ABSTRACT

In this study, we investigated the cytolytic activity of peripheral blood T-cells (PBT) obtained from nine patients with primary lung cancer treated by surgical adjuvant adoptive immunotherapy (AIT) with lymphokine-activated killer cells and low-dose recombinant interleukin 2 at the time of rebound lymphocytosis (24-48 h after AIT). In eight of nine patients, non-specific cytotoxicity of peripheral blood lymphocytes significantly increased as compared with that of pre-AIT peripheral blood lymphocytes. However, purified PBT showed much less activity to kill tumor cells although they increased in number and were activated well in terms of increases in the expression of HLA-DR and interleukin 2 receptor (IL-2R). The cytolytic activity of post-AIT PBT was significantly enhanced when they were targeted to Fc receptor-bearing tumor cells (K562 or Daudi) with anti-CD3 (NU-T3) or anti-T-cell receptor (TCR)β (WT31) monoclonal antibody in all five patients examined. Phenotypically, the targeted cytotoxicity was predominantly mediated by CD8+ cells. The results indicated that in vivo-activated PBT by AIT could not exhibit direct cytotoxicity, but they acquired cytolytic potential, the effect of which was expressed by targeting to tumor cells.

INTRODUCTION

Since the discovery of the LAK4 phenomenon (1), IL-2 has been used for cancer therapy, either alone or in combination with ex vivo-generated LAK cells (2-5). Several investigators reported that LAK activity is successfully induced in PBL by IL-2/LAK therapy (4, 6). Recently, CD56+ NK cells have been proposed as a circulating in vivo cytotoxic effector because most of in vivo-induced LAK activity is mediated by CD56+ NK cells while PBT acquire hardly substantial cytotoxicity (7-11) and become hypo-responsive to exogenous antigens and mitogen (12, 13), following IL-2 administration. However, there are several reports (14, 15) showing that LAK activity is also induced in T-cells by stimulation with IL-2 for several days in vitro. For improvement of efficacy of AIT, it is important to clarify whether in vivo-activated PBT can acquire cytolytic activity or not. This study showed that most in vivo LAK activity following AIT using ex vivo-induced LAK cells and s.c. injection of IL-2 (16-18) is mediated by CD8+ T-cells and NK cells (19) with a half of each daily dose for 6 consecutive days. Lymph node lymphocytes were aseptically prepared from regional lymph nodes at the time of surgery and used as a source of LAK cells (16). The lymph node lymphocytes were subsequently cultured using a dialysis-perfusion culture device, which we have recently developed (17), and infused back to patients on the second day of administration of IL-2. Numbers of infused LAK cells ranged from 3 x 10⁹ to 1.1 x 10¹⁰/patient (mean, 7 x 10⁹).

Preparation of PBL. Heparinized peripheral blood samples were obtained just before the start of AIT and 2 days after the finish. PBL were isolated from each blood sample by centrifugation on Ficoll-Hypaque gradients (LSM; Organon Teknika, Durham, NC) and by subsequent removal of plastic-adherent monocytes.

Antibodies. FITC-conjugated and/or FITC-unconjugated NU series of murine mAbs were kindly provided by Nichirei Co., Tokyo, Japan. The mAbs used were: NU-T3 (anti-CD3), NU-Tpan (anti-CD5), NU-T4b (anti-CD4), NU-Tac (anti-CD8), and NU-B2 (anti-CD20). FITC-conjugated and FITC-unconjugated Leu-11a (anti-CD16) and Leu-19 (anti-CD56), phycoerythrin-conjugated Leu-3b (anti-CD3), anti-IgD (anti-Tac) and anti-HLA-DR, and both FITC-conjugated and FITC-unconjugated WT31 (anti-TCRβ) were purchased from Becton-Dickinson, Mountain View, CA. Goat anti-mouse IgG + IgM were purchased from Tago, Inc., Burlingame, CA.

Isolation of Lymphocyte Subsets. Subpopulations of PBL were isolated by a panning method (24). Briefly, PBL were incubated with mAb for 40 min at 4°C. After 3 washings with Hank’s balanced salt solution.
the cells were put onto goat anti-mouse immunoglobulin-immobilized dishes and incubated at 4°C for 1 h. Negative (unbound) cells were collected and positive (bound) cells were detached by a jet stream of Hanks' balanced salt solution through a 27-gauge needle. For purification of PBT, NU-Tpan (anti-CD5) was used to preserve the expression of CD3-TCR complex.

