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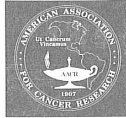

# Cancer Research

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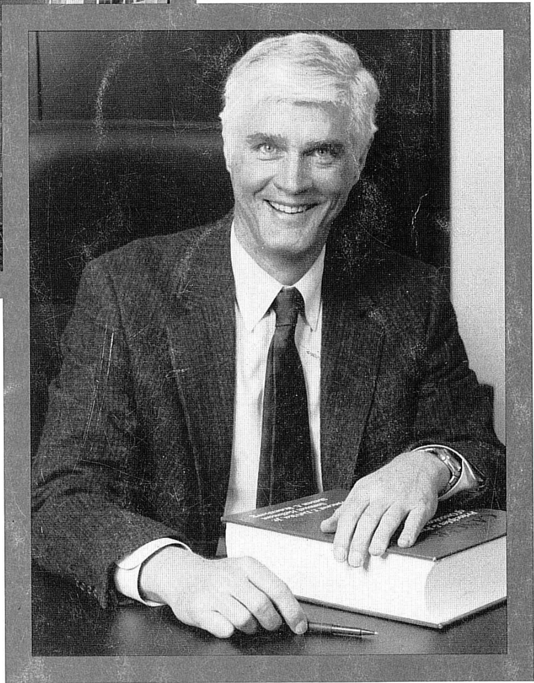

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# Enhancement of the Selectivity and Antitumor Efficacy of a CC-1065 Analogue through Immunoconjugate Formation

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## ABSTRACT

**Bis-indolyl-(*seco*)-1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one compounds are synthetic analogues of CC-1065 that are highly cytotoxic toward a broad spectrum of tumor cell lines. One of these compounds, called DC1, was conjugated to antibodies *via* novel cleavable disulfide linkers. Conjugates of DC1 with murine mAbs anti-B4 and N901 directed against tumor-associated antigens CD19 and CD56, respectively, proved to be extremely potent and antigen selective in killing target cells in culture. DC1 conjugates with humanized versions of anti-B4 and N901 antibodies were also constructed and demonstrated to be as cytotoxic and selective as the respective murine antibody conjugates. The anti-B4-DC1 conjugate showed antitumor efficacy in an aggressive metastatic human B-cell lymphoma survival model in SCID mice and completely cured animals bearing large tumors. Anti-B4-DC1 was considerably more effective in this tumor model than doxorubicin, cyclophosphamide, etoposide, or vincristine at their maximum tolerated doses.**

## INTRODUCTION

A continuing challenge in designing new anticancer chemotherapeutic drugs is to develop agents possessing superior antitumor efficacy combined with reduced toxic side effects. One widely used approach toward this goal has been to target conventional anticancer drugs to tumor sites by conjugating these drugs to antibodies that have selective affinity for tumor cells (1–14). In general, these antibody-drug conjugates proved to be only modestly cytotoxic for cells *in vitro* with $IC_{50}$ values ranging from $10^{-7}$ M to $10^{-5}$ M, and most of them were significantly less potent than the respective drugs in nonconjugated form.

We (15) and others (5) have reported recently about novel antibody-drug conjugates that had greatly increased potency. These conjugates were at least 100-fold more cytotoxic to cultured cells than immunoconjugates of clinically used anticancer drugs such as doxorubicin, melphalan, mitomycin C, cisplatin, bleomycin, and *Vinca* alkaloids. Furthermore, in contrast to the unconjugated drugs, these immunoconjugates killed cells in an antigen-selective manner.

In an extension of this work, we report here the preparation and testing of immunoconjugates comprising a new class of cytotoxic drugs that have a broad spectrum of activity and are highly cytotoxic for a wide variety of tumor cell types. These compounds are bis-indolyl-(*seco*)-CBI[3] derivatives, which, like adozelesin (16) are synthetic analogues of CC-1065. The mode of action of these cytotoxic drugs is DNA alkylation at adenine bases after binding with high affinity to the minor groove of the DNA in AT-rich regions (17–19).

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[3] The abbreviations used are: CBI, 1,2,9,9a-tetrahydrocyclopropa[*c*]benz[*e*]indol-4-one; CPI, 1,2,8,8a-tetrahydro-7-methylcyclopropa[*c*]pyrrolo[3,2*e*]indol-4(5*H*)-one; SAMB, *N*′-succinimidyl 4-*N*-(2′-acetylthiopropionyl)-*N*-methyl-4-aminobutyrate; MST, median survival time; MTD, maximum tolerated dose; *seco*-CBI, 1-chloromethyl-5-hydroxy-1,2-dihydro-3*H*-benz-indole.

