Arch. Pharm. Res. Vol. 17, No. 3, pp. 194-198, 1994

Antitumor Effect of ^{212}Bismuth-conjugated Anti-IL-2R Monoclonal Antibody(2E4) on a IL-2 Receptor Positive Tumor EL4J3.4

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(Received January 17, 1994)

The antitumor effects of the 2E4 and anti-Tac, monoclonal antibodies directed to IL-2 receptor (IL-2R), conjugated with α-particle emitting radionuclide bismuth-212., were compared. The ^{212}Bi-2E4 demonstrated specific cytotoxicity to EL4J3.4, a IL-2R$^+$ cell line, than to EL4J, a IL-2R$^-$ cell line in thymidine incorporation assay. ^{212}Bi-2E4 exerted the maximal antitumor effect in that % T/C in C57BL/6 mice implanted with EL4J3.4 ascitic tumor was 331% at the concentration of 50 μCi, while that of ^{212}Bi-anti-Tac was 258% at 100 μCi.

Key words: Antitumor effect, IL-2 receptor-directed monoclonal antibodies, ^{212}Bi-2E4, ^{212}Bi-anti-Tac, EL4J, EL4J3.4

INTRODUCTION

The p55 interleukin-2 receptor (IL-2R) is expressed on activated lymphoid cells but not on precursor or non-activated lymphoid cells and normal tissues (Lorberboum-Galski *et al.*, 1988) Hence, application of anti-IL-2R directed immunotherapy makes possible the treatment of certain cancers, autoimmune diseases and prevention of graft rejection (Waldmann, 1986) by eliminating the hyperactive IL-2R positive cells. Recently, several laboratories have engaged in the development of specific monoclonal antibodies (mAb) directed to the human IL-2 rceptor (IL-2R) and found that the conjugates of mAb with radionuclide or toxins exert strong cytotoxic effects against the IL-2R positive tumor cells (Bacha *et al.*, 1988; Ruegg *et al.*, 1990; Kozak *et al.*, 1990a; Kozak *et al.*, 1990b). For preparation of the immunoconjugates, anti-Tac, RPC-5 and 2E4 were used as monoclonal antibodies (Tseudo *et al.*, 1989), pseudomonas exotoxin and A chain of toxin ricin as toxins (Fitzgerald *et al.*, 1984), and 212-Pb, 212-Bi, 205/206-Bi, 111/131-I as radionuclides (Jones *et al.*, 1992). The mAbs conjugated with α-particle emitting radionuclides were known to be more cytotoxic reagents because of releasing high energy (≈ 8 million eV; 1 eV=1.602×10^{-19} J) to nucleus of the cells (Macklis et al, 1988), while the toxin conjugates have such short comings as poor internalization or passage. Kozak (Kozak *et al.*, 1985), one of the authors, and his collaborators, reported that anti-Tac alone was unable to inhibit the proliferation or protein synthesis of most leukemic T-cell lines, but ^{212}Bi-conjugated anti-Tac efficiently reduced protein synthesis of HUT-102B2, IL-2 receptor positive cells by more than 80% without affecting IL-2 receptor negative cells at the dose of 6 rad/ml. Uchiyama (Uchiyama *et al.*, 1981) and his coworkers, Depper (Depper *et al.*, 1983) and his collegues, by using anti-Tac as a mAb directed against the human p55 IL-2 R subunit, demonstrated that alloactivated cytotoxic T lymphocytes (CTL) were in the IL-2R positive population and could be eliminated by anti-Tac plus complements after activation of T cell. It was also proved that anti-Tac conjugated either with phycoerythrin (Pseudomonas exotoxin) or Bismuth-212 is effective *in vitro* in specifically eliminating cytotoxic effector cells (Kozak *et al.*, 1990). Recently it was reported that a rat IgG2c anti-murine p55 IL-2R monoclonal antibody (2E4) could be successfully chelated and radiolabelled either with indium-111 or bismuth-205/206 and that both conjugates had similar blood clearance and tissue distributions indicating that indium and bismuth were not prematurely eluting from the chelate-antibody complex (Jones *et al.*, 1992).

