A New In Vitro Model of Specific Targeting Therapy of Cancer: Retargeting of PWM-LAK Cells with Bispecific Antibodies Greatly Enhances Cytotoxicity to Hepatocellular Carcinoma

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SAIJO, S., KUDO, T., KATAYOSE, Y., SAEKI, H., CHIBA, N., SUZUKI, M., TOMINAGA, T. and MATSUNO, S. *A New In Vitro Model of Specific Targeting Therapy of Cancer: Retargeting of PWM-LAK Cells with Bispecific Antibodies Greatly Enhances Cytotoxicity to Hepatocellular Carcinoma.* Tohoku J. Exp. Med., 1996, 178 (2), 113-127 — For the purpose of establishing a new in vitro model of adoptive immunotherapy, we synthesized two kinds of bispecific antibodies (BsAbs), i.e., (OK $\times$ L) BsAbs constructed with both OKT-3 (anti-CD3) and L-7-6 (anti-HCC), and (3G $\times$ L) BsAbs constructed with 3-G-8 (anti-CD16) and L-7-6 antibodies. These two BsAbs, having pairs of binding arms on their single molecule, showed similar binding to target cells as the parental monoclonal antibodies (OKT-3, 3-G-8 and L-7-6), when examined with FACS. Newly devised in vitro cytotoxicity tests revealed that LAK or PWM-stimulated LAK (PWM-LAK) cells did not show any significant cytotoxic activity to HCC cells, while both effector cells equally showed greatly enhanced cytotoxicity to HCC even at a low effector/target (0.3) in the presence of BsAbs (OK $\times$ L) for the efficient retargeting of the effector cells. Inasmuch as PWM-LAK cells proliferate in vitro 3-5 times faster than LAK cells, adoptive immunotherapy using PWM-LAK cells in combi-

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Abbreviations used in this paper: BsAb, bispecific antibody; HCC, hepatocellular carcinoma; LAK, lymphokine-activated killer; rIL-2, recombinant interleukin-2; PWM, pokeweed mitogen; STT, specific targeting therapy; DTT, dithiothreitol; DTNB, 5,5'-dithio-bis (2-nitrobenzoic acid); SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; PWM-LAK, PWM-stimulated LAK; MoAb, monoclonal antibody; PBMNC, peripheral blood mononuclear cell; MTT, 3-(4,5-dimethyl thiazo-2-yl)-2,5-diphenyl tetrazolium bromide; FBS, fetal bovine serum; BSA, bovine serum albumin; FACS, fluorescence activated cell sorter.

nation with (OK $\times$ L) BsAbs should be very promising. ——— bispecific antibody; PWM-LAK cells; adoptive immunotherapy; hepatocellular carcinoma

Many clinical trials using lymphokine activated killer (LAK) cells for cancer immunotherapy have been carried out since the report of successful activation of peripheral blood lymphocytes with IL-2 (Grimm et al. 1982). However, the clinical results so far obtained have proved much worse than expected from the results of in vitro experiments (Rosenberg et al. 1985; Okuno et al. 1986; Ishikawa et al. 1988; Rosenberg 1988). This might be attributed to the following: First, the killing potential of individual LAK cells is not sufficiently strong to effectively lyse target cells: Second, accumulation of LAK cells in tumor tissues is less than satisfactory because of a low affinity for tumor cells.

In order to obtain better results with LAK therapy, one approach might therefore be to improve the targeting and killing activity of LAK cells. For this purpose, construction of bispecific antibodies (BsAbs) directed to both T cell receptor (TCR) and tumor-associated antigens has been performed. Addition of BsAb to effector cells results in their cytotoxic activity being augmented (Perez et al. 1985; Staerz et al. 1985; Staerz and Bevan 1986). Many kinds of BsAb have been now produced (Nitta et al. 1989, 1990a; Mezzanzanica et al. 1991; Hsieh-Ma et al. 1992) and compared to LAK therapy alone. A specific targeting therapy (STT) consisting of LAK and BsAb in combination has shown much better clinical results, especially in cases of malignant glioma (Nitta et al. 1990b) and ovarian cancer (Bolhuis et al. 1992). These results have encouraged us to apply BsAb to the LAK treatment of hepatocellular carcinoma (HCC), for which recurrence is very frequently observed after radical surgery. Therefore, we have tried to develop a new in vitro STT model for HCC, as an approach of postoperative adjuvant therapy, and also as the basis of a multidisciplinary therapy for patients with inoperable HCC.