## MATERIALS AND METHODS

**Bis-indolyl-seco-CBI (DC1-derivatives 1a and 1b) and SAMB 3.** *Seco*-CBI was prepared as described previously (20) and converted to DC1 derivatives **1a** and **1b** by methods that will be described separately.[4] SAMB **3** is a new bifunctional cross-linking agent that enables the introduction of sterically hindered sulfhydryl groups on an antibody. Details of the synthesis of SAMB will be described elsewhere.[4]

**mAbs.** Murine mAbs anti-B4 (anti-CD19; Ref. 21) and N901 (anti-CD56; Ref. 22) were purified by methods described previously for anti-B4 (23). The humanized versions of anti-B4 and N901, called h-anti-B4 and hN901, were generated at ImmunoGen by using variable domain resurfacing (24) and grafting of complementarity determining regions,[5] respectively, on the framework of a human IgG1 antibody molecule.

**Conjugation of DC1-derivative 1b with mAbs.** To introduce sulfhydryl groups, antibodies (10 mg/ml) were first incubated at 30°C with a 14-fold molar excess of SAMB **3** in 0.1 M potassium phosphate buffer (pH 7.0), containing 2 mM EDTA, for 30 min, followed by treatment with hydroxylamine (50 mM) for 15 min at 30°C. The modified antibody (typically containing between 5 and 6 thiol groups/molecule of antibody, as determined by an Ellman's assay; Ref. 25) was separated from low molecular weight compounds by gel filtration through a Sephadex G25 column equilibrated in 5 mM sodium acetate buffer (pH 4.7), containing 50 mM NaCl and 1 mM EDTA. The modified antibody was then diluted to 1 mg/ml, and the pH was adjusted to 7.5 by the addition of 0.1 M sodium phosphate buffer (pH 7.5), containing 20% dimethylacetamide and 1 mM EDTA and then incubated immediately at 37°C with DC1 derivative **1b** (1.5 equivalents/sulfhydryl group, from a stock solution of 10 mg/ml in dimethylacetamide) for 80 min. Any unreacted thiol groups on the antibody were then converted to thioethers by treatment with *N*-ethylmaleimide (0.075 mM) for 15 min at 37°C. The conjugate was purified by mixing with Porapak Q (Millipore Corp., Milford, MA) chromatography resin (0.4 ml settled resin/mg antibody) for 2 h at room temperature to remove unconjugated drug and subsequent dialysis at ambient temperature for 24 h into PBS containing 20% propylene glycol. The content of free drug in the antibody-DC1 conjugate preparations was determined as follows: (*a*) low molecular weight compounds were separated from conjugate by passing the conjugate solutions through a Sep-Pak C-18 cartridge (Waters Chromatography, Milford, MA); and (*b*) recovered small molecular weight compounds were then analyzed and quantitated by HPLC on a reverse-phase $C_{18}$ column (BAKERBOND; J. T. Baker, Inc., Phillipsburg, NJ). The conjugates contained <0.25% free drug (lower detection limit of the assay). Conjugates containing an average of 4–5 drug molecules linked/antibody molecule were prepared by this method as calculated from absorbance measurements by using the extinction coefficients $\epsilon_{280\ nm} = 2.24 \times 10^5\ M^{-1}cm^{-1}$ for antibody, and $\epsilon_{280\ nm} = 4.17 \times 10^4\ M^{-1}cm^{-1}$, and $\epsilon_{314\ nm} = 4.17 \times 10^4\ M^{-1}cm^{-1}$ for drug. Conjugate solutions (0.5 mg/ml) were quickly frozen in liquid nitrogen and then stored at −20°C.

**Cell Lines.** Human cell lines Namalwa (Burkitt's lymphoma, ATCC CRL 1432), MOLT-4 (acute lymphoblastic leukemia, ATCC CRL 1582), NALM-6 (non-B non-T acute lymphoblastic leukemia; Ref. 26), and SW2 (human small cell lung carcinoma; Ref. 27) were maintained as exponentially growing cultures in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum and 2 mM L-glutamine.

**Binding Studies.** Relative affinities of antibodies and their DC1 conjugates for their cognate antigens were determined in competition binding assays on isolated plasma membranes (28). The proportion of inactive anti-B4 (IgG

[4] R. V. J. Chari. Synthesis and antitumor activity of disulfide-containing benzannelated analogues of CC-1065, manuscript in preparation.

[5] M. Roguska and B. Guild, unpublished data.



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incapable of binding to CD19-expressing cells) in samples of native anti-B4 antibody and anti-B4-DC1 was determined by the method described elsewhere (29). Samples (0.6 ml) of anti-B4 (1.0 nM) or anti-B4-DC1 (0.8 nM) in Eagle's MEM for suspension cultures supplemented with 2.5% pooled human serum of AB type and 10 mM HEPES buffer (pH 7.2) were incubated with CD19-expressing Namalwa cells or CD19-negative MOLT-4 cells in several successive incubations ($4 \times 10^7$ fresh cells/incubation) until the samples were depleted of all the conjugate that was capable of binding to the cells. The concentration of the remaining immunoconjugate was determined by an ELISA (30) using anti-B4 antibody and anti-B4-DC1 for generating the standard curves.

