Radioimmunotherapy with Alpha-Particle–Emitting Immunoconjugates

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Alpha particles are energetic short-range ions whose higher linear energy transfer produces extreme cytotoxicity. An α-particle–emitting radioimmunoconjugate consisting of a bismuth-212–labeled monoclonal immunoglobulin M specific for the murine T cell/neuroectodermal surface antigen Thy 1.2 was prepared. Analysis in vitro showed that the radioimmunoconjugate was selectively cytotoxic to a Thy 1.2$^+$ EL-4 murine tumor cell line. Approximately three bismuth-212–labeled immunoconjugates per target cell reduced the uptake of [^{3}H]thymidine by the EL-4 target cells to background levels. Mice inoculated intraperitoneally with EL-4 cells were cured of their ascites after intraperitoneal injection of 150 microcuries of the antigen-specific radioimmunoconjugate, suggesting a possible role for such conjugates in intracavitary cancer therapy.

RADIONUCLIDES THAT EMIT α-PARTICLES have a number of physical characteristics that make them attractive candidates for radioimmunotherapy (*1*). Since the path length of an α-particle with an energy of 5 to 8 MeV is on the order of 40 to 80 μm, the effective treatment radius is limited to several cell diameters from the atom that emits the particle, and nonspecific irradiation of distant tissues is eliminated. The high linear energy transfer (LET) of such energetic particles (~100 keV/μm) and the limited ability of cells to repair the damage to DNA from α-particle irradiation contribute to their extraordinary cytotoxicity (*2*). Indeed, at doses on the order of 100 to 200 cGy, α radiation may be 5 to 100 times as toxic as γ or β radiation (*3*). In addition, α-particle–mediated cell killing is essentially insensitive to conditions of necrotic hypoxia, which may compromise the clinical efficacy of low LET radiations (*4*).

Bismuth-212 (^{212}Bi) is an α-emitting radioisotope that is available via a ^{224}Ra generator system (*5*). This isotope has a short physical half-life (60.55 minutes) and decays either through ^{208}Tl or ^{212}Po to stable ^{208}Pb. Kozak and colleagues have demonstrated that ^{212}Bi can be bound to a monoclonal antibody (mAb) conjugated to the chelating agent diethylenetriaminepentaacetic acid (DTPA) (*6*), an approach previously used for labeling antibodies with ^{111}In (*7*).

We have used cyclic DTPA dianhydride to prepare a ^{212}Bi-labeled murine immunoglobulin M (IgM) mAb to the surface antigen Thy 1.2. This radioimmunoconjugate (RIC) recognizes one of the allelic forms of the murine antigen Thy 1, which is present on the surface of normal and malignant murine T cells and related tissues (*8*). We report here that this ^{212}Bi-labeled RIC is highly efficient at eradicating Thy 1.2$^+$ EL-4 murine lymphoma cells in vitro and in vivo and this cytotoxicity is antigen selective.

Anti–Thy 1.2 IgM and nonspecific bovine IgM were conjugated with five to eight molecules of DTPA per antibody molecule as described (*9*) and radiolabeled with ^{212}Bi (*10*). We evaluated the ability of these α-emitting RICs to deliver cytocidal doses of radiation to the Thy 1.2$^+$ EL-4 target cells in vitro by using a [^{3}H]thymidine incorporation assay as an indicator of cell proliferation (Fig. 1).

When compared to the untreated control, the ^{212}Bi-labeled anti–Thy 1.2 RIC was highly efficient at inhibiting [^{3}H]thymidine incorporation. At RIC doses of ≥0.2 μCi per 10^5 cells (≥1 μCi/ml), essentially complete inhibition (≥98%) of cell proliferation was observed [median inhibition constant (IC_{50}) = 0.85 μCi/ml]. This corresponds to an antibody dose of 16 to 80 ng per 10^5 cells (two to ten times mAb excess). Parallel experiments had shown that at a dose of the unconjugated anti–Thy 1.2 mAb of up to 10 μg per 10^5 cells, the amount of inhibition of cell proliferation was insignificant. In contrast, the nonspecific ^{212}Bi-labeled RIC showed limited cytotoxicity, with no demonstrable effects when 10^5 cells were incubated with up to ~2.5 μCi (12.5 μCi/ml). However, a gradual decrease in [^{3}H]thymidine uptake through the range of 2.5 to 15 μCi per 10^5 cells (12.5 to 75 μCi/ml) was observed (IC_{50} = 35 μCi/ml).

