Cure of SCID Mice Bearing Human B-Lymphoma Xenografts by an Anti-CD74 Antibody-Anthracycline Drug Conjugate

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ABSTRACT

Purpose: The purpose of this research was to test the therapeutic efficacy of an anthracycline-antibody conjugate for the treatment of human B-cell lymphoma in a preclinical animal model.

Experimental Design: Doxorubicin (dox) conjugates of the murine and humanized versions of the anti-B-cell antibody LL1, targeting CD74, were prepared, along with a nonspecific control dox-antibody conjugate, targeting carcinoma embryonic antigen. Antibody conjugates carried approximately 8–10 drug molecules attached site-specifically at thiols of reduced interchain disulfide bonds. Conjugates were tested, initially in vitro, and then for therapeutic efficacy in a systemic model, using a lethal i.v. dose of Raji cells in SCID mice.

Results: Dox-LL1 conjugates were shown to be stable and 3-fold more effective in vitro against the human B-cell Burkitt’s lymphoma line, Raji, compared with the nonspecific control conjugate that did not target CD74 or B cells. When SCID mice were given an i.v. dose of 2.5 million Raji cells, they would die of disseminated disease within 15–25 days postinjection. A single dose of dox-LL1 conjugate, 117–350 μg, given 5 days to 14 (advanced disease) days after injection of the Raji cells resulted in cure of most animals out to 180 days after injection of the cells, whereas animals in treatment control groups were not cured. The dose of dox-LL1 found useful in this work corresponds with a significantly lower drug dose than reported previously with other drug-antibody conjugates.

Conclusion: CD74 appears to be a uniquely useful target antigen for delivery of drugs, effecting cures of animals with single, low doses of conjugate.

INTRODUCTION

Conjugates of monoclonal antibodies (MAbs) with drugs or toxins have been investigated for many years as a potential approach to delivering these agents more specifically to cancers (1). One such agent, Mylotarg (gemtuzumab ozogamicin), a conjugate of the anti-CD33 antibody with the highly potent cytotoxic drug, calicheamicin, has gained Food and Drug Administration approval for treatment of CD33-positive acute myeloid leukemia in patients over age 60 in first relapse (2). Other drug-MAb conjugates are currently in development for the treatment of various solid tumors. Pursuing a similar strategy of using a supertoxic drug, a MAb conjugate of a maytansinoid drug (C242-DM1) was described, and shown to eradicate large colon tumor xenografts in athymic mice (3). Liu et al. (3) contended that MAb conjugates of conventional chemotherapy drugs, such as doxorubicin (dox; Ref. 4) or vinblastine (5), had inadequate potency to elicit a clinical effect, and showed that C242-DM1 cured animals at a 50-fold lower dose than an earlier-described dox-BR96 MAb conjugate (4). Subsequently, the dox-BR96 failed to show significant efficacy in a Phase II clinical study in breast cancer patients (6).

All of the above conjugates used an antibody that internalizes into target cells, and this is considered necessary for efficacy. Our two anti-B-cell antibodies, termed LL1 (anti-CD74; II; invariant chain) and LL2 (epratuzumab; anti-CD22; Ref. 7), have demonstrated, in contrast with other antibodies, such as against CD20, rapid internalization of radiolabels targeted to lymphomas (8, 9). The LL1 antibody exhibits the highest rate of accumulation inside a target B cell of any of the MAbs we have tested, with 8 × 10^6 LL1 molecules per cell per day bound, internalized, and catabolized (10).

CD74 is a type-II transmembrane chaperone molecule that associates with HLA-DR, inhibiting binding of antigenic peptides to the class-II antigen presentation structure (11). It is expressed on the surface of cells (12) and directs transport from the surface to an endosomal compartment within the cell (13, 14) where rapid proteolysis occurs (15). Its roles in MHC class II synthesis and antigen presentation have been reviewed (16), and it has also been demonstrated on a T-cell lymphoma (HUT 78), a melanoma (SK-MEL-37), and a colonic carcinoma (HT-29) cell line after IFN-γ treatment (17), as well as on 48 of 126 clinical samples of gastric cancer (18) and several renal cell carcinoma cell lines (19).

We have shown potent activity of LL1 conjugates of radionuclides against CD74+ B cells, particularly using Auger electron emitters (20, 21), presumably because of its property of rapid internalization. This, with the other factors above, encouraged us to test this MAB for drug delivery. We decided to test an anthracycline for several reasons. Anthracyclines are water-soluble and not too hydrophobic, making them readily compatible with aqueous protein solutions. They have several chemically reactive groups. Many hundreds of anthracycline...
analogue are known (22), with dox, epirubicin, daunorubicin, and idarubicin approved in the United States for various cancer indications. Analogue up to 1000 more toxic than dox itself, such as 2-pyrrolinodoxorubicin, have been described and used in preclinical targeting studies (23). Finally, dox itself has been proposed to act by several mechanisms, including inhibition of topoisomerase II, intercalation into DNA, and effects on cell membranes (22, 24).