***In Vitro* Cytotoxicity Assays.** The method for the determination of surviving fractions of cell cultures by back extrapolation of the exponential growth curve is similar to that described previously (Ref. 31 and references therein). Cell cultures that had been exposed to a drug or a conjugate for a specified period of time were washed once with prewarmed medium and resuspended in fresh growth medium at a density of about 2–3 × $10^5$ cells/ml. The cells were then cultured and counted daily by using a Coulter Counter. The cultures were diluted with fresh medium to $3 \times 10^5$ cells/ml when they reached a density above $6 \times 10^5$ cells/ml. The daily increase in the cell density was used to calculate an increase of the number of cells in the culture assuming no dilution. An estimate of the number of surviving cells was made by extrapolation of exponential growth curves of treated cultures (in semilogarithmic plots) to the end of the treatment period. The surviving fractions were calculated as the extrapolated number of surviving cells in treated culture:number of cells in the control culture ratio. One unique feature of DC1-containing compounds and their antibody conjugates is that the process of cell death induced by these agents is slow, and the cells undergo up to four divisions before dying (data not shown). Therefore, to obtain accurate surviving fractions of cells, the proliferation of cell cultures treated with DC1 derivatives or their immunoconjugates was monitored for at least eight doubling times.

***In Vivo* Toxicity Studies.** Systemic toxicity of DC1 immunoconjugates was evaluated in female CD-1 mice. Animals received either a single i.v. (through tail vein) bolus injection (acute toxicity study, eight animals/dose) or daily i.v. bolus injections for 5 consecutive days (subacute toxicity study, ten animals/dose) of a conjugate in PBS containing 20% propylene glycol and human serum albumin (1 mg/ml). Toxicity was assessed by observing animals for weight loss and survival. The maximum tolerated dose under subacute dosing conditions was also determined in female CB-17 SCID mice obtained from the Department of Radiation Oncology, Massachusetts General Hospital, Boston, MA.

**Antitumor Activity of Conjugates.** The therapeutic efficacy of immunoconjugates with DC1 was evaluated in 7-week-old female SCID mice bearing a human lymphoma (Namalwa) xenograft (32). Animals were injected i.v. (through tail vein) with $4 \times 10^6$ Namalwa cells. It has been shown previously that tumors metastasize to ovaries, to the leptomeninges of the brain, bone marrow of the vertebra, the dura matter of the spinal cord, and to muscles surrounding the spinal column. Control animals received no treatment because we observed previously that treatment with PBS did not alter the survival of the tumor-bearing mice. The median survival time of untreated mice was 23–24 days. Treatment with immunoconjugates or unconjugated drugs was initiated 7-days posttumor inoculation unless indicated otherwise. The extent of cell kill (log cell kill) *in vivo* was estimated as described previously (32, 33). Mice that were alive on day 120 of the studies were sacrificed and examined histopathologically for signs of residual tumor by Dr. Roderick T. Bronson (School of Veterinary Medicine, Tufts University, Boston, MA).

**Determination of Serum Concentrations of anti-B4-DC1.** Serum concentrations of anti-B4-DC1 in SCID mice bearing Namalwa tumors were determined 1-h post i.v. administration of the conjugate at a dose of 5 mg/kg by an ELISA method by using CD19-expressing Namalwa plasma membrane-coated plates to bind the conjugate and $Eu^{3+}$-labeled anti-murine IgG to detect the conjugate as described elsewhere (28).

## RESULTS

We modeled DC1 after adozelesin **2**, which is a synthetic analogue of the natural product CC-1065 (Fig. 1). Adozelesin has been found to be as cytotoxic as CC-1065 but without having the delayed toxicity in animals of CC-1065 (16). The alkylating portion of adozelesin consists of the CPI unit, which we replaced in DC1 with *seco*-CBI (20). Bis-indolyl-CBI compounds are at least as cytotoxic as the corresponding bis-indolyl-CPI compounds (34, 35) and, importantly, CBI, and its open-ring prodrug form, called *seco*-CBI, are more stable in aqueous solutions and easier to synthesize than CPI. *seco*-CBI readily converts into the active form, CBI, under physiological conditions with a half-life of about 3.5 h in human plasma at 37°C (data not shown). We, therefore, incorporated *seco*-CBI into DC1. Finally, to enable linkage of DC1 to an antibody *via* a disulfide bond, we replaced the terminal unsubstituted benzofuran unit of adozelesin by an indole ring bearing a reactive pyridyldithiopropionamido substituent at the C-5 position.