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However, there has been no attempt to conjugate 2E4 with bismuth-212 and to test the antitumor activity of ^{212}Bi-2E4. In the present study we intended to demonstrate the antitumor activity and the binding integrity of ^{212}Bi-2E4 against EL4J3.4, a p55 IL-2R positive thymoma cell line, and to compare the in vivo activities between ^{212}Bi-anti-Tac and ^{212}Bi-2E4.

MATERIALS AND METHODS

Animals

C57BL6 mice (6 week old females) were obtained from Charles River Laboratories, Kingston, NY, housed in microisolators with filter tops and kept on laminar-flow shelves. Food and water were provided *ad libitum*.

Cell Lines

EL4J, EL4J3.4 and 5.1.2 cell lines were used in this study. EL4J is a subline of the EL4 murine thymoma cell line (provided by Dr. T. Taniguichi, Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan) that is negative for IL-2 receptor expression. EL4J3.4 is the thymoma line (a generous gift of Thomas Malek, Univ. of Miami, Miami, FL, USA) which has been transfected with the murine p55 IL-2R subunit and shown to express approximately 1000 to 3000 high affinity IL-2R(Saragovi *et al.*, 1987), 5.1.2 is a murine splenic T cell line from MRL-lpr/lpr mice. They were maintained in RPMI 1640 (Gibco Lab., Grand Island, NY) containing 10% FCS (Reheis Chem. Co., Phoenix, AZ), 4×10^{-4}% 14.3m Mercapthoethanol (Sigma,MO,USA), 1% 100× glutamine and 1% 100× penicilline/streptomycin (Sigma).

Antibodies

The mAbs used in this study were anti-Tac and 2E4. The mAb anti-Tac is a murine IgG_{2a} antibody that is directed to the human P55 IL-2R subunit and the mAb 2E4 is a rat IgG_{2c} antibody to murine p55 IL-2R subunit. Anti-Tac and 2E4 were purified either by protein-A Sepharose or by DEAE-52 cellose (Whatman) followed by sodium sulfate precipitation (Sigma).

FACS Analysis of IL-2 Receptor Positive & Negative Cell Lines

Hank's balanced salt solution without phenol red (Sigma) containing 0.1% NaN_3 and 3% fetal calf serum was used as the staining buffer. EL4J, EL4J3.4 and 5.1.2 (1×10^6 cells) were added into each tubes with 100 µl of 2E4 (1st Ab) and incubated on ice bath for 30 min. Then they were washed twice and incubated with 100 µl of FITC-conjugated goat anti-rat IgG (Tago, Burlingame, CA, USA) for 30 min. After wash the cells were resuspended in 250 µl of staining buffer and analyzed on a FACS machine (Ormerod, 1990).

Bismuth-212 Labeling of the Anti-Tac and 2E4 Monoclonal Antibodies

Bismuth-212 was linked to 2E4 and anti-Tac monoclonal antibodies by use of a bifunctional chelating agent, N-[2-amino-3-(p-isothiocyanatophenyl)propyl]-trans-cyclohexane-1,2-diamine-N,N'N'N",N"pentaacetic acid [(A), p-SCN-Bz-CHX-B-DPTA](Chelate/mAb of 1. 3), The chelating agent, CHXB-DPTA was synthesized and coupled to the mAbs as previously described (Brechbiel *et al.*, 1991). Typically, 8-19 mg of antibodies at 7 mg/ml was dialyzed against HEPES buffer (azide-free) for six hours. The antibody preparations were then conjugated to CHXB-DPTA ligand. Free ligand was separated from conjugated antibody by serial dialysis.

