Specific Targeting of Chlorambucil to Tumors With the Use of Monoclonal Antibodies [1,2]

Mark J. Smyth, [3] **Geoffrey A. Pietersz,** [3] **Brendan J. Classon,** [3] *and* **Ian F. C. McKenzie** [3,4]

ABSTRACT—The concept of attaching cytotoxic drugs, such as the alkylating agent chlorambucil (CBL), to "tumor-specific" antibodies for the treatment of cancer is attractive, inasmuch as the specificity of CBL could be increased and its systemic toxicity reduced. To this end, CBL was activated by *N*-hydroxysuccinimide to produce an active ester derivative that was covalently coupled to monoclonal antibodies reactive with murine cell surface antigens. Up to 30 molecules of CBL were specifically bound per molecule of antibody, without impairing the alkylating activity of CBL and with minimal loss of antibody activity. The in vitro cytotoxicity of the conjugate was tested by the inhibition in [^{3}H]thymidine incorporation into tumor cells, which demonstrated the conjugate to be specifically cytotoxic toward antibody-reactive cell lines, having more activity than the free drug. In vivo treatment of (C57BL/6 × BALB/c)F_1 mice bearing a murine thymoma with CBL-antibody conjugates gave prolonged survival times and greater inhibition of growth of established tumors than was obtained with free antibody or CBL alone. The study is one of the first examples of the greater toxicity of a drug coupled to antibody, inasmuch as most drugs when coupled to antibody lose activity. CBL-monoclonal antibody conjugates may, therefore, provide a means of specifically attacking tumors, which could be therapeutically useful.—JNCI 1986; 76:503-510.

The vast majority of cytotoxic agents in use today have the potential to damage all proliferating cells, although numerous attempts have been made to improve their selective cytotoxicity. Ehrlich's original concept of the "magic bullet" in which antibodies are used to target specifically toxins to tumors is now being reexamined. With the production of MoAb that have high tumor reactivity, toxic materials such as isotopes, antibiotics, alkylating agents, plant toxins, and antimetabolites are being coupled and targeted both in vitro and in vivo (*1*). Many laboratories have been examining the use of radiolabeled antibodies with some success, but further studies are required (*2*). Considerable research has been performed with ricin-antibody conjugates, which appear valuable for the in vitro treatment of bone marrow cells, although these conjugates have limited in vivo potential (*3*). By contrast, there is little published on the use of drug-antibody conjugates because most drugs are complex organic molecules that are coupled to antibodies with difficulty (*4-6*) and in most cases there is a severe loss of drug activity after coupling so that the drug-antibody conjugate is far less toxic than the free drug. For example, we have recently coupled DOX (*6*) and MTX (*7*) to MoAb, and although the drug-antibody conjugates were active, they had only one-tenth of the activity of the free drug (even though the drug-antibody conjugates were highly specific). Thus by coupling to antibody, drugs and toxins have increased target specificity, but there is a concomitant sacrifice of activity. We now describe the covalent coupling of CBL to MoAb by using an active ester derivative. In contrast to the other drugs studied, the conjugates, which had high drug-to-antibody ratios, had greater in vitro and in vivo activity than free CBL.

MATERIALS AND METHODS

Tumor cells.—BW5147 (*8*) and several clonal variants of the murine thymoma ITT(1)75NS (*9*) were used. The ITT(1)75NS.E3 cell line (E3) was obtained after three successive rounds of fluorescence-activated cell sorting of ITT(1)75NS cells stained with monoclonal Ly-2 antibodies; the most fluorescent cells (≈5%) were selected for growth and subsequent sorting. The ITT(1)75NS.D1 cell line (D1) was obtained by sorting the *least* fluorescent cells from the same thymoma cell line [ITT(1)]. As shown herein, the Ly-2 antigen densities of these lines are very different (text-fig. 1). These murine cell lines were maintained in vitro in DMEM, supplemented with 10% heat-inactivated newborn calf serum (Flow Laboratories, Inc., Sydney, Australia), 2 m*M* glutamine (Commonwealth Serum Laboratories, Melbourne, Australia), 100 IU penicillin/ml (Commonwealth Serum Laboratories), and 100 µg streptomycin/ml (Glaxo Laboratories, Melbourne). The clonal variant E3 was maintained by serial passage in ascitic fluid in (C57BL/6 × BALB/c)F_1 (B6CF_1) mice. For in vivo experiments the cells from the ascitic fluid were washed and centrifuged (1,500 rpm for 5 min) twice in DMEM and PBS (pH 7.3), resuspended in PBS, and injected sc or ip into B6CF_1 mice.

