell Preparation. Single-cell suspensions of lymphocytes and tumor cells were prepared from surgical specimens of 16 primary RCC patients using collagenase and DNase, as reported previously (6, 7). Nontumorous tissues surrounding the tumor and the central necrotic area of the tumor were both carefully excised before cell preparation. The tumor samples were washed with Hanks' balanced salt solution before digestion to minimize possible PBL contamination in the TIL preparations. Pathological examination of the tumor specimens confirmed the diagnosis of malignancy. No patients had received prior chemotherapy, radiotherapy, or biotherapy. Concurrently, single-cell suspensions were prepared from nontumorous areas of the resected kidney of each patient using collagenase and DNase. These cell suspensions and heparinized peripheral blood obtained from each patient at the time of surgery were prepared from nontumorous areas of the resected kidney of each patient using collagenase and DNase. These cell suspensions and heparinized peripheral blood obtained from each patient at the time of surgery were used to confirm the identities of lymphocytes in certain experiments. Substantial numbers of TIL were recovered using collagenase and DNase, as reported previously (6, 7). Nontumorous kidney samples were washed with Hanks' balanced salt solution before digestion to minimize possible PBL contamination in the TIL preparations. Pathological examination of the tumor specimens confirmed the diagnosis of malignancy. No patients had received prior chemotherapy, radiotherapy, or biotherapy. Concurrently, single-cell suspensions were prepared from nontumorous areas of the resected kidney of each patient using collagenase and DNase. These cell suspensions and heparinized peripheral blood obtained from each patient at the time of surgery were applied to a Ficoll-Hypaque cushion to obtain viable lymphocytes and tumor cells (6). Cell viability exceeded 80%. Lymphoid cells were discriminated from tumor cells or macrophages by cell size and morphology for human lymphocytes (anti-CD3, -CD4, -CD8 mAb) (Becton Dickinson Co., Mountain View, CA) were used to confirm the identities of lymphocytes in certain experiments before use as effector cells. Substantial numbers of TIL were recovered using these methods in all cases investigated. The majority of cells were cryopreserved at −170°C in 90% FCS and 10% dimethyl sulfoxide at 5 x 10⁶ cells/vial. Cryopreserved cells from the majority of patients or freshly prepared cells from patients 1, 2, 3, and 10 were used for LDA. There was no significant difference in the results obtained here between the frozen samples and the fresh samples. LDA, LDA of TIL, PBL, and lymphocytes from the nontumorous kidney from each patient was carried out at the same time under the same conditions as reported previously (6). This method to establish lymphocyte clones from tumor tissues was modified from methods originally described by Moretta et al. (19, 20). Briefly, different numbers (8, 4, 2, 1, and 0.5 cells/well) of fresh TIL, PBL, or lymphocytes from nontumorous kidney were incubated in 96-well U-bottomed microplates in RPMI (GIBCO, Grand Island, NY) supplemented with 10% FCS, 200 units/ml rIFN-2, kindly provided by Hoffman-LaRoche Inc. (Nutley, NJ), 10 μg/ml phytohemagglutinin (Sigma Chemical Co., St. Louis, MO) in the presence of irradiated (7000 rads) PBMC from two different healthy donors (2 x 10⁶ cells/PBMC/0.2 ml/well). Seven and 14 days later, one-half of the medium was replaced with RPMI supplemented with 10% FCS and 200 units/ml IL-2 and 2 x 10⁶ irradiated PBMC (per well) from two different healthy donors. From 3 to 4 weeks after the initial incubation, each well was scored macroscopically as well as microscopically for growth. The scores of the proliferating microcultures were used to estimate the clonogenic efficiency of lymphocytes. The same lot of FCS was used throughout this series of experiments.

Immunofluorescence Techniques. Double-color analysis of surface antigens on freshly isolated cells or cloned cells were performed with PE-conjugated anti-CD3 mAb and FITC-conjugated anti-CD16 mAb, or with PE-anti-CD4 and FITC-anti-CD20 mAb, as reported previously (6). FITC-anti-Leu-7 mAb and PE-anti-Leu-19 (CD56) mAb were also used for the analysis.