**MATERIALS AND METHODS**

**Cell Lines and MAbs.** Raji Burkitt’s B-lymphoma cells were obtained from the American Type Culture Collection (Rockville, MD), and were grown in RPMI 1640 containing 12.5% fetal bovine serum (Hyclone, Logan, UT), supplemented with glutamine, pyruvate, penicillin, and streptomycin (Life Technologies, Inc., Grand Island, NY). MAb murine LL1 (mLL1) to CD74 (an IgG1) was described previously (7) and was prepared on protein A columns by standard procedures. A nonreactive control MAb, murine MN-14 (mMN-14; anticarcinoembryonic antigen; Ref. 25) was also used.

**Conjugate Preparation.** In a typical procedure, 4(4-N-maleimidomethyl)cyclohexane-1-carboxyl hydrazide hydrochloride (33.2 mg) and dox (9.8 mg) were mixed in 2 ml of DMSO and warmed for 30 min at 50°C. The desired intermediate was purified from starting materials on a C-18 reverse-phase high-performance liquid chromatography column (19 x 300 mm), and lyophilized for storage. MAbs (16 mg; 0.1 µmol) were partially reduced by incubating them 40 min at 37°C in 40 mM DTT in 0.1 M sodium phosphate buffer (pH 7.5) containing 5 mM EDTA. They were purified on spin-columns of Sephadex [in 0.1 M sodium acetate buffer (pH 4.4)], and through a short column of Bio-Beads SM-2 (Bio-Rad, Hercules, CA), equilibrated in the same buffer. They were purified on spin-columns of Sephadex G50/80 in 50 mM acetate buffered saline (pH 5.3) and 2 mM EDTA. The number of thiol groups on the MAb was determined by the Ellman assay (26). For conjugation, MAb-SH and activated dox were incubated together on ice for 15 min, with dimethylformamide as cosolvent at a final concentration of 15–20%. The product was purified on G50/80 Sephadex [in 0.1 M sodium acetate buffer (pH 4.4)], and through a short column of Bio-Beads SM-2 (Bio-Rad, Hercules, CA), equilibrated in the same buffer. A typical conjugate analyzed for an average of 8–10 dox molecules per unit of MAb. Conjugates were lyophilized for long-term storage, and characterized by matrix-assisted desorption ionization-time of flight mass spectral analysis and UV absorbances at 280 and 496 nm, with the 496 nm reading indicative of the anthracycline concentration, as measured by comparison to a standard curve.

**In Vitro Toxicity.** Lymphoma target cells (3.75 x 10^5/well) were plated in 24-well plates in 1.5 ml of medium in the presence of MAb conjugates, over 2 days, at the concentrations indicated. At day 2, after the cell count, the entire contents of each well were transferred to T30 flasks containing 20 ml of media, to maintain the cells in exponential growth. Toxicity was quantitated by viable cell counts, using trypan blue staining to identify dead cells. Cultures were maintained for 21 days, allowing a single viable cell to be readily detected. The functional percentage cell kill was calculated from the growth curves, based on the time required for 16-fold cell multiplication, as described previously in detail (20).
duced MAbs ensured coupling solely at free thiols, while consistently generating a substitution ratio of around 8–10 dox/MAb, without protein aggregation (Fig. 1, bottom). Dox:MAb substitution ratios were determined by measuring UV absorbances of the conjugates at λ 280 and 496, comparing the λ 496 values to standard solutions of dox, and correcting the λ 280 observed values for the contribution of the anthracycline ring, hence calculating both protein and anthracycline concentrations. We confirmed these estimates by matrix-assisted desorption ionization-time of flight mass spectral analyses of the conjugates, in comparison with unsubstituted MAbs.

Potency and specificity were tested in vitro by comparing toxicity of the dox-mLL1 conjugate to a nonreactive MAb conjugate prepared in a similar manner at the same substitution ratio (dox-mMN-14). Fig. 2 shows the toxicity of dox-mMN-14 and dox-mLL1 against Raji cells after a 2-day incubation with the conjugates. The specific antibody conjugate appeared ~3-fold more potent than the nonreactive dox-MAb conjugate.

**Therapy Studies in Tumor-Bearing SCID Mice.** Conjugates were tested in a model of non-Hodgkin’s lymphoma, by administering 2.5 × 10⁶ Raji cells i.v. into SCID mice. If left untreated, mice die of disseminated disease, generally within 18–25 days postinjection of cells. The highest dose of conjugate given to each animal was 350 μg of dox-MAb. In preliminary work, doses up to 850 μg of dox-mLL1 per animal showed little toxicity, and we decided on the 350 μg dose because it would probably be antigen saturating. Comparison to an untreated control group of animals showed a remarkable effect for the dox-mLL1 conjugate (Fig. 3). Nine of 10 mice were cured with a single 117 μg dose of dox-mLL1, and significant life-extension over untreated animals was obtained even with a single 39 μg dose of dox-mLL1. In a specificity test, comparing dox-mLL1 and non-CD74 binding dox-mMN-14, 90% of the mice treated with dox-mLL1 remained tumor free at the termination of the experiment, at 6 months (Fig. 4). In comparison, little or no therapeutic effect was seen with an equivalent dose of unsubstituted mLL1 (data not shown).