The anti-B4 antibody was modified with the cross-linking reagent SAMB **3** and then treated with hydroxylamine to deprotect the thiol groups. These antibody-bound thiol groups reacted readily with DC1 derivative **1b** via a disulfide bond exchange to yield an antibody-DC1 conjugate (Fig. 1). Immunoconjugates containing, on the average, 4–5 DC1 molecules/anti-B4 antibody molecule were prepared and are referred to as anti-B4-DC1 in this report. Binding studies on CD19-expressing cell membranes demonstrated that such conjugates preserved nearly all of the binding activity of native anti-B4 antibody (Fig. 2). The apparent affinity was only 2–3-fold lower, which, in principle, can be explained by either a complete inactivation of 50–75% of the antibody molecules during their modification and conjugation to DC1, or by a general 2–3-fold decrease in the avidity. We, therefore, determined the percentage of immunoreactive conjugate in the preparation. A sample of anti-B4-DC1 was incubated successively with two batches of CD19-expressing Namalwa cells. After each incubation, the remaining conjugate in solution was determined by an ELISA (Table 1). Anti-B4-DC1 behaved similarly to anti-B4 antibody, and after two incubations, <6.3% of the initial amount of conjugate was present in solution (the detection limit). We concluded that at least 93% of the conjugate had bound to CD19-positive Namalwa cells. The binding was antigen specific because similar incubations with the CD19-negative cell line MOLT-4 did not significantly lower the initial concentrations of conjugate in the solution.

Anti-B4-DC1 proved to be highly potent, and killed cultured CD19-expressing Namalwa cells after an exposure of as short as 15 min (Fig. 3) with an $IC_{50}$ value of $3 \times 10^{-10}$ M (0.21 ng/ml) of DC1 (equivalent to $7.5 \times 10^{-11}$ M conjugate). A 15-min exposure to a higher concentration of the conjugated DC1 of $4 \times 10^{-8}$ M (equivalent to $1 \times 10^{-8}$ M conjugate) eradicated >99.999% cells (the detection limit of the assay). The effectiveness of cell kill further increased with a prolongation of the exposure time to 24 or 72 h, with $IC_{50}$ values of $6 \times 10^{-11}$ M and $5 \times 10^{-11}$ M, respectively (Fig. 3). Remarkably, concentrations as low as $4 \times 10^{-9}$ M (24-h exposure) and $1.2 \times 10^{-9}$ M (72-h exposure) were sufficient to kill 5 logs of cells. In marked contrast, at all exposure times measured, the conjugate was essentially nontoxic for antigen-negative MOLT-4 cells with $IC_{50}$ values >$4 \times 10^{-8}$ M DC1 (equivalent to $1 \times 10^{-8}$ M conjugate). The effectiveness of anti-B4-DC1 was not limited to the target cell line Namalwa because it was effective in killing NALM-6, another CD19-positive cell line with an $IC_{50}$ value of $8 \times 10^{-11}$ M DC1 ($2 \times 10^{-11}$ M conjugate) after a 24-h exposure (Fig. 3). This cytotoxic effect was abolished in the presence of nonconjugated anti-B4 antibody (Fig. 3), again demonstrating antigen specificity of the cytotoxic effect. In contrast to anti-B4-DC1 conjugate, unconjugated DC1-derivative **1a** was equally cytotoxic for Namalwa and MOLT-4 cells ($IC_{50}$ of $2 \times 10^{-11}$ M after a 24-h exposure; data not shown).

The effectiveness of DC1 when incorporated into immunoconju-



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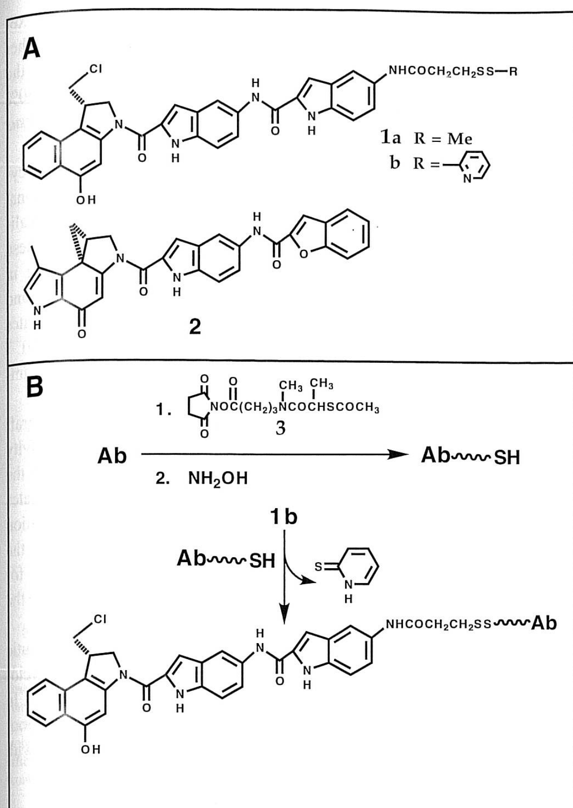


Fig. 1. *A*, structures of adozelesin 2 and bis-indolyl-*seco*-CBI drugs 1a and 1b. *B*, preparation of antibody-DC1 conjugates.

gates is not limited to the anti-B4 antibody. A conjugate prepared with the murine anti-CD56 antibody N901 directed against small cell lung cancer cells (36) was found to be equally potent in an antigen-selective manner toward its target cells (Table 2). Importantly, for future clinical development, we also demonstrated that DC1 conjugates of humanized versions of anti-B4 and N901 antibodies were as potent and specific as their respective murine antibody conjugates (Table 2).