Mice.—B6CF_1 mice were produced in the Department of Pathology, The University of Melbourne.

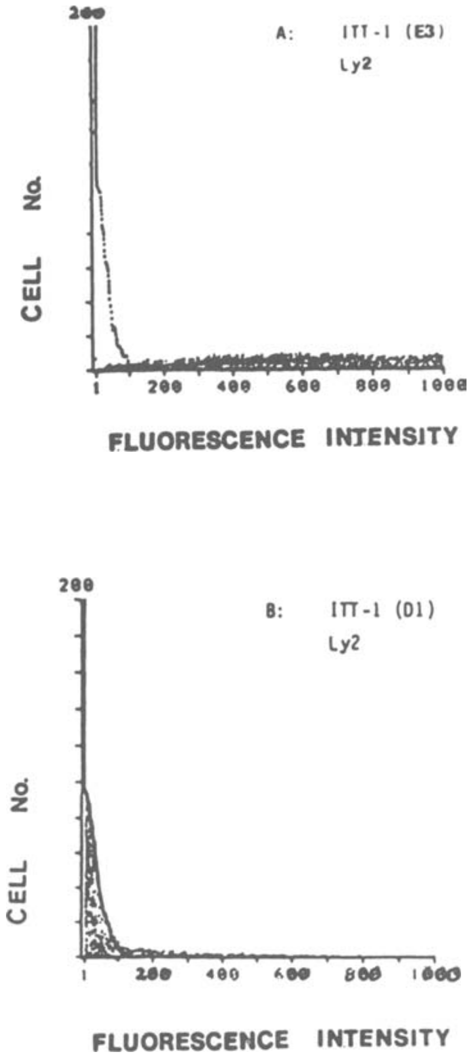
ABBREVIATIONS USED: CBL = chlorambucil; DMEM = Dulbecco's modified Eagle medium; DMF = dimethylformamide; DOX = doxorubicin; MoAb = monoclonal antibody (antibodies); MTX = methotrexate; NHS = *N*-hydroxysuccinimide; PBS = phosphate-buffered saline.

[1] Received April 22, 1985; accepted October 15, 1985.

[2] Grant support for this study was provided by the Anti-Cancer Council of Victoria and the National Health and Medical Research Council of Australia.

[3] Research Centre for Cancer and Transplantation, Department of Pathology, The University of Melbourne, Parkville, Victoria, 3052, Australia.

[4] *Address reprint requests to* Dr. McKenzie.



TEXT-FIGURE 1.—Flow cytometric analysis of two different ITT-1 thymoma cell lines. A=E3 variant selected for high Ly-2 expression (*see* "Materials and Methods"). B=D1 variant selected for low Ly-2 expression. Cell number is shown on the *ordinate* and fluorescence intensity is shown on the *abscissa;* measurements with preamplifier gains A and B=2.85.

Antibodies.—The following MoAb were produced and characterized in this department: 1) anti-Ly-2.1 reactive with the murine Ly-2.1 specificity (*10*), 2) anti-Ly-24.2, and 3) 250-30.6 reactive with human colon and colon carcinoma cells (*11*). The MoAb were isolated from ascitic fluid by precipitation with 40% ammonium sulfate, dissolution in PBS, and dialysis with the same buffer. These crude preparations were adsorbed onto protein-A-Sepharose (Pharmacia Inc., Piscataway, NJ) and washed extensively with PBS (pH 7.3), and the antibodies were eluted with either 0.2 *M* glycine-HCl (pH 2.8) or citrate buffers (pH 5.0, 4.0, 3.0). Following neutralization with saturated Tris, MoAb were dialyzed against PBS, aliquoted, and stored at −78°C. The antibody activity was determined by rosetting with sheep anti-mouse immunoglobulin (*12*).