Since the α-particle emitted during the decay of ^{212}Bi has a mean energy of 7.83 MeV (*11*), one can calculate a total dose of 0.28 cGy/min for 1 μCi/ml (*12*). Therefore, the IC_{50} dose for the ^{212}Bi-labeled anti–Thy 1.2 RIC is 0.94 cGy, whereas that for the nonspecific RIC is 38.5 cGy (the cells were incubated for 4 minutes with either RIC). Thus, the antigen specificity of the RIC contributes a factor of ~40 to its in vitro cytotoxicity. In contrast, the IC_{50} of EL-4 cells for ^{137}Cs γ radiation when measured by the same [^{3}H]thymidine incorporation assay is ~100 cGy. Consequently, the relative radiotoxic effectiveness of the nonspecific ^{212}Bi-labeled IgM (^{212}Bi-IgM) compared to ^{137}Cs is 2.6, and that of ^{212}Bi-labeled anti–Thy 1.2 RIC is 106. The IC_{50} dose of 38.5 cGy is consistent with the D_o (the dose required to reduce the number of clonogenic cells to 37% of their former value) found for other mammalian cells (*12*) when the α-particle is free in solution. The low calculated absorbed dose (0.94 cGy) for the ^{212}Bi-labeled specific antibody and its high

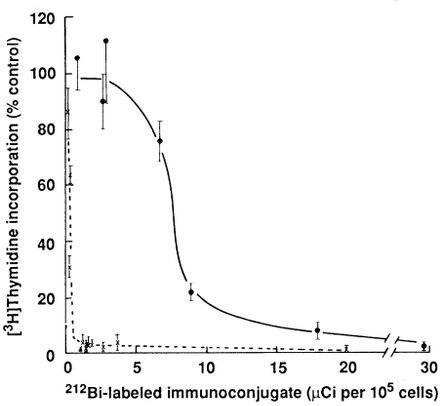

Fig. 1. Cytotoxicity and antigen specificity of ^{212}Bi-labeled RICs. Aliquots of 10^5 EL-4 cells (5×10^5 cells per milliliter) were incubated for 4 minutes on ice with increasing concentrations of ^{212}Bi-labeled immunoconjugate [either antigen-specific ^{212}Bi-labeled anti–Thy 1.2 (x–––––x) or antigen-nonspecific ^{212}Bi-labeled IgM RIC (●——●)]. These assays were carried out under conditions of antibody excess. The mixture was then diluted to 15 ml in phosphate-buffered saline (PBS) and allowed to stand on ice for 15 minutes with constant agitation. The cells were washed, counted, suspended in RPMI medium (containing 10% fetal calf serum) at 10^4 cells per milliliter, and plated in 96-well microtiter plates (0.1 ml per well). Cells were incubated at 37°C for 3 days and were then exposed for 4 hours to [^{3}H]thymidine (1 μCi per well). The cells were harvested and washed, and DNA-incorporated [^{3}H]thymidine activity was assayed. Each data point represents the mean ± standard deviation of three to six duplicate assays. Data from multiple experiments were pooled.

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radiotoxic effectiveness indicate that the dose to the target cells is mainly derived from the cell-bound ^{212}Bi-labeled anti–Thy 1.2 RIC rather than from the extracellular medium.

To calculate the number of ^{212}Bi atoms needed to inhibit EL-4 proliferation, we incubated EL-4 cells with the RIC under conditions of antigen excess. The results (Fig. 2) indicate that the binding of ^{212}Bi-labeled anti–Thy 1.2 was linear with increasing antibody concentration. Analysis of [^{3}H]thymidine incorporation showed that approximately three ^{212}Bi atoms per cell inhibited cell proliferation by ≥98%. This is in good agreement with the results obtained with the α emitter ^{211}At (*12*).