MATERIALS AND METHODS

Monoclonal antibodies

For construction of BsAb, the following monoclonal antibodies (MoAbs) were used: Two MoAbs, against CD3 (OKT-3, mouse IgG2a) and against CD16 (3-G-8, mouse IgG1), directed to effector cells, and L-7-6 (mouse IgG1), developed by Tanno et al. (1995) in our laboratory, directed to HCC-associated antigens. The L-7-6 MoAb was obtained by immunizing BALB/c mouse with human HCC cell line (HuH-7) and reacts mainly with HCC, esophageal cancer, and glioma cells. Its precise specificity is being reported elsewhere (Tanno et al. 1995). The MoAbs were purified from sera and ascitic fluid of mice inoculated with hybridoma cells by caprylic acid treatment combined with the ammonium sulfate method (McKinney and Parkinson 1987). For the FACS analysis, TS2/18.1.1 (anti-CD2), OKT-3 (anti-CD3), OKT-8 (anti-CD8), and 3-G-8 (anti-CD16) MoAbs were used.

Production of BsAb

First, OKT-3 MoAb was digested to $F(ab')_2$ with trypsin in 0.1 M citrate buffer (pH 4.1) at 37°C for 2 hr. Then, $F(ab')_2$ fragments were reduced by DTT (0.5 mM), the reaction stopped by addition of DTNB, and OKT-3 Fab'-S-NB fragments separated from excess reagent by gel filtration. L-7-6 was digested with preactivated papain in 0.1 M acetate buffer (pH 5.5) to generate $F(ab')_2$ at 37°C for 6 hr. The fragments were also reduced with DTT and thus generated Fab-SH fragments with free SH groups were separated by gel filtration (Brennan et al. 1985; Nitta et al. 1989). Since Fab'-SH-NB fragments bind Fab-SH specifically, Fab'-S-NB fragments of OKT-3 MoAb and Fab-SH fragments of L-7-6 MoAb were then mixed at a molar ratio of 1:1, and incubated at room temperature for 4 hr. The resulting preparation was applied to a TSK-2000SW column (Tosoh, Tokyo) of an FPLC system (Pharmacia Biotech, Uppsala, Sweden) to remove the unreactive fragments and obtain (OK $\times$ L) BsAb. BsAb (3G $\times$ L) was prepared as follows. $F(ab)_2$ fragments of 3-G-8 MoAb were obtained by digestion with preactivated papain, and reduced with DTT. The reduction was stopped by addition of DTNB and the Fab-S-NB fragments were separated by gel filtration chromatography. Fab-S-NB (3-G-8) and Fab'-SH (L-7-6) were then mixed in a 1:1 ratio, and incubated for 4 hr. The reconstituted BsAb (3G $\times$ L) was purified by FPLC.

Cell lines

Three human HCC lines (Li-7, HuH-7, and HT-17), reactive with L-7-6 MoAb, were used as BsAb target. Li-7 and HuH-7 cell lines were obtained from Cancer Cell Repository, Tohoku University, and HT-17 cell line was established by Tanno in our laboratory. A human pancreatic carcinoma cell line PK-8 and a lung adenocarcinoma cell line LK-1 were used as negative controls. Mouse fibroblast cell lines (L929, LAG) were also used for control purposes. These cell lines were all cultured in RPMI-1640 supplemented with 10% FBS.

Effector cells

Peripheral blood mononuclear cells (PBMNCs), isolated from heparinized blood of a healthy volunteer by density gradient (Lymphoprep) centrifugation, were cultured for 48 hr in RPMI-1640 supplemented with 10% FBS and 100 U/ml recombinant human IL-2 (Shionogi Pharmaceutical Co., Osaka) at a cell density of 1×10^6 /ml, in a culture flask (178891; A/S NUNC, DK-4000, Roskilde, Denmark), where bottom was pretreated with OKT-3 MoAb (10 μ g/ml) for induction of LAK cells. The LAK cells were expanded in culture medium containing 100 U/ml rIL-2. PWM-LAK cells were induced as reported previously (Kimoto et al. 1992). Briefly, PBMNCs were incubated with RPMI-1640 containing 1,000 U/ml of rIL-2, 5 U/ml of heparin as an anticoagulant, 10% of fresh autologous plasma

and 1 $\mu\text{g}/\text{ml}$ PWM (Sigma, St. Louis, MO, USA) at the cell density of $1 \times 10^6/\text{ml}$ with a static culture system for 48 hr. Then, the cells were washed by centrifugation to remove PWM, and cultured in the above medium free of PWM. The culture scale was expanded gradually with rIL-2 containing medium according to the increase of cell number.