Preparation and quantitation of conjugate.—An active ester of CBL was prepared by dissolving 5.0 mg CBL in 100 μl DMF, and NHS (2.2 mg in 200 μl DMF) was added, followed by *N,N*-dicyclohexylcarbodiimide (3.9 mg in 200 μl DMF). The reaction mixture was allowed to stand at room temperature for 1 hour (sealed from light) and for 18 hours at 4°C and discarded after 4 weeks if unused. A solution (10-100 μl) of CBL active ester (0.2-1.8 mmol) in DMF was added to a solution (0.5-1.0 ml) containing 0.5-2.0 mg of affinity-purified MoAb, the mixture was allowed to react for 1 hour at room temperature, and any precipitated protein was removed by centrifugation. Free CBL and any other unreacted starting materials were removed by gel filtration chromatography with the use of a Sephadex G-25 column (PD-10; Pharmacia Inc.). The amount of CBL incorporated in the drug-antibody conjugates was determined by absorbance spectrophotometry at 258 nm after subtraction of the protein contribution following its estimation by the Bradford dye-binding assay (*13*). The alkylating activity of the conjugate was determined by a modification of the method of Epstein et al. (*14*), while the amount of covalently bound CBL was calculated after removal of the physically bound CBL by extraction with ethyl acetate. For noncovalent conjugates a solution (10-100 μl) of CBL (0.2-1.8 mmol) in DMF was mixed with MoAb, producing a noncovalently associated CBL-MoAb complex, which was purified by PD-10 gel filtration. Free drug was prepared by dissolution in 0.5 *M* $NaHCO_3$ before dilution in medium.

Antibody activity.—A rosetting assay (*12*) was used to determine the antibody activity of the drug-antibody conjugates and thus was compared to the activity of antibody that had undergone the same procedures used in the coupling method.

Drug activity.—Two assays were performed to assess drug activity. They differed in the time the drug-antibody conjugate was in contact with the cells.

In the first assay, a 24-hour assay, 100 μl of cells ($1\text{-}5\times10^6$/ml) was added to a 96-well flat-bottom microtiter plate and incubated for 2-3 hours at 37°C. Antibody, free drug, and drug-antibody conjugates were filtered through a 0.2-μm Millipore filter to ensure sterility, and dilutions were performed in sterile PBS; 50 μl of antibody, free drug, or conjugate was added to the cells with the use of duplicate wells per sample; control wells received 50 μl of medium or PBS, and the cells were cultured at 37°C in a 7% CO_2 atmosphere for 24 hours.

In the second assay, a 30-minute assay, 200 μl of cells ($1\text{-}5\times10^6$/ml) was collected in sterile plastic centrifuge tubes, resuspended in sterile drug, antibody, or conjugate, and mixed for 30 minutes at 37°C. The cells were centrifuged (1,500 rpm for 5 min) and then resuspended in growth medium; 100 μl of cells was then seeded into a microtiter plate with the use of duplicate wells per sample and incubated for 16-24 hours; duplicate samples were performed at each concentration chosen.

As a variation of this procedure to assess inhibition by "cold" antibody, cells were preincubated with 100 μg of free antibody before we followed the 30-minute assay procedure where they were exposed to CBL-antibody conjugates.

After the incubation period in all the assays 50 μl of medium containing 1 μCi of [^{3}H]thymidine (sp act=15 Ci/mmol; Amersham International Ltd., Amersham, England) was added, and the plates were incubated for 3-6 hours; cells were then harvested onto a glass fiber filter paper with the use of a cell harvester and dried for 10 minutes at 80°C, and individual samples were separated and counted on a beta counter (Tri Carb 460C; Packard Instrument Co., La Grange, IL). Incorporation of [^{3}H]thymidine was expressed as a percentage inhibition in incorporation of controls. (Growth inhibition was approximately similar to cell cytotoxicity measured by the in vitro uptake of trypan blue.) The standard error for any given point was generated by duplicate determinations and did not exceed 5% for any given experimental point.