Despite this high level of cytotoxicity in vitro, the 60.55-minute physical half-life of the ^{212}Bi imposes certain restrictions on its use as an in vivo therapeutic agent. Clinical studies suggest that injection of RICs into restricted body cavities such as the peritoneum may result in more rapid and specific radiolabeling of intraperitoneal target structures (*13*). The ^{111}In immunoscintigraphy of mice injected either intravenously or intraperitoneally with the anti–Thy 1.2 IgM RIC shows that both intravenous and intraperitoneal injections resulted in rapid, antigen-specific labeling of the splenic tissue (*14*). However, while the intravenous route resulted in high levels of systemic circulating RIC immediately after injection, most of the radioactivity of the anti–Thy 1.2 IgM RIC was confined to the peritoneal cavity for at least 2 hours after an intraperitoneal injection, in accord with the known slow vascular extravasation of such large pentameric IgM molecules (*15*). We sought to exploit the long intracavitary retention time and short physical half-life of the ^{212}Bi-labeled RICs for intracavitary radioimmunotherapy.

By preparing RICs of the IgM class that incorporate an α-particle–emitting radioisotope with a short half-life, we developed a cytotoxic, slowly diffusing RIC that should maximize the dose to intraperitoneal target structures and minimize the nonspecific dose to the bone marrow and other extraperitoneal tissues. We performed a series of experiments in vivo in which C57BL/6 mice were inoculated intraperitoneally with congenic Thy 1.2$^+$ EL-4 tumor cells and then treated with ^{212}Bi-labeled anti–Thy 1.2 or ^{212}Bi-IgM. This tumor cell line grows intraperitoneally, proliferating rapidly, producing ascites, and giving rise to diffuse tumor deposits that coat the intestinal serosa and the peritoneal lining. Animals eventually die of inanition. In the present experiments normal C57BL/6 mice were inoculated intraperitoneally with 10^7 tumor cells. After 24 hours, various doses of either ^{212}Bi-labeled anti–Thy 1.2 or ^{212}Bi-IgM were injected intraperitoneally for a total of two to four injections (0.5 ml each) given over 4 to 8 hours. One group of control animals was injected with a similar quantity of DTPA-conjugated unradiolabeled anti–Thy 1.2 mAb. Animals were observed for 70 days after tumor inoculation (Fig. 3). No attempts were made to determine the actual absorbed radiation dose to the tumor cells.

Untreated animals or animals treated with the unradiolabeled DTPA-conjugated anti–Thy 1.2 mAb invariably died by day 20. Animals treated with 180 to 1000 μCi of ^{212}Bi-IgM showed some prolongation of survival, but all animals eventually died by day 33 (Fig. 3A). In contrast, animals treated with 40 to 100 μCi of ^{212}Bi-labeled anti–Thy 1.2 showed significant prolongation in survival (Fig. 3B), while most of those treated with 150 or 230 μCi were cured of their tumor burden (Fig. 3B). The only obvious acute toxicity in the last-mentioned animals was an occasional lesion at the site of injection, which was sometimes observed 1 to 4 weeks after RIC treatment. These lesions probably represent soft tissue radiation injury due to trapping of some of the radiolabeled RIC during the intraperitoneal injection. Animals injected with >400 μCi of ^{212}Bi-labeled anti–Thy 1.2 or nonspecific RIC showed some classical signs of radiation exposure such as weight loss, diarrhea, and infection.

While we found that the DTPA-conjugated ^{212}Bi-labeled immunoconjugates were able to sterilize a significant intraperitoneal tumor burden, we observed that such conjugates were not highly stable in vivo. Within 2 hours after intraperitoneal injection, up to 30% of the injected dose of ^{212}Bi was found in the renal collecting system and bladder. This rapid urinary excretion pattern is similar to that which has been found after injection of free ^{206}Bi (*16*), indicating the in vivo instability of the DTPA-chelated ^{212}Bi and the need for development of more stable chelates for clinical use.