Analysis of Functions. Twenty-five to 35 proliferating microcultures per specimen (tumor, PBL, or nontumorous kidney) were transferred from 96-well plates into 24-well microculture plates in RPMI with 10% FCS and 200 units/ml IL-2 in the presence of 0.2–1 x 10⁷ irradiated (7000 rads) single-cell suspensions of autologous tumors (frozen uncultured cells containing both tumor cells and TIL). Proliferating microcultures were preferentially harvested from the 96-well plates that contained the fewest numbers of plated lymphocytes. One-half the volume of each well was replenished with fresh medium containing 200 units/ml rIL-2 every 3–5 days, and the cell density was maintained at <1–2 x 10⁶ cells/ml during the entire culture period. These cells were further expanded for their surface antigens (CD3, CD4, CD8, and CD16), and only cells that showed uniform phenotypes were used as potential clonally pure lymphocytes. The total number of proliferating microcultures analyzed was 926. Using these parameters, 862 of which (93%) contained lymphocytes with uniform surface markers such as CD3⁺CD4⁺CD8⁻CD16⁺ or CD3⁺CD4⁺CD8⁺CD16⁺. These results assured a high probability of clonality for lymphocytes used in this study.

Assay for Autologous and Allogenic Tumor Cell Lysis. A 4-h ⁵¹Cr release assay was used to detect cytotoxic activity (6). Lymphocyte clones were chosen for cytotoxicity based upon their uniform antigen expression and sufficient numbers of both effector T-cells and autologous cell targets. Cryopreserved, uncultured tumor cells were used as autologous targets. NK-resistant MEL-21 tumor cells (an established melanoma tumor cell line) were used as autologous tumor target cells (12, 13). Spontaneous release usually did not exceed 30% of maximum release. The percentage of specific lysis from triplicate determinations at a 10:1 effector:target ratio was calculated as described (6). The means ± SD of cpm showing more than 5% lysis in these assays were significant (P < 0.05 by Student's two-tailed t test) as compared to those of spontaneous release and >3 SD above the spontaneous release. Lymphocyte clones showing ≥10% lysis were determined as cytotoxic clones in these experiments.

RESULTS

Clonogenic Efficiency, Cytotoxicity, and Phenotypic Expression of TIL Clones in Individual Tumors. The numbers of proliferating wells per plate in 16 different RCCs, 12 different PBMC, and 11 different nontumorous kidneys were scored. The mean numbers from two plates are shown in Table 1. There was considerable diversity in the clonogenic efficiency of TIL among the 16 patients: very high in one patient (patient 10); relatively high in two patients (patients 15 and 16); moderate in five patients (patients 5, 6, 7, 9, and 14); low in seven patients (patients 1, 3, 4, 8, 11, 12, and 13); and extremely low in the remaining one patient (patient 2). Two hundred eight of 386 TIL clones were suitable for the cytotoxicity assay because of their uniform surface antigen expression and sufficient numbers of both TIL clones (as effectors) and tumor cells (as target cells). These 208 TIL clones from 13 patients were investigated for cytotoxicity against autologous RCC tumor cells and allogenic MEL-21 melanoma targets at an effector:target cell ratio of 10:1. For comparison of the cytotoxicity levels in TIL clones among the patients, an average of percentage of lysis in total TIL clones from each patient was calculated (Fig. 1). Further, the numbers of cytotoxic TIL clones (>10% lysis against autologous tumor cells) among total clones were also demon-
CLONAL ANALYSIS OF LYMPHOCYTES IN RENAL CELL CARCINOMA

Table 1 Clonogenic efficiency of lymphocytes from tumor, peripheral blood, and nontumorous kidney*