We then tested the dox-mLL1 against mice having more advanced disease, and show the results of delaying for longer times before commencing treatment (Fig. 5). Mice were injected with single 350-μg doses of dox-mLL1 at 5, 10, and 14 days after Raji cell injection. Ten of 10 animals given a single 350-μg dose of dox-mLL1 10 days after Raji cell inoculation survived for 180 days, whereas 9 of 10 untreated animals were dead at 23 days after inoculation. Finally, the corresponding dox-humanized LL1 conjugate was tested in parallel with the murine conjugate and other control groups (Fig. 6). At 350 μg/animal, both dox-mLL1 and dox-humanized LL1 cured all 10 of animals in each group, whereas free drug, free humanized LL1, a mixture of the two, or dox-hMN-14 had little or no effect compared with untreated animals.

**DISCUSSION**

Attachment of the drugs at interchain disulfide/thiol groups (28) after limited disulfide bond reduction ensures that substitution is limited by the number of interchain disulfides; 5 of 10 on a murine IgG1 and 4 of 8 on a human framework IgG1. Also, the approach ensures that the dox addends remain remote from the MAb antigen-binding sites. The Raji animal model is similar to one described previously using Daudi cells (27), but the former cell line was found more consistent in growth pattern and
lethality. Initial testing of the dox-mLL1 conjugate was performed using single 350 µg doses/animal. Because no therapeutic effect was seen without treatment, with antibody alone, or with control dox-mMN-14 conjugate, the observed data are due to specific targeting of dox to CD74 by the dox-mLL1. The drug equivalent dose of free dox used in this experiment was ~9 µg. In comparison, the maximum tolerated dose of free dox was reported recently as being 354 µg (309.5 µmol/kg) in mice (29), ~40-fold higher than the dox equivalent dose given here with the dox-mLL1.

The single dose of dox-mLL1 needed to cure animals is as low as 117 µg (equivalent to <3 µg of free dox) when given 5 days after the Raji cells, and is 350 µg (or possibly less) when given as many as 10 days after injection of Raji cells. In comparison, Trail et al. (4) required multiple dosing to cure 100% of tumor-bearing animals using dox-BR96, achieving only 50% cures with a single 21-mg dose of conjugate, which is ~180 times higher than the 117 µg dose used here with dox-mLL1. Liu et al. (3) used maytansinoid conjugates of the C242 MAb (C242-DM1) and prevented tumor growth in animals given COLO 205 tumor cells 7 days previously, by giving five daily doses of ~12 mg/kg (~1225 µg total conjugate in a 20-g mouse), which is ~10-fold higher than our single dose of 117 µg of dox-mLL1.

A comparison can also be made between other described agents used previously to treat systemic B-cell lymphoma. The most successful preclinical work has been with conjugates of anti-B-cell MAbs coupled to toxins rather than drugs. Ghetie et al. (30) obtained an ~43-day life extension of mice given 5 million Daudi cells (untreated mice died at ~30 days) 1 day before treatment with RFB4-dgA (anti-CD22 × deglycosylated ricin A chain), given in four daily portions, at 40% of its LD₉₀ (~240 µg in a 20-g mouse). Newton et al. (31) showed an average life extension of 35 days when animals administered 5 × 10⁶ i.v. Daudi cells were treated 1 or 7 days later with 100 µg each day for 5 days of an LL2-onconase conjugate. A final comparison is also appropriate with respect to Mylotarg. In preclinical therapy experiments against HL-60 200-mm³ xenografts in athymic mice (32), complete tumor regression was seen by day 28 with no tumor regrowth by day 100 (5 mice of 5), at a total dose of ~18 mg/kg of MAb-drug per 20-g mouse (three doses), which is near the stated maximum tolerated dose of 300–400 µg calicheamicin equivalents/kg. In all of these studies, conjugates were given at levels near their maximum tolerated doses to obtain therapeutic effects.

In contrast, with dox-LL1 cures were seen at a single 117-µg conjugate dose, which is <3 µg in terms of drug equivalents and essentially nontoxic. This dox-mLL1/-humanized LL1 data may challenge the concept that ever more toxic moieties are needed to make toxin/drug-MAb conjugates useful therapeutic agents, although even better results may be obtained with a more potent drug than dox. Nevertheless, we believe it is the targeting of CD74 by the LL1 conjugates and the properties of the antigen itself that causes the substantial improvement over the other conjugates used to date.

Immunohistochemistry on a panel of 31 normal human tissues (including heart and liver), as per the Points to Consider requirements of the Food and Drug Administration for MAbs, showed an absence of CD74 on all but B cells (tonsils, thymus, spleen, and lymph node; data not shown), although prior work had also shown CD74 positivity on monocytes (7), and the antigen is likely to be present on dendritic cells, Langerhans cells, and possibly Kupffer cells (20). Nevertheless, we appreciate that low levels of expression of CD74 in normal organs of patients may prove to be dose-limiting despite its general absence by immunohistochemistry. Therefore, this drug immunoconjugate will need to proceed cautiously to clinical evaluation, because the lack of CD74 in the SCID mouse model accentuates the therapeutic index of the agent in this preclinical model.

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