Gansow and colleagues have recently developed a modified form of DTPA in which benzyl-isothiocyanyl and alkyl side chains are attached to the backbone of the DTPA (*17*). Preliminary studies show that ^{212}Bi-labeled immunoconjugates constructed with this bifunctional DTPA analog are significantly more stable in vivo than those constructed with the unmodified DTPA, presumably partly because of the increased che-

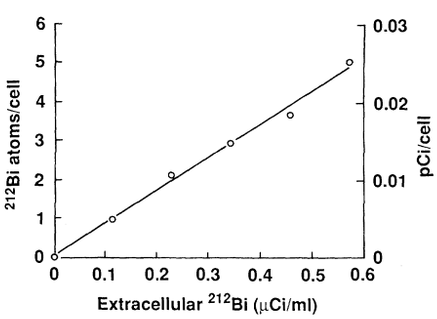

Fig. 2. Target-cell binding of ^{212}Bi-labeled anti–Thy 1.2 RICs. Aliquots of 10^6 cells per 200 μl of medium were incubated with 10, 8, 6, 4, and 2 μl of ^{212}Bi-labeled antibody (8 μg/ml, 2.6 μCi/μg). After 4 minutes at 4°C, the cells were diluted with 10 ml of cold medium and centrifuged, and the supernatant was decanted. The cells were washed, resuspended, and counted, and the radioactive content of aliquots was determined in a gamma counter. [^{3}H]Thymidine incorporation after 3 days in culture was also measured in two samples preincubated with 2 or 6 μl of ^{212}Bi-labeled anti–Thy 1.2, as well as in samples incubated with 10 μl of unlabeled anti–Thy 1.2 or with 10 μl of PBS. All assays were carried out under conditions of antigen excess and in triplicate. Uptake (picocuries per cell) of ^{212}Bi-labeled anti–Thy 1.2 by EL-4 cells is plotted as a function of extracellular radioactive concentration (microcuries per milliliter). The cell-bound activity was used to calculate the number of ^{212}Bi atoms per cell.

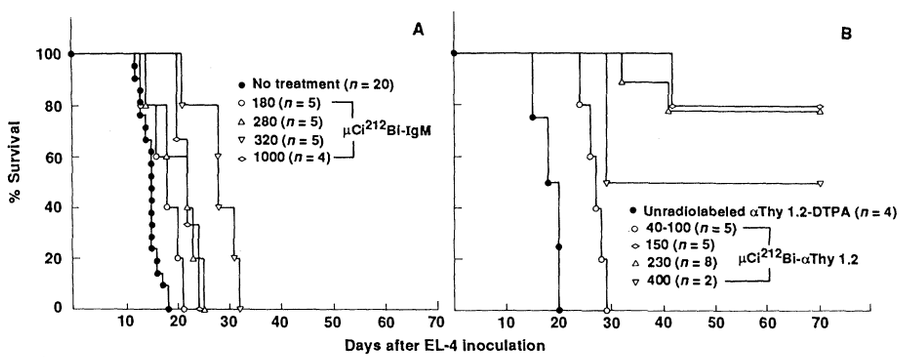

Fig. 3. Intracavitary ^{212}Bi-labeled RIC therapy in mice. C57BL/6 mice were inoculated intraperitoneally with 10^7 Thy 1.2$^+$ EL-4 tumor cells and treated 24 hours later with various radioactive concentrations of ^{212}Bi-labeled nonspecific IgM RIC (**A**) or tumor-specific ^{212}Bi-labeled anti–Thy 1.2 antibody (**B**).

late denticity provided by the additional side arm (*17*). Further studies with this interesting compound are currently in progress.

The results obtained here with ^{212}Bi are not entirely consistent with those showing a prolongation in survival and cure of an experimental ovarian cancer with ^{211}At nonspecific radiocolloid (*18*). The longer 7.2-hour half-life of this α-particle emitter as well as its physical form may be responsible for the observed differences.