In Vitro Induction of PBT-LAK Cells. PBL derived from healthy volunteers were cultured in CM containing IL-2 (40 JRU/ml) for 4 days and separated into T-cell and non-T-cell populations. The IL-2 concentration of 40 JRU/ml approximated the in vivo-achieved concentration during the AIT, according to our previous pharmacokinetic study of administered IL-2 (18).

Tumor Targets. Three human tumor cell lines were used as targets: K562, an erythroleukemia line (25); Raji, a Burkitt's lymphoma B-cell line (26); and Daudi, a Burkitt's lymphoma B-cell line (27). FcR were expressed by more than 90% of K562 and Daudi cells as reported previously (28).

Cytolysis Assay. Cytotoxicity was assessed by the standard 4-h 51Cr-releasing assay. Varying concentrations of PBL (effector cells) were incubated in CM (100 µl) in the presence or absence of mAb (NU-T3, 1 µg/ml, or WT31, 30 ng/ml) in 96-well round-bottomed microtiter plates (Sumitomo Bakelite Co., Tokyo, Japan) for 30 min at 37°C before addition of 51Cr-labeled target cells (5 x 10^5/100 µl/well in CM). After 4 h of incubation at 37°C, the supernatants (100 µl) were harvested and counted with a gamma counter. Maximal and spontaneous 51Cr release values were obtained by incubating target cells with 0.1 N hydrochloric acid and CM alone, respectively. All determinants were made in triplicate and the percentage of lysis was calculated according to the formula

\[
\text{% of cytolysis} = \frac{\text{Experimental} (\text{cpm}) - \text{spontaneous} (\text{cpm})}{\text{Maximum} (\text{cpm}) - \text{spontaneous} (\text{cpm})} \times 100
\]

Data were reported as the percentage of specific cytotoxicity or as lytic units per 10^7 lymphocytes (LU), where one LU caused 20% cytotoxicity.

Cell Proliferation Assay. PBL (5 x 10^5/100 µl/well in CM) were seeded in 96-well flat-bottomed microtiter plates (Sumitomo Bakelite), pulsed with 1 µCi [3H]thymidine/well, and incubated at 37°C for 4 h. Cells in each well were harvested onto glass fiber filters and incorporation of [3H]thymidine was assessed with a Beta Plate System (Aloka Co., Tokyo, Japan). Data were reported as mean dpm of triplicate cultures. Standard deviation of triplicates was always less than 15%.

FACS Analysis. PBL (5 x 10^5) were stained for 30 min at 4°C with FITC- or PE-conjugated mAbs. The stained cells were washed 3 times with phosphate-buffered saline and examined for FITC or PE fluorescence with a FACScan (Becton-Dickinson). Analyses of data were made by counting 1 x 10^5 viable cells.

Statistical Analysis. Statistical analysis was made using the paired Student t test, and P < 0.05 was considered to be significant.

RESULTS

Phenotypic Change of PBL after AIT. A marked lymphocytosis occurred in the peripheral blood of all patients 24-48 h after AIT (Table 1). The degree of lymphocytosis ranged from 2.3- to 5.6-fold (mean, 4.0-fold) over the pretreatment values. The phenotypic analysis revealed that the proportion of CD3+ cells increased significantly after AIT (P < 0.01) (Table 1). Further, in the CD3+ T-cell population, the proportion of HLA-DR+ and IL-2R+ cells increased significantly (P < 0.01). CD4+ and CD8+ subsets increased in proportion similarly. As for NK cell subsets, the proportion of CD16+ cells relatively decreased (P < 0.05) and that of CD56+ cells did not change. The proportion of B-cell subsets (CD20+ cells) decreased significantly after AIT (P < 0.01). However, when the change in the total absolute number was taken into account, the absolute number of NK cells increased and that of B-cells did not change.