<table>
<thead>
<tr>
<th>Patient</th>
<th>TIL</th>
<th>PBMC</th>
<th>Lympocytes from nontumorous kidney</th>
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<tbody>
<tr>
<td></td>
<td>8</td>
<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1 (RC5)</td>
<td>9</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>2 (RC6)</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>3 (RC7)</td>
<td>14</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>4 (RC8)</td>
<td>13</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>5 (RC10)</td>
<td>25</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>6 (RC11)</td>
<td>32</td>
<td>23</td>
<td>11</td>
</tr>
<tr>
<td>7 (RC9)</td>
<td>20</td>
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<tr>
<td>8 (RC19)</td>
<td>11</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>9 (RC27)</td>
<td>33</td>
<td>18</td>
<td>8</td>
</tr>
<tr>
<td>10 (RC-Tr1)</td>
<td>84</td>
<td>67</td>
<td>63</td>
</tr>
<tr>
<td>11 (RC26)</td>
<td>17</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>12 (RC24)</td>
<td>9</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>13 (RC3)</td>
<td>11</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>14 (RC22)</td>
<td>22</td>
<td>13</td>
<td>6</td>
</tr>
<tr>
<td>15 (RC2)</td>
<td>42</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>16 (RC4)</td>
<td>53</td>
<td>30</td>
<td>12</td>
</tr>
</tbody>
</table>

* Methods were described in "Materials and Methods." Values represent the mean numbers of proliferating wells per plate from two different plates. ND, not done.

Fig. 1. Cytotoxicity of TIL clones. TIL clones from 13 different RCCs were tested for their cytotoxicity against autologous tumor cells and allogeneic melanoma (MEL-21) tumor cells (C) in a 4-h 51Cr release assay at the triplicate determinations of an effectortarget cell ratio of 10:1. Averages of percentage of specific lysis by the total TIL clones per patient were described. The total numbers of TIL clones used, and numbers of cytotoxic TIL clones (≥10% lysis against autologous tumor cells) were described. Statistical analysis was performed between the mean (average) ± SD of the percentage of autologous tumor cells in the total TIL clones in one patient to that in another patient. The average of percentage of lysis in the total TIL clones from patients 1, 2, 4, or 13 was significantly higher than any of that from patients 3, 5–9, 11, or 12 (P < 0.01). Student's two-tailed t test. ND, not done. Numbers in parentheses, numbers of clones with ≥10% lysis.

strated (Fig. 1). In 4 of 13 patients, the averages of percentage of autologous tumor cell lysis in total TIL clones exceeded 10%. In these four patients, more than one-third of the TIL clones (6 of 13, 46% in patient 1; 5 of 7, 71% in patient 2; 4 of 11, 36% in patient 4; and 11 of 13, 85% in patient 13) were cytotoxic (≥10% lysis) against autologous tumor cells (Fig. 1). None of these four tumors displayed high or even moderate levels of clonogenic efficiency (Table 1). Five of 26 TIL clones in patient 5 and 3 of 13 clones in patient 10 were cytotoxic against autologous tumor cells, but averages of percentage of lysis in the total clones in these two patients did not exceed ≥10% lysis (Fig. 1). Clonogenic efficiency was moderate in patient 5 and very high in patient 10. Two of either 16 clones in patient 8 or 35 clones in patient 11 were cytotoxic against autologous tumor cells, and averages of percentage of lysis in the total clones were <5% in these patients (Fig. 1). Clonogenic efficiency in these two patients was low (Table 1). Only 1 of either 27 TIL clones in patient 9 or 11 clones in patient 12 was cytotoxic, and in the other 3 patients no TIL clones displayed ≥10% autologous tumor cell lysis (0 of 10 in patient 3, 0 of 17 in patient 6, and 0 of 9 in patient 7) (Fig. 1). The clonogenic efficiency among these five patients varied greatly. These results suggest that there was also considerable diversity in the cytotoxic capability of TIL clones against autologous tumor cells from patient to patient. Either the average of autologous tumor cell lysis in the total clones or the incidence of cytotoxic TIL clones did not correlate with clonogenic efficiency in all patients investigated here. There were no tumors which possessed both a high clonogenic efficiency of TIL clones and a higher average of percentage lysis or higher incidence of cytotoxic TIL clones.