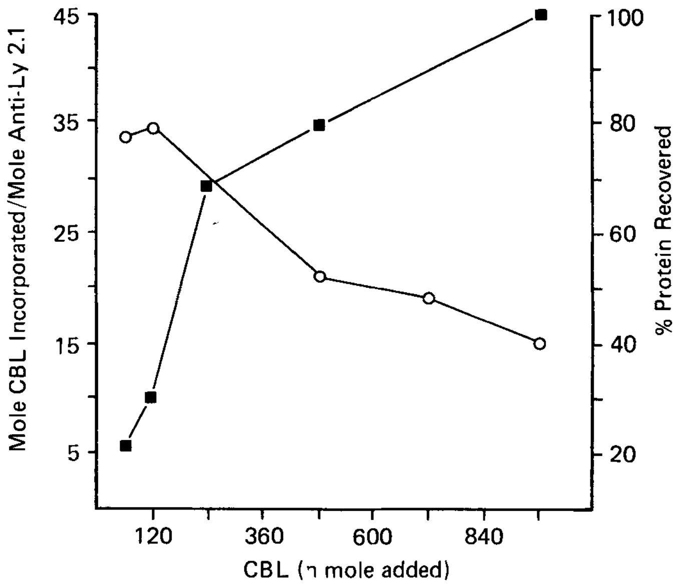
In vivo experiments.—In a survival study, tumor cells were injected ip into B6CF$_1$ mice and 6 hours later a series of ip treatments was begun (*see* below). The percentage survival of mice in each group was plotted as a function of time. In a tumor growth study, tumor cells were injected sc into the abdominal wall of the mouse and allowed to develop into palpable tumors measuring 0.5 cm in diameter before treatment was begun. Mice were then subjected to a series of ip treatments, and the size of the tumors was measured daily with a caliper square; we measured along the perpendicular axes of the tumors, and the data were recorded as mean tumor size (product of two diameters ± standard error of the mean). Experimental groups of 8-10 mice, all of the same sex and age, were used in each experiment.

RESULTS

The rationale of the studies described herein was to establish the conditions for the coupling of CBL to antibody and then to measure the residual drug and antibody activities in the conjugate. If these studies were satisfactory, then in vitro and in vivo studies of the tumor inhibitory activity of the conjugate were to be examined.

Coupling of CBL to Antibody

The MoAb anti-Ly-2.1, which reacts with the murine tumor ITT(1)75NS, was reacted with different amounts of CBL active ester (*see* "Materials and Methods") to produce conjugates that varied in the amount of drug coupled. We found that an increasing amount of CBL could be bound by increasing the amount of active ester of CBL present (text-fig. 2); up to 30 molecules of CBL could be bound without a great loss of protein, but when 30-35 molecules were exceeded, a substantial loss of protein solubility occurred (text-fig. 2). Addition of 120 nmol of CBL active ester to 3 nmol of anti-Ly-2.1 led to an incorporation of 10 molecules of CBL per molecule anti-Ly-2.1 with 80% protein recovery. By con-

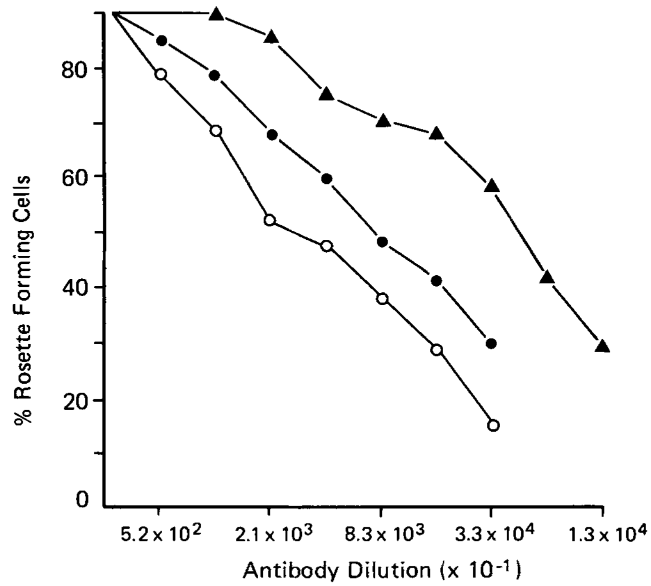


TEXT-FIGURE 2.—Coupling of CBL to anti-Ly-2.1 (0.5 mg). Moles of CBL incorporated per molecule anti-Ly-2.1 (■) and protein recovery (O) are shown as a function of the number of nanomoles of CBL in the reaction mixture (*abscissa*).