Cytolytic Activity of PBL after AIT. The cytolytic activity of PBL was assessed at the time of lymphocytosis after AIT and the results are shown in Table 2. In all patients but one (patient 4), NK activity (against K562, NK sensitive) was augmented and LAK activity (against Raji, NK-resistant) was substantially induced by the AIT. Further, we investigated the cytotoxicity of PBT. PBT were purified by a panning method (more than 95% of CD3+ cells) from freshly isolated PBL following AIT and tested for the cytolytic activity. As shown in Table 3, in all 6 patients examined, in vivo-activated PBT had much lower cytotoxicity than T-cell-depleted PBL (less than 30% CD3+). This result indicates that in vivo-induced nonspecific cytotox-
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Levels of circulating PBL is mainly mediated by non-T-cell population.

Targeted Cytotoxicity of in Vitro-activated PBT. PBL from a normal donor were cultured in vitro for 4 days with 40 JRU/ml of IL-2. The concentration of IL-2 was about 50-fold lower than that for the optimum induction of LAK activity in vitro. After culture, isolated PBT (more than 95% CD3+ cells) were examined for targeted cytotoxicity against FcR+ tumor cells (K562), using anti-CD3 (NU-T3) or anti-TCRαβ (WT31) antibody. As shown in Fig. 1, in vitro-activated PBT had a lower cytolytic activity than non-T-cells. By the addition of NU-T3 (1 μg/ml) or WT31 (30 ng/ml) during the cytotoxicity assay, the cytolytic activity of such PBT against FcR+ K562 cells was significantly augmented. In the induction of targeted cytotoxicity, WT31 was more effective than NU-T3. When the PBT were further cultured for 4 more days with lower concentrations of IL-2 (20, 2, and 0 JRU/ml), the proliferative response, expression of HLA-DR and IL-2R, and cytolytic activity decreased in parallel with the decrease in IL-2 concentration (Fig. 2). However, the targeted cytotoxicity was maintained after culture at lower concentrations or even in the absence of IL-2 (Fig. 2c). On the other hand, nonactivated fresh PBT did not exhibit any cytolytic activity (Fig. 2c).

Targeted Cytotoxicity of PBT Activated by AIT. From the results of the above in vitro PBT activation, it was expected that the targeted cytotoxicity could be detected in in vivo-activated PBT. A representative case (patient 5) was presented in Fig. 3.

Despite the PBT after AIT expressed little cytotoxicity directly against K562 cells, they exhibited a considerable level of targeted cytotoxicity with NU-T3 or WT31 (Fig. 3a). WT31 was more effective for the targeting of in vivo-activated PBT than NU-T3. The targeted cytotoxicity was also observed against NK-resistant Daudi cells (FcR positive) as well as against K562 cells (Fig. 3b). The targeted cytotoxicity was detected in all cases tested (Table 4).

Phenotypic Analysis of Effector-mediating Targeted Cytotoxicity. In patients 8 and 9, the CD4+ subset and the CD8+ subset separated from in vivo-activated PBT were examined for targeted cytotoxicity. After non-T-cells (CD56+ cells, CD16+ cells, and CD20+ cells) were depleted from PBL by a panning, the purified PBT were separated into two subsets by a panning using NU-T11/1 (anti-CD4 mAb). More than 90% of bound cells were CD4+, and more than 70% of unbound cells were CD8+.

In both patients, very high levels of targeted cytotoxicity with...
WT31 were detected in CD8+ cells (Fig. 4). Any cytotoxicity was not detected in CD4+ cells of patient 8 (Fig. 4a), while cytotoxicity of such a T-cell subset in patient 9 was enhanced slightly by targeting from a low level of direct toxicity without targeting (Fig. 4b). These results indicated that the targeted cytotoxicity of in vivo-activated PBT is predominantly mediated by the CD8+ subset.