Cytotoxicity against autologous and allogenic tumor cells and phenotypic expression of each TIL clone were demonstrated in five different tumors which contained ≥20% cytotoxic TIL clones (patients 1, 2, 4, 10, and 13) (Table 2). With the exception of TIL clones from patient 2, almost all cytotoxic TIL clones (≥10% lysis against autologous tumor cells) from the four patients also lysed allogenic tumor cells. Substantial numbers of noncytotoxic TIL clones (<10% lysis against autologous tumor cells) in patient 1 (8 of 13 clones), in patient 4 (3 of 11 clones), and in patient 10 (8 of 13 clones) showed >10% lysis against MEL-21 melanoma cells (Table 2). This discrepancy may be due in part to the higher susceptibility of MEL-21 targets that are resistant to NK cell-mediated lysis but are sensitive to LAK cell-mediated lysis. Alternatively, fresh isolated RCC target cells are relatively resistant to lysis by TIL clones. Although the exact mechanisms involved in this discrepancy are unclear, these results suggest that many TIL clones in these four patients potentially possess MHC-nonrestricted cytotoxicity. In contrast to these TIL clones with MHC-nonrestricted cytotoxicity from the four patients, five cytotoxic TIL clones against autologous tumor cells in patient 2 did not lyse MEL-21 tumor cells (Table 2). Thirteen of 21 clones established from PBL from patient 2 showed >10% lysis of autologous

Table 2 Cytotoxicity and phenotypic expression of TIL clones*

<table>
<thead>
<tr>
<th>Patient</th>
<th>Target cells</th>
<th>% of specific lysis by TIL clone</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>1 (RC5)</td>
<td></td>
</tr>
<tr>
<td>2 (RC6)</td>
<td>Auto-Tu</td>
<td>19° 10 21 17 6 0° 0 32° 4° 0 11° 7°</td>
</tr>
<tr>
<td>4 (RC8)</td>
<td>Auto-Tu</td>
<td>0 8° 38° 20° 0° 5° 0 0 14° 0</td>
</tr>
<tr>
<td>5 (RC-Tr1)</td>
<td>Allo-Tu</td>
<td>0 34 53 36 0 23 0 12 0 25 5</td>
</tr>
</tbody>
</table>

* Cytotoxicity against autologous tumor cells (Auto-Tu) and allogenic tumor cells (Allo-Tu) and phenotypic expression of all TIL clones were demonstrated in 5 different tumors which contained ≥20% cytotoxic TIL clones (patients 1, 2, 4, 10, and 13) (Fig. 1). Values represent mean percentages of lysis mediated by each clone in triplicate determinations against autologous and allogenic tumor cells in a 4-h 51Cr release assay. * Unlettered entries, CD3*CD4*CD8− T-cell clones. * CD3*CD4*CD8+ T-cell clones.

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tumor cells (data not shown), and 12 of these 13 cytotoxic PBL clones also did not lyse allogenic tumor cells. One of three clones from the lymphocytes of nontumorous kidney in patient 2 also lysed only autologous (13% lysis) but not MEL-21 tumor cells (0% lysis).

There was no correlation between cytotoxicity against either autologous or allogenic tumor cells and phenotype expression in the TIL clones investigated here. Partial results are shown in Table 2. Thus, either CD3+CD4+CD8- T-cell clones with a T-helper/inducer marker or CD3+CD4+CD8+ T-cell clones with a T-suppressor/cytotoxic marker lysed autologous and allogenic tumor cells. None of three CD3+CD4+CD8- T-cell clones shown herein were cytotoxic against autologous tumor cells. Also, there was no correlation between cytotoxicity and CD4 or CD8 antigen expression in PBL clones and those from nontumorous kidney (Table 3). Further, cytotoxicity against autologous or allogenic tumor cells did not correlate with CD56 (Leu-19) antigen expression at the T-cell clonal level established from TIL or the others (Table 3). A CD56 marker is recognized and accepted as a lymphocyte activation marker similar to an IL2R+ (CD25) or CD16 marker, and it is well known that CD56 expression on IL-2-activated lymphocytes correlates well with their cytotoxicity at the bulk culture level (21, 22). The majority of these T-cells, regardless of cytotoxic activity, expressed 4B4 antigens and did not express either Leu-7 antigen or 2H4 (Leu-18) antigen (data not shown). Therefore, none of the lymphocyte activation or differentiation markers in T-cell clones tested here had a correlation with cytotoxic activity.