Cytofluorometry

For cytofluorometry, LAK cells, PWM-LAK cells, HCC cells and control cells were incubated with various MoAbs, or BsAbs as first antibodies. The second antibody used was FITC-conjugated goat anti-mouse IgG F(ab')₂ (55518; Cappel Lab., Malvern, PA, USA). Stained cells were observed with an epi-illumination fluorescence microscope (Zeiss standard 18) and analyzed with FACScan cytofluorometer.

Cytotoxicity assay

In this experiment, new cytotoxicity assay method was developed by modifying the previous method (Mosmann 1983; Beun et al. 1993), which used U-well microplates. Target culture cells at subconfluent phase, detouched gently with 0.02% EDTA-PBS solution, were suspended in RPMI-1640 medium supplemented with 10% FBS. Twenty thousand cells (100 μl cell suspension) were distributed to each well of a half-area (A/2) 96-well flat bottom plate (3696; Costar, Cambridge, MD, USA) and cultured overnight to promote adherence to the plate. After removing the culture medium by aspiration, rIL-2, BsAb, and effector cells suspended in RPMI-1640 plus 10% FBS were added to each well. Namely, 25 μl of rIL-2 (100 U/ml), 25 μl of various concentrations of antibody solution and 50 μl of effector cell suspension were given. Effector/target (E/T) ratios tested in this paper were 0.3, 0.6, 1, 2, and 4. All determinations were carried out in triplicate. After culture for 24–72 hr at 37°C, plates were washed with 100 μl of PBS/well twice or three times to remove effector cells and killed target cells. Then, 50 $\mu\text{l}/\text{ml}$ of the culture medium containing 1 mg/ml MTT were added and the plates incubated at 37°C for 4–5 hr. Crystalline formazan was dissolved by adding acid-isopropanol (0.04 N HCl in isopropanol, 50 $\mu\text{l}/\text{well}$), followed by overnight incubation at 37°C. Then, the plates were thoroughly agitated and read on a microplate reader (Bio-Rad model 3550) at 595 nm. As reported previously (Beun et al. 1993), percentage cytotoxicity was calculated as follows: Cytotoxicity (%) = $(1 - \text{OD}_{595} \text{ of experiment} / \text{OD}_{595} \text{ of control}) \times 100$. LAK cells used for MTT cytotoxicity were those cultured for 3–14 days, and PWM-LAK cells used for MTT cytotoxicity were those cultured for 14–21 days.

RESULTS

Characterization of BsAb

By chemical synthesis methods using DTT and DTNB, two kinds of BsAbs,

(OK $\times$ L) produced from OKT-3 and L-7-6, and (3G $\times$ L) produced from 3-G-8 and L-7-6, were obtained. The elution profiles obtained with the products of reaction between Fab-SH (L-7-6) and Fab'-S-NB (OKT-3) on a TSK-2000SW sepharogel column showed first peak equivalent to BsAb at 17 min, and second peak (unreactive Fab and Fab') at 20 min. The average yield of the BsAb obtained from Fab-SH and Fab'-S-NB with the procedure was in the range of 70%. Profiles of the main products of the respective reaction analyzed by SDS-PAGE (10%) under non-reducing condition are shown in Fig. 1, with purified IgGs from OKT-3 and L-7-6, F(ab')₂ fragment of OKT-3 MoAb, F(ab)₂ of L-7-6 MoAb, Fab' fragments of OKT-3 MoAb, Fab fragments of L-7-6 MoAb, and BsAb (OK $\times$ L) constructed between Fab-SH (L-7-6) and Fab'-S-NB (OKT-3) being examined. The BsAb obtained by thiol-disulfide interchange using DTNB showed a molecular mass (Mr) of 100 kDa.