Bismuth-212 was obtained from radionuclide generator and the procedures for chelate coupling and radiolabeling are described in detail elsewhere (Parenteau *et al.*, 1992). The generator was eluted with 0.75 ml of 2 M HCl to produce ^{212}Pb-Bi, which was evaporated to dryness by vacuum centrifugation (Savant), redissolved in 250 µl of 0.1 M HCl, and ^{212}Pb was quantitatively absorbed on a 1×2.5 mm column of AG-50 WX4 (200-400 mesh) H^+ form resin (Bio-Rad) and rinsed with 2 ml of deionized water. After 2 hr, ^{212}Bi was eluted with 0.15 ml of 0.1 M HI. For protein incorporation, the ^{212}Bi/HI solution (≈3 mCi; 1 Ci=37 GBq) was reduced to 50 µl and brought to PH 4-4.5 with 0.3 M phosphate/0.10 M acetate, PH 6.9, and immediately underwent reaction for 15 min with 30 µg of anti-Tac. The resulting ^{212}Bi-labeled anti-Tac and 2E4 were purified by high performance liquid chromatography on a TSK-3000 column with 0.1 M phosphate-buffered saline eluant. Protein specific activity was 40 µCi/µg(1 Ci=37 GBq) was achieved. ^{212}Bi-CHXB-2E4 and ^{212}Bi-CHXB-anti-Tac will be referred to hereafter as ^{212}Bi-2E4 and ^{212}Bi-anti-Tac.

3H-2E4 Competitive Binding Assay

Binding integrity of 2E4 after conjugation with CHXB DTPA was evaluated in competitive binding assay. Unmodified 2E4 and CHXB-2E4 were compared in their ability to compete with a nonsaturating (10 ng) amount of 3H-2E4 binding to 5.1.2 cells(10^6 cells) (Jones *et al.*, 1992). Tritium labeling of 2E4 to specific activity was done according to Tack and co-workers (Tack *et al.*, 1980). Cell-associated 3H-labeled 2E4 and 2E4-CHXB were measured by centrifugation through 1 M sucrose and the pellet was resuspended in 0.1 ml of 0.15 M phosphate buffered saline and counted in a scintillation counter (Kozak *et al.*, 1985).

Inhibition of DNA Synthesis with ^{212}Bi-2E4

Cytotoxicity of ^{212}Bi-2E4 was evaluated after treatment of EL4J3.4 (a IL-2R$^+$ cell line) and EL4J (a IL-2R$^-$ cell line) by using in vitro killing assay. DNA synthesis of cells treated by ^{212}Bi-2E4 was assayed by [^{3}H] thymidine incorporation 48 hr after treatment of 2×10^5 cells per ml per well in a 96-well plate (Costar, Cambridge, MA, USA). After treatment with various doses of ^{212}Bi-2E4, 200 µl of the cell suspension was aliquoted into a 96 well round bottomed plate (Costar). Cells were washed three times with 0.15 M phosphate-buffered saline by centrifugation and aspiration of supernatants. Cells were then resuspended in 100 µl of RPMI 1640 medium (GIBCO) supplemented with 5% dialysed fetal calf serum. Cells were pulsed for 4 hr with 0.5 µCi of [^{3}H] thymidine per well (New England Nuclear), harvested on a Skatron (Sterling, VA, USA) Mash unit, and counted in a liquid scintillation counter. The experiment was carried out in triplicate and the resulting values were then expressed as a percentage of the [^{3}H] thymidine incorporated into the medium control cell lines that were not treated with ^{212}Bi conjugates (Krejcarek *et al.*, 1977).

In Vivo Antitumor Assay of ^{212}Bi-2E4

As for a tumor implantation, EL4J3.4 (3×10^6 cells/mouse) was injected i.p. into C57BL/6 mice 3 days after i.p. injection of 0.5 ml pristane prime(6,10,14-tetramethyl pentadecane, Sigma) and then various doses (0-200 µCi/mouse) of ^{212}Bi-2E4 was injected intravenously once 3 days after the tumor inoculation, while ^{212}Bi-anti-Tac was used as a positive control. The changes of survival time were monitored every 2 or 3 days for 63 days. Each experimental groups and control group consisted of 5 mice and 10 mice repectively.