Comparison between TIL and PBMC or Lymphocytes from Nontumorous Kidney at the Clonal Level. We compared the clonogenic efficiency (Table 1) and the cytotoxic activity (Fig. 2) in TIL clones to those of PBL or lymphocytes from nontumorous kidney in 12 patients. The phenotypic expressions of freshly prepared lymphocytes from tumor, peripheral blood, or nontumorous kidney used here as sources of lymphocytes for LDA were not largely different from each other. They consisted of 65–75% CD3+ T-cells, 35–50% CD4+ T-cells, 20–25% CD8+ T-cells and 5–10% CD3-CD16+ NK cells. For 6 of 12 patients, clonogenic efficiency of TIL was lower than that of PBL (type I; patients 1, 2, 4, 5, 11, and 12), while the opposite was true for the remaining 6 patients (type II) (Table 1). In both type I and type II classifications, 1 of 6 patients (patient 2 in type I and patient 8 in type II) had a very low frequency of proliferating lymphocytes, regardless of the lymphocyte sources. In the other 10 cases, however, there was a difference between the proliferation frequencies between TIL and PBL to some extent (Table 1). The clonogenic efficiency of lymphocytes from nontumorous kidney was usually lower than that of PBL or TIL.

Substantial numbers of TIL clones from 3 of 6 type I tumors were cytotoxic against autologous tumor cells, and averages of percentage of lysis in the total clones were ≥10% of lysis in all the three cases (type Ia; patients 1, 2, and 4), while a majority of clones from the remaining three cases of type I were noncytotoxic for autologous tumor cells and averages of percentage of lysis in the total clones were <5% (type Ib; patients 5, 11, and 12) (Figs. 1 and 2). PBL clones from all these Type Ia patients contained more cytotoxic clones and averages of % lysis in the total clones were significantly higher than PBL clones from all Type Ib patients (Fig. 2). Very few TIL clones from all six type II cases were cytotoxic against autologous tumor cells, and averages of percentage of lysis in the total clones were <5% in 4 of 6 cases; those in 2 of 6 cases were 5% (patient 8) and 7.5% (patient 10). PBL clones from these two cases also showed 6% (patient 8) and 10% (patient 10) lysis as averages in the total clones. In the other 4 type II cases, PBL clones did not demonstrate significant cytotoxicity against autologous tumor cells. The levels of cytotoxicity mediated by clones from nontumorous kidney primarily correlated well with those of TIL clones, although they were usually lower than those of TIL clones (Fig. 2).

From these results, we identified three different patterns of TIL-mediated anti-autologous tumor response: type Ia (3 or 12 cases), a lower frequency of proliferating TIL (as compared to PBL) and high cytotoxicity (≥10% as an average percentage of lysis in the total clones) against autologous tumor cells; type Ib (3 of 12), a lower frequency of proliferating TIL and low cytotoxicity; and type II (6 of 12), a higher frequency of proliferating TIL and low cytotoxicity. There were no significant differences in surface markers between clones from TIL, PBL, and lymphocytes from the nontumorous kidney (Fig. 3). CD4+CD8- T-helper/inducer clones predominated in all cases (70–85%). There was a low but substantial proportion of CD4-CD8- T-cytotoxic/suppressor clones (15–25%). The frequency of double-negative (CD4-CD8-) clones was usually very low (0–10%). Surface markers of TIL clones in type I and type II clones were also not significantly different, although the percentage of CD8+ T-cells in type I was slightly higher than that in type II. Furthermore, there were no significant differ-

Table 3 Cytotoxicity and phenotypic expression of lymphocyte clones from tumor, peripheral blood, and nontumorous kidney in one patienta