Reactivity of BsAb (OK $\times$ L) with effector cells and HCC cells was analyzed by FACScan (Fig. 2). LAK cells (panel 1, 3) or HuH-7 cells (panel 2, 4) reacted with 5 μ g/ml of BsAb (OK $\times$ L) (panel 3, 4) or intact OKT-3 IgG (panel 1) or intact IgG L-7-6 (panel 2), followed by incubation with FITC-conjugated goat anti-mouse IgG F(ab')₂. When BsAb (OK $\times$ L) was used as first antibody, both LAK cells and HuH-7 cells showed positive FACS pattern peaks, essentially similar to those when OKT-3 or L-7-6 MoAb was used as first antibodies. Similar findings were obtained when BsAb (3G $\times$ L) was tested (data not shown). From these results, it was confirmed that both BsAbs, (OK $\times$ L) and (3G $\times$ L), possess

Fig. 1. Analysis of MoAbs, MoAb fragments and BsAb on SDS-PAGE (10%). Applied samples were intact OKT-3 IgG (lane 1); intact L-7-6 IgG (lane 2); F(ab')₂ fragments of OKT-3 MoAb digested by pepsin (lane 3); F(ab)₂ fragments of L-7-6 MoAb digested by preactivated papain (lane 4); Fab' fragments of OKT-3 MoAb (lane 5); Fab fragments of L-7-6 MoAb (lane 6); BsAb (OK $\times$ L) (lane 7). Standard markers are shown in the left margin.

Fig. 2. Reactivity of BsAb (OK $\times$ L) analyzed by FACScan. LAK cells (panels 1, 3), or HuH-7 (panels 2, 4) reacted with 5 μ g/ml BsAb (OK $\times$ L) (panels 3, 4), intact OKT-3 IgG (panel 1), or L-7-6 IgG (panel 2), followed by incubation with FITC-conjugated goat anti-mouse IgG F(ab')₂. Shaded areas represent negative controls for each panel.

two binding arms to two different epitopes.

Proliferation of effector cells

LAK cells were induced by stimulation of PBMNCs with immobilized OKT-3 MoAb for an initial 48 hr, and PWM-LAK cells were induced by stimulating

Fig. 3. Proliferation of LAK cells ($\circ$ — $\circ$) and PWM-LAK cells ($\bullet$ — $\bullet$). Culture media for the respective cells were changed every 2–3 days, when cell numbers were counted.

PBMNCs with PWM ($1 \mu\text{g/ml}$) for this length of time. Then, both LAK cells and PWM-LAK cells were washed by centrifugation, and cultured with the respective culture media containing rIL-2. Culture media were changed every 2–3 days, when cell numbers were counted to examine proliferation of the cells. In Fig. 3, growth curves of LAK cells and PWM-LAK cells are shown. In the case of LAK cells, cell number after 14 day cultivation had increased about 30 times, and LAK cells after 21 day cultivation were about 100 times more numerous than at the start of cultivation. In the case of PWM-LAK, cell number of 21 days' cultivation had increased about 450 times. PWM-LAK cells proliferated 3 to 5 times more rapidly than LAK cells during the culture period between 2 to 3 weeks in line with the previous report (Kimoto et al. 1992).

Effector cells

Phenotype of effector cells was analyzed using TS2/18.1.1 (anti-CD2), OKT-3 (anti-CD3), OKT-8 (anti-CD8), and 3-G-8 (anti-CD16) MoAbs (Fig. 4). More than 95% of LAK and PWM-LAK cells during the culture period were CD2 positive. In the LAK cell case, 82% were CD3 positive after the initial 3 days' cultivation and more than 90% after 7 days' cultivation. During the cultivation,

Fig. 4. Phenotype of effector cells analyzed by FACScan. LAK cells after 3 day (panels 1, 2, 3, 4), 7 day (panels 5, 6, 7, 8), and 12 day cultivation (panels 9, 10, 11) and PWM-LAK cells after 18 day cultivation (panels 12, 13, 14, 15) were tested for their reactivity with anti-CD2 (panels 1, 5, 12), anti-CD3 (panels 2, 6, 9, 13), anti-CD8 (panels 3, 7, 10, 14) and anti-CD16 MoAbs (panels 4, 8, 11, 15).