To determine the dose range of anti-B4-DC1 for efficacy studies in human tumor xenograft models in SCID mice, we first evaluated its systemic toxicity. Under acute (a single i.v. dose) and subacute (one bolus dose/day for 5 days) conditions in CD-1 mice, $LD_{50}$ values for anti-B4-DC1 were 225 $\mu$g DC1/kg (equivalent to 14 mg/kg of anti-B4-DC1 conjugate) and 465 $\mu$g DC1/kg cumulative dose over 5 days (equivalent to 29 mg/kg of anti-B4-DC1 conjugate), respectively. The MTD for anti-B4-DC1 in both SCID mice and CD-1 mice was found to be 80 $\mu$g DC1/kg/day $\times$ 5. The dose-limiting toxicities of anti-B4-DC1 were hematological effects, such as the early depression of circulating leukocytes, followed by recovery, and lesions in renal tubules and lungs. The renal lesions were resolving on day 29 after first injection of conjugate.

The antitumor efficacy of murine anti-B4-DC1 was evaluated in SCID mice bearing a disseminated aggressive human lymphoma xenograft (32) that was lethal for nontreated mice in 22–26 days. Mice were i.v. inoculated with 4 $\times$ $10^6$ human Namalwa cells, and the tumor was allowed to establish for 7 days before therapy. Animals were then treated on 5 consecutive days with anti-B4-DC1 (80 $\mu$g/kg/day of DC1, equivalent to 5 mg/kg/day of conjugate), or the isotype-matched, nonbinding conjugate N901-DC1 (5 mg/kg/day of conjugate), or a mixture consisting of anti-B4 antibody (5 mg/kg/day) and unconjugated DC1 derivative **1a** (80 $\mu$g/kg/day). The control group of mice that was left untreated had a MST of 23 days, and all animals were dead by day 26 (Fig. 4*A*). Mice treated with anti-B4-DC1 showed significantly prolonged survivals with a 2.7-fold longer MST of 62 days. The nonbinding conjugate N901-DC1, or the mixture of anti-B4 antibody and drug **1a**, had only modest therapeutic effects, prolonging the MST to 42 and 30 days, respectively. The increased therapeutic effect of the nonbinding conjugate of DC1 compared to the nonconjugated drug may be due to the slow release of the drug from the conjugate over an extended period of time.

Inoculation of SCID mice with as few as 4 $\times$ $10^3$ Namalwa cells killed all mice within 42 days with an MST of 39 days (32). In the experiment shown in Fig. 4*A*, the tumor burden was estimated to be 7 $\times$ $10^7$ Namalwa cells at the beginning of treatment (based on their *in vivo* doubling time of 1.7 days; Ref. 32). This direct comparison shows that anti-B4-DC1 with an MST of 62 days eradicates more than 4 logs of tumor cells. The tumor cell kill is at least 6 logs when calculated by the method of Corbett and Valeriote (33). Anti-B4-DC1 was similarly effective when administered every other day or every third day (in both cases, MST values were between 77 and 81 days; data not shown).

To better understand the meaning of the *in vivo* therapeutic effects of anti-B4-DC1 in the xenograft tumor model, we treated mice bearing the same tumors with chemotherapeutic drugs that are known to be clinically effective against lymphoma (Fig. 4*B*). Drugs were used at their previously determined MTDs (causing transient body weight loss of 20%). Higher doses resulted in systemic toxicity manifested by body weight loss of >20% and early death (within 3 days after the last injection) of several animals. Optimal treatment schedules, which resulted in the maximal therapeutic effect, were determined by varying doses, schedules (daily, every other day, every third day, or every fourth day), and the total number of injections (between a single injection and up to three injections) were evaluated in this survival model for each drug. The most effective treatment schedules at MTD for cyclophosphamide (100 mg/kg/day $\times$ 3 every 2 days, i.p.), vincristine (1 mg/kg/day $\times$ 3 every 2 days, i.v.), etoposide (15 mg/kg/day $\times$ 3 every 2 days, i.v.), or doxorubicin (3 mg/kg/day $\times$ 3 every 4 days, i.v.) had only a modest prolongation of survival of the tumor-bearing mice with MST values of 44, 37, 32, and 28 days, respectively, in comparison to untreated mice that died with an MST of 23 days. Anti-B4-DC1 (80 $\mu$g DC1/kg/day $\times$ 5 every day i.v.) with an MST of 62 days was, therefore, significantly more efficacious than any of these chemotherapeutic agents.