<table>
<thead>
<tr>
<th>No. of Clones</th>
<th>% of lysis vs. Auto-Tu</th>
<th>Surface antigen (%</th>
<th>% of lysis vs. Auto-Tu</th>
<th>Surface antigen (%</th>
<th>% of lysis vs. Auto-Tu</th>
<th>Surface antigen (%</th>
<th>% of lysis vs. Auto-Tu</th>
<th>Surface antigen (%</th>
<th>% of lysis vs. Auto-Tu</th>
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<tbody>
<tr>
<td>TIL CD4 CD8 CD56</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td>&gt;98 &lt;1</td>
<td>2.2</td>
<td>8</td>
<td>3</td>
<td>&gt;98 &lt;1</td>
<td>2.5</td>
<td>9</td>
</tr>
<tr>
<td>PBL CD4 CD8</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td>&gt;98 &lt;1</td>
<td>&lt;1</td>
<td>12</td>
<td>11</td>
<td>&lt;1</td>
<td>12.5</td>
<td>9</td>
</tr>
<tr>
<td>Lymphocytes from nontumorous kidney CD4 CD8 CD56</td>
<td>1</td>
<td>0</td>
<td>14</td>
<td>&gt;98 &lt;1</td>
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<td>&lt;1</td>
<td>12.5</td>
<td>9</td>
</tr>
</tbody>
</table>

* All lymphocyte clones established from tumor, peripheral blood, and nontumorous kidney in one patient (patient 10, RC-Trl) were tested for cytotoxicity against autologous and allogenic tumor cells and phenotype expression (CD3, CD4, CD8, CD56, and Leu-7 antigens). Values represent mean percentage of lysis in triplicate determinations by a 4-h "Cr release assay, and percentage of positive cells by immunofluorescence techniques using flow cytometry. All lymphocyte clones were CD3 antigen positive (>98%) and Leu-7 antigen negative (<3%). ND, not done.
phenotypes before and after activation with IL-2 (18), ability to
differences in the immunological properties of TIL in each
RCC at the bulk culture level with respect to their surface
phenotypes between TIL and PBL in patients with
cell clones or double-negative clones, regardless of the sources
clones; DN, primarily CD3*CD4~CD8~ clones. There are very few
clones; CDS* indicates CD3*CD4~CD8*CD16~ T-cytotoxic/suppressor T-cell

There were no fundamental qualitative differences in immu
nological properties of RCC-TIL in comparison to those of
PBL with respect to clonogenic efficiency, phenotypic
expression, and cytotoxicity.

The cloning efficiency in this study was significantly lower
than that reported in manuscripts by Moretta et al. (19). Modi
fication of the original LDA method used in this study may be
partly responsible for this difference. Further, lymphocyte
sources are different. Purified T-cell fractions from PBMC of
healthy volunteers were used in their studies, while whole
PBMC, single-cell suspensions of tumor, and nontumorous
kidney from patients with RCC were used in this study. All
samples, including peripheral blood, were taken from patients
under general anesthesia and surgery. Surgical procedures re
quired clamping off of the blood supply for at least 30–60 min
prior to removal of the kidney, probably compromising the
ability of lymphocytes to respond to lymphokines.

The majority of autologous TIL clones from the RCCs studied
here lysed both autologous and allogenic tumor cells. One
exceptional case [patient 2 (Table 2; Fig. 1)] contained TIL
clones that lysed autologous, but not allogenic, tumor cells.
Five of 7 TIL clones, 13 of 21 PBL clones, and 1 of 3 lympho
cyte clones from nontumorous kidney in this patient were
cytotoxic (≥10%) against autologous tumor cells. Except for
one PBL clone, all the cytotoxic clones from this patient
(patient 2) did not lyse allogenic tumor cells. Although IL-2-
activated melanoma TIL usually display cytotoxicity restricted
to autologous tumor cells, IL-2-activated RCC-TIL demon
strate predominantly MHC-nonrestricted cytotoxicity (6). Only
one autologous tumor target (MEL-21) was used to measure
alloreactivity in this experiment, whereas most experiments
required more than three different allogenic targets to detect
the specificity of functions (6, 7). We could not repeat the
experiment with TIL clones from patient 2, owing to an insuf
cient supply of effector clones. It is uncertain from this limited
experiment whether all these cytotoxic lymphocyte clones from
patient 2 represent autologous tumor-specific cytotoxic T-lym
phocytes, although a T-cell clone from ascitic fluid of RCC
have been reported to display such a specificity (23).