CD8 positive cells increased (33.2% on day 3, 49.7% on day 7, 60.5% on day 12) gradually, while CD16 positive cells decreased (55.5% on day 3, but negligible later than 7 days). PWM-LAK cells of 18 days' cultivation were 95% CD2 positive, more than 90% CD3 positive, 82.9% CD8 positive, and 5–6% CD16 positive.

Cytotoxicity assay

Cells in the cytotoxicity assay observed under the inverted microscope are shown in Fig. 5. Target cells (HuH-7) cultured for 72 hr showed a pavement-like appearance (left). When HuH-7 cells and LAK cells (E/T ratio was 4) were cocultured in the presence of BsAb (OK $\times$ L, 1 μ g/ml) for 72 hr, HuH-7 cells became attached to the surrounding LAK cells (center). Right picture shows the morphology after aspiration of supernatant containing LAK cells, destroyed HuH-7 cells and BsAb from the same well shown in the central picture. Only a few residual HuH-7 cells are evident in the well, and this correlated well with the MTT in vitro cytotoxicity assay value.

Effect of BsAb concentration on cytotoxicity

Various concentrations of BsAb (0.1 μ g/ml, 0.5 μ g/ml, 1.0 μ g/ml) were tested for cytotoxicity against two HCC lines (HT-17, Li-7). LAK cells after 3 day cultivation chosen as effector cells showed similar effective killing of HCC lines when BsAb concentrations ranging from 0.1 μ g/ml to 1.0 μ g/ml were added to the

Fig. 5. Phase-contrast micrographs of cells used in the cytotoxicity assay. Target cells (HuH-7 cells) cultured for 72 hr without effector cells and BsAb (left). HuH-7 cells and LAK cells after 7 day cultivation (E : T = 4 : 1) were cocultured for 72 hr in the presence of BsAb (OK $\times$ L), 1 μ g/ml) (center). After aspirating the supernatant of the well shown in the center, only a few residual HuH-7 cells were found in the well (right).

assay system. From these results, the concentration of BsAb for the following experiments was fixed at $1.0 \mu\text{g/ml}$.

Culture period of effector cells influences cytotoxicity

In order to determine the effect of culture period used for induction of effector cells, four kinds of effector cells (LAK cells of 3 day, of 6 day, of 14 day cultivation, and PWM-LAK cells after 18 day cultivation) were tested simultaneously for cytotoxicity against the two HCC lines, in the presence of BsAb (3G $\times$ L, upper panels of Fig. 6) or BsAb (OK $\times$ L, lower panels of Fig. 6). As shown in the upper two panels, only LAK cells after 3 day cultivation were sufficiently cytotoxic for the two HCC lines in the presence of BsAb (3G $\times$ L). In contrast, as shown in the lower two panels, the four effector cells, irrespective of culture period, demonstrated effective killing in the presence of BsAb (OK $\times$ L). These results were considered to reflect the phenotype expression of effector cells, for most effector cells expressed CD3, while CD16 expression was limited to LAK cells after 3 day cultivation, as shown in Fig. 4.

Influence of the reaction time on the cytotoxicity assay

In Fig. 7, effects of reaction time on the cytotoxicity assay were determined.

Fig. 6. Effects of BsAb (3G $\times$ L) (upper two panels) and BsAb (OK $\times$ L) (lower two panels) on cytotoxic activity of various effector cells in the 72 hr MTT assay. Effector cells were LAK cells after 3 day ($\circ$ — $\circ$), 6 day ($\bullet$ — $\bullet$), or 14 day cultivation ($\triangle$ — $\triangle$), and PWM-LAK cells after 18 day cultivation ($\blacktriangle$ — $\blacktriangle$). BsAb was added at the concentration of $1.0 \mu\text{g/ml}$. Target cells were HT-17 (left two panels), and Li-7 (right two panels).

Fig. 7. Kinetics of MTT cytotoxicity. Effects of reaction time for the cytotoxicity assay were determined using LAK cells after 7 day cultivation as effector cells and BsAb (OK $\times$ L, 1.0 μ g/ml). Target cells were HT-17 (left) and Li-7 (right). Reaction times were 24 hr (●—●), 48 hr (△—△), and 72 hr (▲—▲). Control (○—○) was tested without BsAb, incubation for 72 hr.