DISCUSSION

We have recently designed a regimen of the surgical adjuvant adoptive immunotherapy for cancer, which features the prolonged (6 consecutive days) s.c. administration of low-dose IL-2 in combination with a transfusion of ex vivo-generated LAK cells from lymphocytes of regional lymph nodes (16–18). The present study was undertaken to clarify an in vivo immunomodulatory effect of the regimen. The immunotherapy successfully caused a marked lymphocytosis and in vivo induction of LAK cells. The rebound lymphocytosis after the immunotherapy was accompanied by the increase in number and proportion of T-cells expressing the HLA-DR antigen and the IL-2R. However, in vivo-induced LAK activity resided predominantly in the non-T-cell population in all the cases tested. These results are consistent with reports by other investigators that in vivo-induced LAK activity is mediated by CD56+CD16+ or CD56+CD16− non-T-cells (7–10).

There have been several reports showing that T-cell-mediated nonspecific killing can be induced when T-cells are targeted via TCR-related molecules such as CD3 antigen to tumor cells (28–34). For the induction of targeted cytotoxicity, however, several investigators reported that the induction of targeted cytotoxicity from resting PBT needs prestimulation by IL-2 (20, 31) or anti-CD3 antibody (29) or both (34) for several h (20, 31) to several days (29, 34). Garrido et al. (34) reported that only 2% of resting PBT can exhibit targeted cytosis without prestimulation. In the present study, we showed that PBT could exhibit a targeted cytotoxicity after culture for 4 days with a concentration of IL-2 (40 JRU/ml) 50-fold lower than that for optimal induction of LAK activity, and the targeted cytotoxicity was maintained at high level for several days in the absence of IL-2. Such in vitro phenomenon were also observed in vivo during the lymphocytosis following AIT. The AIT was so effective as to induce cytolytic potential in circulating PBT, the effect of which was expressed by targeting via TCR-related molecules. This information is thought to be therapeutically advantageous. A bispecific antibody recognizing both CD3 or TCR of T-cells and components on tumor cells may enable conversion of in vitro-activated polyclonal PBT to tumor-specific effectors by mimicking a ligand-receptor relationship without major histocompatibility complex restriction. Rebound lymphocytosis following AIT or IL-2 therapy can provide abundant PBT capable of being effectors in targeted cytotoxicity and this LAK/IL-2 therapy is expected to offer a favorable chance to the therapy with bispecific antibodies.

Table 4. Targeted cytotoxicity of in vivo-activated PBT

<table>
<thead>
<tr>
<th>Case</th>
<th>Medium</th>
<th>NU-T3</th>
<th>WT31</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>41</td>
<td>77</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
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<td>67</td>
<td>250</td>
</tr>
<tr>
<td>7</td>
<td>25</td>
<td>ND</td>
<td>177</td>
</tr>
</tbody>
</table>

*a LU/10^6 effector cells against K562.
*b ND, not determined.

WT31 (30 ng/ml) induced targeted cytotoxicity more efficiently than NU-T3 (1 μg/ml) in all experiments. In the system of anti-CD3 mAb and targets (K562 and Daudi) bearing FcR, Sewenter et al. (35) observed that targeted cytotoxicity is induced by various IgG subclasses, but they did not describe quantitative efficiency. In our study, the difference of IgG subclasses (WT31, IgG1; NU-T3, IgG2a) cannot be ruled out as the background of different efficiencies in targeted cytotoxicity, although a difference of cross-linked site on T-cells may be a more plausible reason.

Targeted cytotoxicity of in vivo-activated PBT was attributed to the CD8+ T-cell subset. In the in vitro experiments, Martin et al. (36) showed that the induction of targeted cytotoxicity is correlated with the synthesis of PFP and that the PFP content of CD8+ cells increases about 20-fold upon stimulation with anti-CD3 or IL-2, while that of CD4+ cells increases about 2-fold. Similarly, Smyth et al. (20) demonstrated that stimulation with IL-2 alone for several h can induce PFP mRNA exclusively in CD8+ T-cells and that the generation of targeted cytotoxicity in T-cells is accompanied by the expression of PFP mRNA. Therefore, we are now analyzing PFP mRNA of in vivo-activated PBT, as well as mRNA of various cytokines, to clarify the in vivo effect of LAK/IL-2 therapy.

REFERENCES

Cytolytic Potential of Peripheral Blood T-Lymphocytes following Adoptive Immunotherapy with Lymphokine-activated Killer Cells and Low-Dose Interleukin 2

Ichiro Yoshino, Tokuiro Yano, Mitsuhiro Murata, et al.


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