Animals treated on day 7 postinoculation of tumor cells had a large tumor burden of 7 $\times$ $10^7$ cells and were not completely cured. We then wished to determine if an earlier initiation of anti-B4-DC1 therapy at the time of a lower tumor burden could achieve complete cures of mice. Treatment of animals with anti-B4-DC1 (80 $\mu$g/kg/day $\times$ 5 of DC1) that were again inoculated i.v. with 4 $\times$ $10^6$ Namalwa cells was, therefore, begun on day 2, 3, or 5 postinoculation, representing tumor burdens of 9 $\times$ $10^6$, 1.4 $\times$ $10^7$, and 3 $\times$ $10^7$ cells, respectively (Fig. 4*C*). More than one-half of the animals (6 of 8 and 5 of 8) that were treated 2 or 3 days after inoculation of tumor were alive on day 120 and showed no signs of toxicity or tumor growth. The mice were then sacrificed on day 120 and were examined histopathologically for signs of residual tumors. No evidence of tumors was found, and the animals were considered cured. In the group of eight mice that were treated on day 5 after tumor inoculation, two animals were cured, and the remaining six animals had a much prolonged MST of 73 days.



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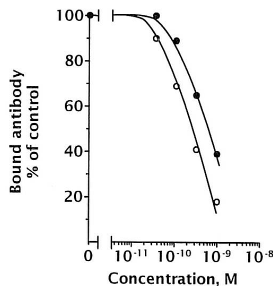


Fig. 2. Competition binding. Assays measure the competition of binding of biotin-labeled anti-B4 by anti-B4-DC1 conjugate (●) or by anti-B4 antibody (○). Solutions containing biotin-labeled anti-B4 antibody (0.2 nM) and various concentrations of competing anti-B4-DC1 or anti-B4 antibody were incubated (18 h at ambient temperature) with CD19-expressing Namalwa cell membranes immobilized in 96-well plates. Positive controls lacked competing antibody, whereas negative controls lacked biotin-labeled antibody. Plate-associated biotin-labeled anti-B4 was then quantified with $Eu^{3+}$-labeled streptavidin and plotted as a percentage of the fluorescence of the positive control sample *versus* the concentration of competing reagent.

Table 1 *Removal of immunoreactive material from samples of anti-B4 antibody and anti-B4-DC1 conjugate*

| Cell line | Antibody or conjugate | Fraction of immunoreactive antibody remaining in the supernatant[a] |
|---|---|---|
| Namalwa (CD19-positive) | anti-B4 | <0.063[b] |
| Namalwa (CD19-positive) | anti-B4-DC1 | <0.063[b] |
| MOLT-4 (CD19-negative) | anti-B4 | 0.92 |
| MOLT-4 (CD19-negative) | anti-B4-DC1 | 0.99 |

[a] Antibody remaining in the supernatant after 2 cycles of treatment with cells as a fraction of the initial concentration. The initial concentrations of anti-B4 and anti-B4-DC1 were 1 and 0.8 nM, respectively.

[b] Lower limits of detection in these experiments as determined by the standard curves.

## DISCUSSION

Bis-indolyl-*seco*-CBI belongs to a group of synthetic analogues of CC-1065, such as adozelesin and carzelesin, which possess the high cytotoxic activity of CC-1065, but lack its delayed systemic toxicity. These drugs are DNA alkylators that bind tightly to the minor groove of DNA and then react covalently with adenine bases. To confer cellular specificity to bis-indolyl-*seco*-CBI while maintaining its potency, we prepared several immunoconjugates with a bis-indolyl-*seco*-CBI derivative called DC1. Interestingly, when target cells were exposed to these conjugates, the cytotoxic effects were only observed after several cell divisions after the treatment. This response is similar to that reported for cells exposed to CC-1065 analogues (37), suggesting to us that the conjugated and nonconjugated DC1 kill cells in an analogous manner.

Antibody-DC1 conjugates proved to be extremely potent ($IC_{50}$ in the range of $10^{-10}$ M DC1) and antigen selective in killing tumor cells *in vitro* and were able to eradicate greater than five logs of cells from cultures of antigen-positive cells. High potency may be an important feature in achieving efficacious levels at tumor sites *in vivo*. In cancer therapy, it has been well established that at least a 2-log or greater reduction in the number of tumor cells is necessary to achieve a clinical remission and even a greater cell kill is required to prevent relapse (38). In general, previously reported conjugates of antibodies with conventional anticancer drugs had $IC_{50}$ values of $10^{-7}$-$10^{-5}$ M (1–4, 14, 39–43) and were effective in treatment of a number of xenograft tumors in mice. In contrast, for those few drug immunoconjugates that have been evaluated in human clinical trials, the peak circulating levels achieved were in the range of their *in vitro* $IC_{50}$ values (between $10^{-6}$ and $10^{-7}$ M; Refs. 44–48), and little therapeutic effect has been observed thus far (1, 44–48). Some of the possible reasons for the higher antitumor efficacy of drug conjugates in xenograft models compared to human trials may be the much higher doses used in the animal studies; and the better delivery of the targeting antibodies to the xenograft tumor sites in animals [10–50% tumor localization of injected i.v. dose (1, 49) in animals *versus* 0.1–0.0002% in humans (50)].