trast, addition of 480 nmol of CBL active ester led to an incorporation of 35 molecules with a 55% protein recovery. Thus a compromise is sought between higher CBL-to-MoAb incorporation ratios and lower protein recoveries when one is establishing satisfactory coupling conditions; CBL-anti-Ly-2.1 conjugates that were further tested in vitro and in vivo had been 10 and 30 molecules of CBL incorporated per molecule of anti-Ly-2.1. By extracting the physically bound CBL with ethyl acetate, we found that 80% of the CBL content of the conjugate was *covalently* bound. Only 10% of the physically bound CBL in noncovalent CBL-MoAb conjugates could not be extracted with ethyl acetate by the same procedure. Conjugates retained over 90% of their alkylating activity. Thus large amounts of CBL could be covalently bound to antibody with some loss of protein; however, the activity of drug and antibody in the conjugates required measurement.

Antibody Activity

The titers of antibody before and after conjugation were measured by the rosetting method (text-fig. 3) and were determined as the dilution at which 50% of the E2 target cells demonstrated rosettes. Anti-Ly-2.1 conjugates containing 25 and 35 molecules of CBL had antibody titers of 1:15,000 and 1:10,000, respectively, while the nonconjugated antibody titer was 1:50,000. Thus there is clearly some loss of antibody activity due to the conjugation procedure. However, the titers of the coupled antibody were sufficiently high to allow experiments to proceed.



TEXT-FIGURE 3.—Antibody titer measured as the percent rosette-forming cells vs. antibody dilution of anti-Ly-2.1 conjugates on ITT(1)75NS target cells. Serial dilutions were performed on a 0.25 mg/ml solution of either neat (no CBL) anti-Ly-2.1 (▲) or anti-Ly-2.1 with 25 (●) or 35 mol CBL/mol conjugate (○).

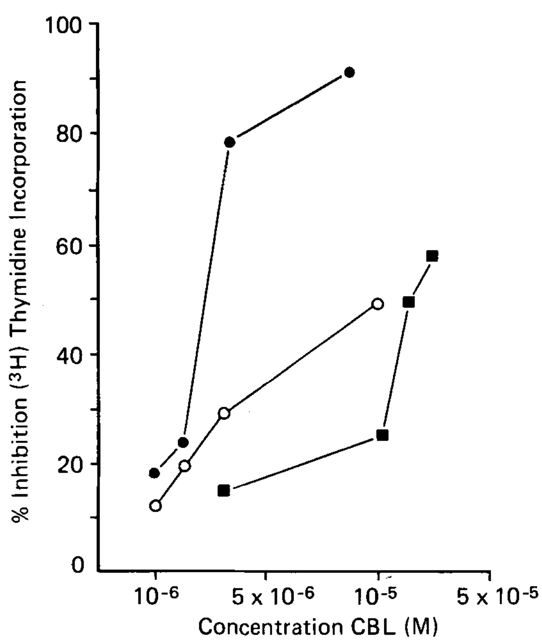
Cytotoxicity In Vitro

The cytotoxicity of anti-Ly-2.1 conjugates was tested on Ly-2^+ E2 target cells and compared with that of free CBL or CBL-anti-Ly-2.1 bound noncovalently (text-fig. 4). The activity of the bound drug was considerably increased over that of free CBL, with 50% inhibition in [^{3}H]thymidine incorporation occurring at 2.6×10^{-6} *M* for the anti-Ly-2.1 conjugate compared to 1.7×10^{-5} *M* for free CBL, i.e., a fivefold increase in activity when CBL was specifically targeted to the cell. In addition, CBL noncovalently bound to anti-Ly-2.1 was twice as cytotoxic toward E2 as free CBL alone. The MoAb used in this study was found to have no in vitro cytotoxic action on target cells in the absence of complement.