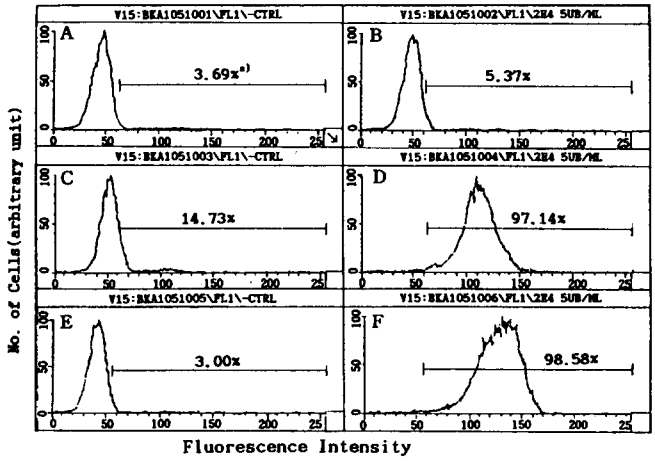

Fig. 1. IL-2 receptor positivity of tumor lines piror to *in vivo* administration. The cells were incubated with 2E4 (B, D and F) or medium (A, C and E) followed by FITC-conjugated F(ab')$_2$ fragment of Goat anti-Rat IgG H & L and then FACS analysis was done. EL4J is a murine thymoma cell line which is negative for IL-2R expression (A & B). EL4J3.4 is the murine thymoma line transfected with the murine p55 IL-2R (C & D). 5.1.2 is a IL-2R$^+$ murine splenic T cell line from MRL-lpr/lpr mice (E & F).
a) Percentage of the cells showing the fluorescence intensity marked by the bar.

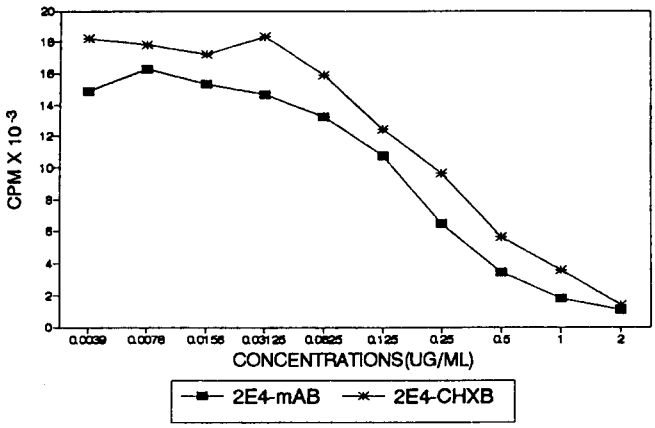

Fig. 2. Sensitivity of competitive binding assay was demonstrated by comparing unmodified 2E4 MAb and CHXB-2E4 MAb(C/P=1.35). A murine IL-2R$^+$ splenic T cell line, 5.1.2 (10^6 cells) was incubated with 5.5 ng of ^{3}H-2E4 alone, or with varying doses of 2E4 MAb and 2E4-CHXB. Cell-associated ^{3}H-labeled 2E4 was measured by centrifugation through 1M sucrose and the pellet was resuspended in 0.1 ml of 0.15 M phosphate-buffered saline and counted in a scintillation counter.

RESULTS AND DISCUSSION

In order to verify the IL-2R expression of EL4J3.4 tumor cell line prior to in vivo and in vitro experiments, EL4J3.4, EL4J, and 5.1.2 (IL-2R$^+$) cells were incubated with 2E4 and then with FITC-conjugated goat anti-rat IgG H & L. On FACS analysis, the percentages of FITC-labeled (IL-2R$^+$) cells of EL4J, EL4J3.4 and 5.1.2 were 5.37, 97.14 and 98.58, respectively (Fig. 1). From these results we could confirm that EL4J3.4 and 5.1.2 are a IL-2R positive cell lines, and EL4J is a IL-2R negative cell line.

Next we examined the immunoreactivity of 2E4-CHXB(Chelate/mAb of 1.35). As shown in Fig.2, the binding of 2E4-CHXB to EL4J3.4 was similar to that of 2E4 indicating that 2E4-CHXB certainly retains the immunoreactivity of 2E4. These results imply that 2E4-CHXB incorperated with α-particle emitting radionuclide bismuth-212(^{212}Bi-2E4) could bind to IL-2R of the cell without losing its immunoreactivity.