Recently, novel immunoconjugates incorporating more potent cytotoxic agents (at least 100-fold more cytotoxic than conventional anticancer drugs) have been reported. Maytansinoids (15, 51), calicheamicins (5) and morpholinodoxorubicin (8) were utilized in these conjugates. Immunoconjugates containing DC1 are comparable in their *in vitro* potency to those containing maytansinoids (15) and appear to be somewhat more potent than calicheamicin conjugates ($IC_{50}$ values of $3 \times 10^{-10}$ M after a 15-min exposure *versus* $6 \times 10^{-9}$ M after a 7-min exposure, respectively). Morpholinodoxorubicin immunoconjugates described thus far (8) appeared not to be active.

Survival of SCID mice bearing a human lymphoma xenograft (Namalwa cells; Ref. 32) was used to examine the antitumor activity of anti-B4-DC1. We chose this model for several reasons: (*a*) the cancer is aggressive, highly metastatic, and quickly disseminates throughout the body; (*b*) the tumor model mimics the clinical situation because most cancer patients die of disseminated disease; (*c*) the survival of patients is the most direct and widely used criterion for therapy evaluation; and (*d*) survival models allow the study of the effects of therapy on minimal residual tumor burden.

In a direct comparison, anti-B4-DC1 showed a greater therapeutic effect in this model than did the anticancer drugs doxorubicin, cyclophosphamide, etoposide, and vincristine when used at their MTD. In an experiment, where animals were treated on day 7 after tumor inoculation, the median increase in life span in animals treated with

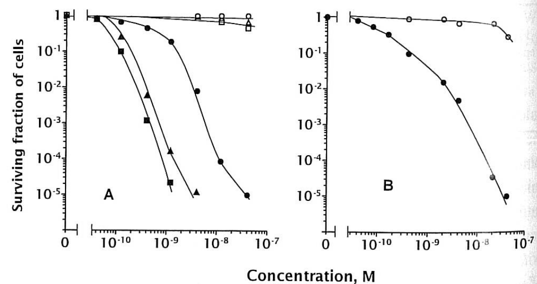


Fig. 3. *In vitro* cytotoxicity and specificity of anti-B4-DC1. *A*, *in vitro* cytotoxicity of anti-B4-DC1 for CD19-positive Namalwa cells (●, ▲, and ■) or CD19-negative MOLT-4 cells (○, △, and □). Cells were exposed to various concentrations of anti-B4-DC1 for 15 min (● and ○), 24 h (▲ and △), or 72 h (■ and □). The surviving fractions of cells were then determined by the growth back-extrapolation assay as described elsewhere (31). The anti-B4-DC1 used in this experiment had 4.0 DC1 molecules linked/antibody molecule, and the results are plotted in terms of DC1 concentration. *B*, *in vitro* cytotoxicity of anti-B4-DC1 for CD19-positive NALM-6 cells after a 24-h exposure was measured in the absence (●) or in the presence of excess ($1 \times 10^{-6}$ M) anti-B4 antibody (○). The surviving fractions of cells were determined by the growth back-extrapolation assay (31).

Table 2 *In vitro* cytotoxicity and specificity of antibody-DC1 conjugates

| Conjugate | Cell line: Target | Cell line: Nontarget | $IC_{50}$, M[a]: Target cells A | $IC_{50}$, M[a]: Nontarget cells B | Specificity factor B/A |
|---|---|---|---|---|---|
| Anti-B4-DC1 | Namalwa | MOLT-4 | $8.0 \times 10^{-11}$ | $>4 \times 10^{-8}$ | >500 |
| h-Anti-B4-DC1 | Namalwa | MOLT-4 | $1.5 \times 10^{-10}$ | $>4 \times 10^{-8}$ | >260 |
| N901-DC1 | SW2 | Namalwa | $1.2 \times 10^{-10}$ | $2.4 \times 10^{-8}$ | 200 |
| hN901-DC1 | SW2 | Namalwa | $9.6 \times 10^{-11}$ | $3.9 \times 10^{-8}$ | 410 |

[a] Cells were exposed to the conjugates for 24 h, and the surviving fractions of cells were then determined by back extrapolating the exponential growth curves (see "Materials and Methods"). $IC_{50}$, inhibiting concentration of drug that kills 50% of cells.