The experiments described in this report demonstrate that ^{212}Bi-labeled α-particle–emitting immunoconjugates exhibit high levels of antigen-selective cytotoxicity in vitro, requiring very few ^{212}Bi atoms per target cell to reduce [^{3}H]thymidine incorporation to background levels. These RICs are capable of antigen-specific cure of mice inoculated with malignant ascites.

Our curative therapy of an intraperitoneal murine lymphoma using a radionuclide with a short physical half-life coupled to an antibody of the IgM isotype that remains confined to the peritoneum for relatively long periods of time represents an attempt to design a class of RICs that may eventually prove valuable in the treatment of intraperitoneal malignancies such as ovarian carcinoma and metastatic carcinomatosis from gastrointestinal cancer. The high LET and 5- to 7-cell-diameter path length of the α-particle ejected from the ^{212}Bi nucleus make this radionuclide ideal for the treatment of tumors in which most clonogenic cells are accessible to antibody binding, and especially for the treatment of micrometastases. The short half-life of the isotope limits doses to nontarget structures in those cases where rapid antibody binding to target tissues can be achieved. Further progress in this field will be made by the judicious design of RICs that match the physical and chemical characteristics of the radionuclide and the antibody with the immunologic and clinical characteristics of the proposed tumor target.

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HIV-1 Production from Infected Peripheral Blood T Cells After HTLV-I Induced Mitogenic Stimulation

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The human immunodeficiency virus type 1 (HIV-1) and human T-cell leukemia virus type I (HTLV-I) are two distinct human retroviruses that infect T cells. Recent epidemiologic studies have identified a cohort of individuals that are coinfected with both viruses. It is reported here that human peripheral blood leukocytes infected with HIV-1 in vitro can be induced to produce large quantities of HIV-1 after mitogenic stimulation by noninfectious HTLV-I virions. It is also shown that HTLV-I virions may exert this effect prior to, immediately following, or well after the cells are infected with HIV-1. These results provide further impetus for epidemiologic studies of dually infected individuals to determine whether HTLV-I may act as a cofactor for acquired immunodeficiency syndrome (AIDS).

THE HUMAN IMMUNODEFICIENCY virus type 1 (HIV-1) infects cells bearing the CD4 molecule, which include certain T cells and macrophages (*1*). The human T-cell leukemia viruses types I (HTLV-I) and II (HTLV-II) cause various forms of T-cell leukemia and lymphoproliferative disorders (*2*) and can immortalize peripheral blood T cells in vitro (*3*). Since some patients are infected with HIV-1 as well as HTLV-I or HTLV-II (*4–6*), we investigated whether HTLV would augment HIV production in vitro.

HTLV-I containing supernatants and particles prepared by high-speed centrifugation are mitogenic for T cells without directly infecting these cells (*7, 8*). Gradient-purified virions of HTLV-I and HTLV-II are similarly mitogenic (*9*). Human sera containing antibodies to HTLV-I as well as a monoclonal antibody to the HTLV-I envelope glycoprotein gp46 neutralize the virus-induced proliferation of T cells (*8*), suggesting that gp46 mediates this effect. Both $CD4^+$ and $CD8^+$ T cells respond to HTLV-I stimulation (*8, 9*). Because HTLV-I is mitogenic for the same cells ($CD4^+$) that may be infected by HIV-1, we tested whether HTLV-I induced proliferation of HIV-1–infected peripheral blood leukocytes (PBL) results in the increased production of HIV-1.

As shown in Table 1, HIV-1–infected PBL increased production of HIV-1 when exposed to HTLV-I (*10, 11*). A similar increase in HIV-1 production occurred when PBL were stimulated with phytohemagglutinin (PHA) (*12*). The increased production of HIV-1 was not due to HTLV-I–induced T cell transformation, because HTLV-I cell-free virions are generally not infectious (*13*). Furthermore, no immortalization of PBL incubated with purified HTLV-I virions occurred under these conditions.

The cumulative amount of HIV-1 produced by HTLV-I–stimulated PBL depended on the concentration of HTLV-I used to stimulate the cells (Fig. 1). The highest concentration of HTLV-I virions tested

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