Specific cytotoxicity of ^{212}Bi-2E4 was examined by ^{3}H-thymidine uptake assay using EL4J3.4 and EL4J as target cell lines (Fig. 3). The CPM of EL4J and EL4J3.4 cultured in the ^{212}Bi-2E4 free media was 15126 and

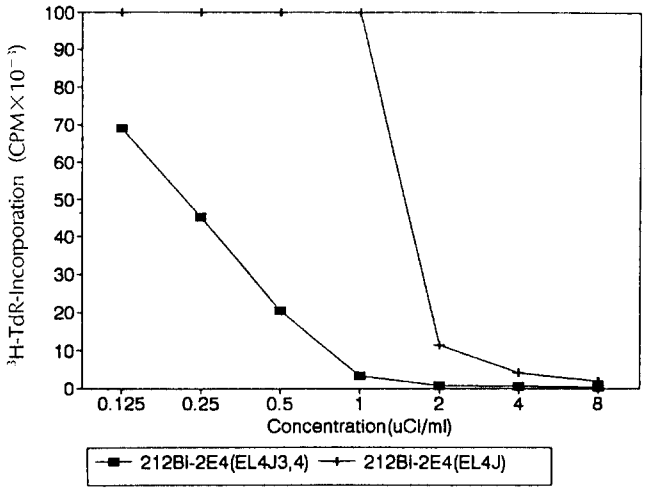

Fig. 3. The specific cytotoxicity of ^{212}Bi-2E4 was compared on EL4J3.4 and EL4J cell lines by measurement of thymidine incorporation. Increasing concentrations of ^{212}Bi-2E4 were added to IL-2R$^+$ Cells (EL4J3.4) or IL-2R-cells(El4J). EL4J and EL4J 3.4 were plated at 10^5/well in round bottom microtiter plates with increasing concentrations of ^{212}Bi-2E4. After a 24 hr incubation, cells were washed three times with PBS and resuspended in RPMI 1640 and pulsed for 12 hr with 0.5 μCi/well of [^{3}H] Thymidine (Sp. act. 64 Ci/mmol; New England Nuclear, Dupont, DE).

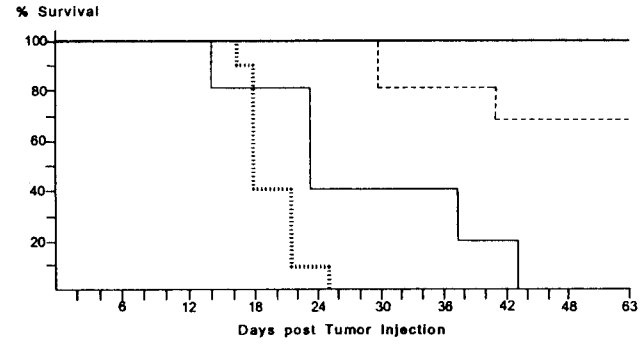

Fig. 4. Effect of ^{212}Bi-2E4 therapy on survival of C57BL/6 mice injected i.p. with EL4J3.4 ascites tumor. EL4J3.4 cells (3×10^6) were implanted i.p. and 3 day later PBS (control IIIII, n=10), 100 μCi of ^{212}Bi-2E4 (---, n=5), 50 μCi of ^{212}Bi-2E4 (—, n=5), and 25 μCi of ^{212}Bi-2E4 (—, n=5) were injected i.v. into the veins of C57BL/6 mice.

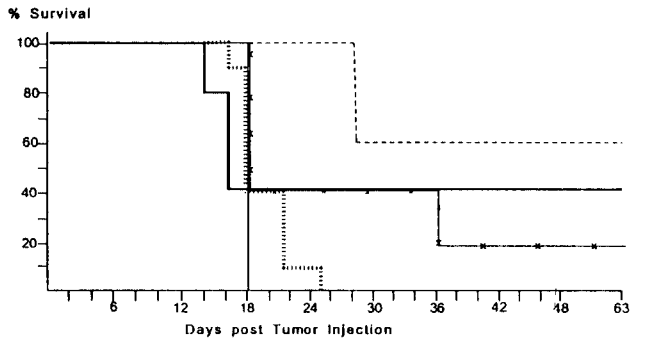

Fig. 5. Effect of ^{212}Bi-Tac therapy on survival of C57BL/6 mice injected i.p. with EL4J3.4 ascites tumor. EL4J3.4 cells (3×10^6) were implanted i.p. and 3 day later PBS (control IIIII, n=10), 200 μCi of ^{212}Bi-αTac2 (—, n=5), 100 μCi of ^{212}Bi-αTac (---, n=5), 50 MCi of ^{212}Bi-αTac (-*-, n=5) and 25 μCi of ^{212}Bi-αTac (—, n=5) were injected i.v. into the veins of C57BL/6 mice.