LAK cells after 7 day cultivation were tested for their cytotoxicity to HT-17, and Li-7 cells in the presence of BsAb (OK $\times$ L) for various assay periods (24 hr, 48 hr and 72 hr). A negative control (LAK cells without BsAb) is also included in the figure. In the course of reaction period from 24 hr to 72 hr, gradual increase of cytotoxicity was observed. Based on these results, the reaction time for cytotoxicity was usually fixed to 72 hr.

Specificity of BsAb determines the killing activity of effector cells

The pancreas cancer cell line (PK-8) and lung adenocarcinoma cell line (LK-1), not reactive with BsAb (OK $\times$ L), were used as target cells to confirm the specificity of effector cells. In this assay, neither PK-8 cells nor LK-1 cells were killed, though HCC cells were efficiently lysed. These results clearly indicate that the cytotoxicity specificity is determined by the specificity of BsAb.

Intact IgG MoAb does not enhance the cytotoxicity of effector cells

Purified, intact MoAbs, OKT-3, or L-7-6, were tested for their enhancing effects on cytotoxicity (Fig. 8). LAK cells of 7 day cultivation were tested for their cytotoxicity to HT-17 cells (left) and Li-7 cells (right), in the presence of 1 μ g/ml BsAb (OK $\times$ L), or 10 μ g/ml OKT-3 together with 10 μ g/ml L-7-6 MoAb. In the presence of BsAb, LAK cells effectively killed the HCC cell lines. However, in the presence of both of the MoAbs, even at 10 μ g/ml, effective cytotoxicity could not be observed. These results show the necessity for BsAb, possessing two binding arms directed to effector cells and to HCC, respectively on

Fig. 8. Addition of intact MoAbs (OKT-3 and L-7-6) to the MTT assay does not enhance LAK activity. MTT assays (72 hr) of LAK cell action were performed in the presence of 10 μ g/ml intact OKT-3 plus 10 μ g/ml intact L-7-6 ($\triangle$ — $\triangle$), with (OK $\times$ L) BsAb (1.0 μ g/ml) ($\bullet$ — $\bullet$) or without BsAb ($\circ$ — $\circ$). Target cells were HT-17 (left), and Li-7 (right).

the single molecule.

Cytotoxicity of PWM-LAK cells is also enhanced by BsAb

In the presence of 1 μ g/ml BsAb (OK $\times$ L), PWM-LAK cells of 15 days' cultivation were tested for cytotoxic activity against the three HCC cell lines (panel 1, HT-17; panel 2, HuH-7; panel 3, Li-7 in Fig. 9) under the same condi-

Fig. 9. PWM-LAK activity is remarkably enhanced by the addition of (OK $\times$ L) BsAb. Cytotoxicity was tested in the 72 hr MTT assay using PWM-LAK cells with 1.0 μ g/ml (OK $\times$ L) BsAb ($\bullet$ — $\bullet$) or without BsAb ($\circ$ — $\circ$). Target cells were HT-17 (left), HuH-7 (center), and Li-7 (right).

tions with Fig. 6. PWM-LAK cells showed remarkable cytotoxic activity, more than 80% cytotoxicity being obtained for all three HCC cell lines when the E/T ratio was 4 (Fig. 9). Since PWM-LAK cells proliferate more rapidly than LAK cells (Fig. 3), and the cytotoxic activity of PWM-LAK cells ranked with that of LAK cells, use of PWM-LAK cells is advantageous.