In all 6 type I cases, the averages of autologous tumor cell
lysis in the total TIL clones were higher than those in total
PBL clones (Fig. 2), whereas the clonogenic efficiency of TIL
was lower than that of PBL (Table 1). In contrast, in all 6 type
II cases, the frequency of proliferating TIL was higher than
that of PBMC (Table 1), and the average in the total TIL clones
were slightly lower than those in the total PBL clones (Fig. 2).
The results are inconsistent with the previous report, which
described the reduced clonogenic efficiency of TIL as compared
to that of PBL (24). One explanation for the discrepancy

DISCUSSION

Three (patients 10, 15, and 16) (19%) tumors contained TIL
with relatively high clonogenic efficiency and 4 (patients 1, 2,
4, and 13) of 13 (31%) tumors had TIL with relatively high
cytotoxicity at the clonal level. TIL from no tumors studied
here displayed both high proliferation and high cytotoxicity at
the clonal level. The same culture conditions, including the
same lots of FCS and phytohemagglutinin, were used through-
out the experiments, suggesting that the differences were not
simply due to culture conditions. In addition, since care was
taken to remove both the central necrotic area of the tumor and
the adjacent surrounding normal tissue, these immunological
differences studied here among individual tumors may reflect
differences in the immunological properties of TIL in each
patient.

There were no fundamental qualitative differences in immu
nological properties between TIL and PBL in patients with
RCC at the bulk culture level with respect to their surface
phenotypes before and after activation with IL-2 (18), ability to
proliferate in culture with IL-2, and their patterns of cytotoxic
ity (MHC-nonrestricted cytotoxicity) after activation with IL-
2 (6, 11–18). We have described here that even at the clonal
level, there may be no fundamental qualitative differences be
between TIL and PBL with respect to their surface phenotypes,
clonogenic efficiency, and patterns of cytotoxicity. Conse
quently, this paper did not provide any immunological findings
to support the unique properties of RCC-TIL in comparison to
others.
between our results and the others is that the different methods, as well as different sources of lymphocytes, were used in the studies. Purified CD2+ T-cells were used in their experiments, while whole populations were used in this experiment. In our study, heparinized peripheral blood was drawn from the patients at the time of surgery, whereas peripheral blood was drawn from normal healthy donors in the others. Another explanation is that TIL from some RCC tumors have a greater ability to proliferate at the clonal level than do PBL. This explanation is supported by the data showing the magnitude of IL-2-dependent proliferation of TIL to be significantly higher than that of PBL (＞1000-fold increase versus 10–100-fold increase) (6, 16). It may be important to note, regardless of the exact mechanisms involved in this discrepancy, that there may be no immunological defect or suppression in the proliferative capability of renal cell carcinoma TIL or their cytotoxicity as compared to those of PBL under the conditions used in this experiment.

Averages of percentage lysis in the total TIL clones from 2 of 3 type Ia (patients 1 and 4) were 2 times higher than those in the total PBL clones (Fig. 3). In these two cases, the numbers of CD8+ clones from TIL (15 of 31 clones in patient 1, or 10 of 21 clones in patient 4) were considerably higher than those from PBL (2 of 43 in patient 1, or 6 of 27 in patient 2). None of the other 10 cases showed such differences, and overall there was no significant difference in surface phenotypes of clones among the three different lymphocyte sources (Fig. 3). These results suggest that CD8+ T-cells with potential MHC-nonrestricted cytotoxicity were concentrated in some RCCs.

Three different patterns of TIL-mediated antitumor response were noted among 16 patients with RCC. These results provide clear evidence for immunobiological diversity among patients with RCC, although the significance of these differences in biological therapy is unclear.

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Clonal Analysis of Lymphocytes from Tumor, Peripheral Blood, and Nontumorous Kidney in Primary Renal Cell Carcinoma

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