Table I. Survival time of C57BL/6 mice injected i.v. with ^{212}Bi-2E4 and ^{212}Bi-αTac after inoculation of EL4J3.4

Traetment	Survival Time	MST[a]	P value[b]
PBS	16, 18, 18, 18, 18, 18, 21, 21, 21, 25	19	
100 μCi ^{212}Bi-2E4	31, 43, 63, 63, 63	53	<0.001
50 μCi ^{212}Bi-2E4	63, 63, 63, 63, 63	63	<0.001
25 μCi ^{212}Bi-2E4	14, 21, 21, 37, 43	27	N.S[c]
200 μCi ^{212}Bi-αTac	14, 16, 16, 63, 63	34	N.S
100 μCi ^{212}Bi-αTac	28, 28, 63, 63, 63	49	<0.01
50 μCi ^{212}Bi-αTac	18, 18, 21, 37, 63	31	N.S
25 μCi ^{212}Bi-αTac	18, 18, 18, 18, 18	18	N.S

EL4J3.4 cells (3×10^6 cells/ml) were injected i.p. to pristane-primed C57BL/6 mice. Various doses of ^{212}Bi-2E4 and ^{212}Bi-αTac were injected into the veins of mice 3 days after tumor inoculation. Thereafter, the survival time of each group was monitored twice a week for 63 days.; The number of PBS-treated control was 10 and other group was 5 mice each other.[a] Mean suvival time.

[b] Significantly different from the data of PBS-treated control (EL4J3.4).

[c] Statistically not significant.

20018, respectively. This means that the two cell lines are rapidly growing cell lines. ^{212}Bi-2E4 did not inhibit the thymidine incorporation of EL4J cells up to 1 μCi/ml, which means that ^{212}Bi-2E4 did not bind EL4J cells possibly due to the lack of the ^{212}Bi-2E4 binding site (IL-2R) on EL4J cells. In contrast, ^{212}Bi-2E4 strongly inhibited the incorporation of thymidine by EL4J3.4 cells which express the IL-2R$^+$. Especially at 1 μCi/ml, ^{212}Bi-2E4 showed more remarkable cytotoxicity against EL4J3.4 than against EL4J.

The cytotoxic effect of ^{212}Bi-2E4 against EL4J3.4 ascitic tumor cells in C57BL6 mice bearing the IL-2R positive EL4J3.4 cell was examined. The mean survival time (MST) of the control mice was 19 days while those of the mice treated with 100, 50, 25 Ci of ^{212}Bi-2E4 were 53 days ($p<0.001$), 63 days ($p<0.001$) and 27 days (not significant at $p<0.05$), respctively (Fig. 4). From the fact that 50 μCi of ^{212}Bi-2E4 was more effective than 100 μCi, it was supposed that 100 μCi might be slightly toxic, while 50 μCi was well tolerated. Therefore, it is reasonable to assume the most effective dose to be between 50 μCi and 100 μCi of ^{212}Bi-2E4.

MST of mice treated with 100 μCi of ^{212}Bi-anti-Tac was 49 days ($p<0.001$), whereas those of mice treated

with higher or lower doses of ^{212}Bi-anti-Tac were statistically not significant (at $p<0.05$) as compared to that of the control (Fig. 5 and Table 1). Therefore, in case of ^{212}Bi-anti-Tac, 100 μCi of it appeared as the most effective dose. ^{212}Bi-anti-Tac might be toxic at the concentration of 200 μC, while 25 μCi was not effective at all. From these results it is clear that ^{212}Bi-2E4 is more effective *in vivo* than ^{212}Bi-anti-Tac at all concentrations tested.

ACKNOWLEDGEMENTS

S. H. Kim thanks the Korea Science and Engineering Foundation (KOSEF) for postdoctoral funds and Dr. Robert W. Kozak and Linda Johnes Tiffany for their encouragements and excellent advices on the experiments.

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