DISCUSSION

Immunotherapy using LAK cells has been applied for the treatment of malignant diseases. The efficacy of adoptive immunotherapy using LAK cells and IL-2 has been reported for a number of malignancies, including the renal cell carcinoma and malignant melanoma, but clinical results so far obtained with most malignancies have been worse than expected (Ettinghausen et al. 1988; Rosenberg 1988). One method to improve LAK immunotherapy is based on the idea enhancing the targeting of LAK cells, since sufficient cytotoxicity might be obtained if appropriate binding between LAK cells and target cells is achieved. In case of lectin dependent cell-mediated cytotoxicity in which lectin helps binding of effector cells to target cells, strong target cell lysis is obtained if effector cells are activated. In our preliminary study in which PWM (1 μ g/ml) was added to the LAK assay system (cytotoxicity tests), LAK cells killed not only HCC cell lines, but also mouse fibroblast cell lines (L929, LAG) quite effectively (data not shown). Percent cytotoxicity for two fibroblast cell lines ranged 70–80%, even at a low E/T ratio (0.3). This suggests that LAK cells possess enough potential to lyse tumor cells both in vitro and in vivo, if they are appropriately retargeted. This is the principle behind specific targeting therapy (STT) (Nitta et al. 1990a) and for the present study. We prepared BsAbs chemically according to Nitta's method (Nitta et al. 1990a, b). The resultant (OK $\times$ L) BsAb and (3G $\times$ L) BsAb, both retained the antibody specificity and activity of the original MoAbs (OKT-3, 3-G-8, and L-7-6). Moreover, calculated as protein amounts, BsAb yield ranged 20–30% of the original MoAbs, and since the preparation method is relatively simple compared to other approaches (Lanzavecchia and Scheidegger 1987; Kostelny et al. 1992), it appears to have practical advantage.

In the present study, phenotype analysis disclosed that most of our LAK cells express CD2, and CD3, indicating that they were categorized to T-LAK, though early LAK cultures (after 3 days' culture) contained CD16 positive cells (55%). Positiveness for cell markers correlated well with the cytotoxicity to HCC cells, that is, LAK cells demonstrated remarkable cytotoxicity in the presence of (OK $\times$ L) BsAb, while only early LAK cells could show cytotoxicity to target cells in the presence of (3G $\times$ L) BsAb. PWM-LAK cells, mostly expressing CD2, CD3 and CD8, showed remarkable cytotoxicity to HCC cells in the presence of (OK $\times$ L) BsAb. Perforin, which is considered as the main killing molecule of LAK cells, was observed similarly in both LAK cells and PWM-LAK cells by immunostaining (data not shown). Since PWM-LAK cells show a remarkable cytotoxic

activity comparable to LAK cells in the presence of BsAb, even at low E/T ratio (0.3) (Fig. 9), and we can obtain 3–5 times greater number of PWM-LAK cells from the same amount of blood (Fig. 3), they are particularly suitable for STT. In addition, it is also advantageous that autologous plasma, instead of FBS, can be used for the culture medium in the PWM-LAK case.

In our preliminary experiment, when both ($OK \times L$) BsAb and ($3G \times L$) BsAb were added to the LAK assay system, we did not obtain the expected synergistic effect. This contrasts with the remarkable synergism reported for the two BsAbs ($CD3 \times CD30$ and $CD28 \times CD30$) in the xenografted Hodgkin's tumor (Renner et al. 1994). The possibility of trispecific antibody that uses cooperative signaling via the TCR/CD3 complex and CD2 has also been described (Tutt et al. 1991) and further combination studies of STT are clearly warranted.

In the present investigation, a modification of the previous MTT assay (Beun et al. 1993) was made by using half area (A/2) microplates, instead of U-well plates. We could obtain reliable, and reproducible results, for well-to-well difference became much smaller by better attachment of target cells to the plate. The concentration of rIL-2 was selected as 25 U/ml from preliminary experiments which showed a plateau in cytotoxicity in the presence of rIL-2 more than 25 U/ml. Compared with standard ^{51}Cr release assay of relatively short duration, our assay system may reflect more accurately the process that has taken place in vivo, for it includes cytostatic effects of effector cells, direct cytotoxicity and apoptosis. It is conceivable that recycling of the effector cells in the presence of rIL-2 and free BsAb should occur during 72 hr of the assay.

Before the clinical trials, independent of the great efficacy of STT in vitro, in vivo experiment, such as STT in tumor-grafted SCID mouse, is necessary. For measurement of an efficacy of STT, distribution analysis of BsAb-targeted PWM-LAK cells in the tumor-grafted SCID mice by using fluorescence-labeled PWM-LAK cells or immunohistological study will give us good information. Two recent papers (Takahashi et al. 1993; Renner et al. 1994), which reported the successful adoptive immunotherapy in this animal system, do moreover provide encouragement.

In conclusion, we succeeded in constructing in vitro model of adoptive immunotherapy using PWM-LAK cells with BsAb. This STT might be very hopeful for HCC, since local infusion of effector cells and BsAb to HCC tissues would be possible through the hepatic artery by a catheter as reported previously (Keilholz et al. 1994).

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