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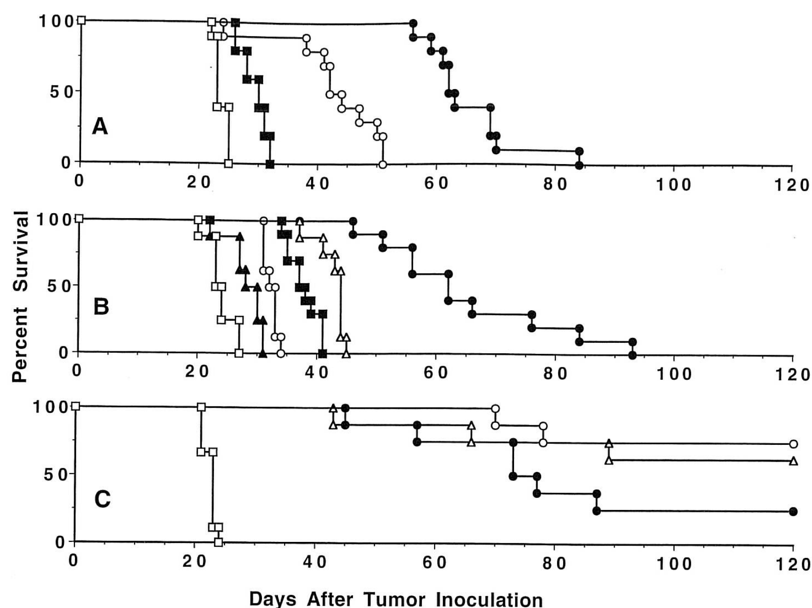


Fig. 4. *In vivo* antitumor efficacy and specificity of anti-B4-DC1 and of conventional chemotherapeutic drugs. *A*, antitumor efficacy and specificity of anti-B4-DC1. SCID mice (10 animals/group) were i.v. inoculated with 4 × $10^6$ Namalwa cells. Animals were left untreated (control group, □) or were treated i.v. on day 7 after tumor inoculation with a mixture of anti-B4 (5 mg/kg/day × 5 every day) and unconjugated DC1 (80 μg/kg/day × 5 every day) (■), or with the isotype-matched but nonbinding conjugate, N901-DC1 (○) at a DC1 dose of 80 μg/kg/day × 5 every day, or with anti-B4-DC1 (●) at a DC1 dose of 80 μg/kg/day × 5 every day. *B*, comparison of the therapeutic efficacy of anti-B4-DC1 with that of conventional anticancer drugs. SCID mice (8–10 animals/group) were i.v. inoculated with 4 × $10^6$ Namalwa cells. Animals were left untreated (control group □), or treated on day 7 after tumor inoculation with one of the following agents: anti-B4-DC1 (●) at a DC1 dose of 80 μg/kg/day × 5 every day, i.v.; doxorubicin (▲), 3 mg/kg/day × 3 every 4 days, i.v.; etoposide (○), 15 mg/kg/day × 3 every 2 days, i.v.; vincristine (■), 1 mg/kg/day × 3 every 2 days, i.v.; or cyclophosphamide (△), 100 mg/kg/day × 3 every 2 days, i.p. *C*, antitumor efficacy of anti-B4-DC1 in SCID mice bearing varying tumor burdens. Mice (8–10 animals/group) were i.v. inoculated with 4 × $10^6$ Namalwa cells. Animals were left untreated (control group □) or treated i.v. with anti-B4-DC1 at a DC1 dose of 80 μg/kg/day × 5 every day, on day 2 (○), day 3 (△), or day 5 (●) after tumor inoculation.

anti-B4-DC1 was 170%, representing more than 6 logs of cell kill *in vivo*, whereas the conventional anticancer drugs increased the life span in mice by 22–91% (Fig. 4) and were capable of killing only between 1 and 3.4 logs of tumor cells. Therapy of mice bearing highly disseminated Namalwa tumors of considerable sizes (up to 1.4 × $10^7$ cells) with anti-B4-DC1 completely cured a majority of animals with no detectable tumors on histopathological evaluation.

Anti-B4-DC1 is effective at lower doses than any previously reported immunoconjugates of anticancer drugs that were evaluated in human xenograft tumor survival models in immunodeficient mice (0.4 mg/kg *versus* 8–90 mg/kg cumulative drug equivalent dose) (4, 39, 52). We believe that high activity at low concentration is an important feature of conjugates for clinical use due to their low efficiency in penetrating solid tumors (1, 50, 53, 54). Furthermore, tumor-associated antigens tend to be expressed in low numbers on the surface of the target cells, in general, between $10^4$ and $10^5$ antigens/cell and rarely exceeding $10^6$ antigens/cell. The serum levels of anti-B4-DC1 at its MTD in mice 1 h after injection was found to be 1.48 ± 0.08 μM (determined by an ELISA; data not shown), which is 18,000-fold greater than its $IC_{50}$ for cultured tumor cells. Moreover, even 1/100th of this concentration of anti-B4-DC1 can eradicate as many as 5 logs of cultured tumor cells. In addition, the normal human tissue distribution of the CD19 antigen targeted by anti-B4 is restricted to cells with the morphology of lymphocytes. Thus, anti-B4 bound strongly to B lymphocytes in the spleen and the follicles of lymph nodes and tonsils. Anti-B4 also bound to cells with the morphology of lymphocytes in tissues such as esophagus, stomach, small intestine, colon, and thymus, which are known to contain some B lymphocytes.[6] Consequently, the minimal cross-reactivity of anti-B4 with normal human tissues and the high potency of anti-B4-DC1 makes us hopeful that therapeutically effective levels of anti-B4-DC1 will be achieved at tumor sites in patients.

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