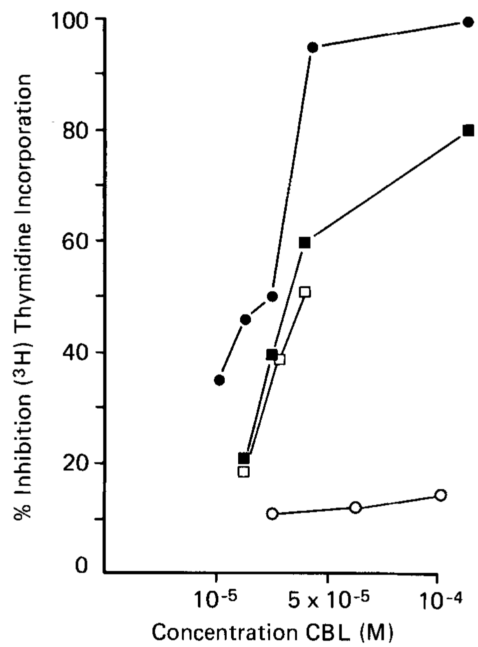
Specific Cytotoxicity

It was, therefore, necessary to show that the inhibition effects produced were specific. Four procedures were performed.

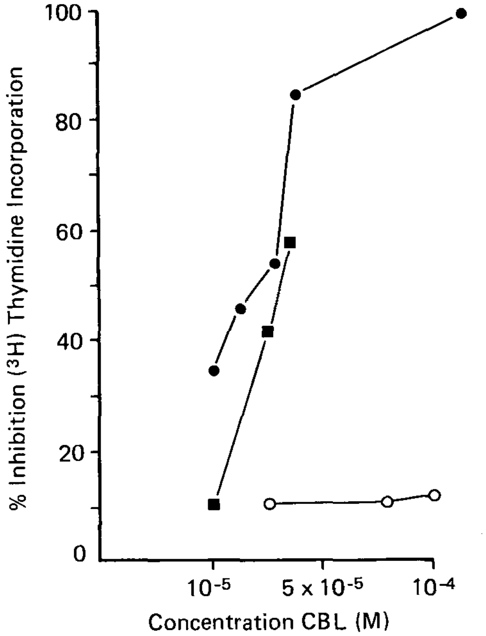
Procedure 1.—The 30-minute assay was designed to demonstrate that conjugates were exerting their cytotoxic effect by specifically binding target cells via antibody at the antigen binding site. (It was reasoned that little free drug would reach cells in 30 min.) Antibody-reactive and nonreactive cell lines were employed here: The anti-Ly-2.1 conjugate was shown to bind the antibody-reactive cell line E2 and to exert its cytotoxicity on these cells after 30 minutes' exposure (text-fig. 5). In this case 50% inhibition in [^{3}H]thymidine incorporation occurred at 2.5×10^{-5} *M* compared with 3.0×10^{-5} *M* for free CBL. To determine whether this inhibition was due to specific binding of the antibody, we used an antibody-nonreactive cell line, BW5147. The two cell lines had basically the same inhibitory response to free CBL; however, the anti-Ly-2.1 conjugate demonstrated selectivity in its action by inhibiting only the Ly-2^+ E2 cell line, and no inhibition of the Ly-2^- BW5147 cell line



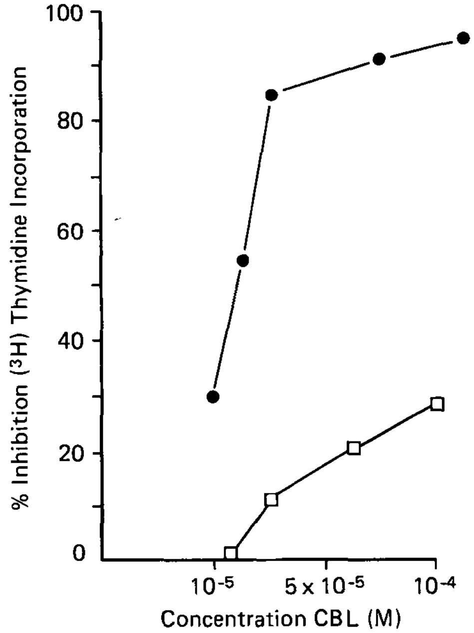
TEXT-FIGURE 4.—Inhibitory effect of CBL on E2 cells in a 24-hr assay (*see* text) with the drug free (■), noncovalently bound to anti-Ly-2.1 MoAb (30 mol CBL/mol conjugate) (○), or covalently bound to anti-Ly-2.1 MoAb (30 mol CBL/mol conjugate) (●).



TEXT-FIGURE 5.—Inhibitory effect of free CBL (□) or CBL-anti-Ly-2.1 conjugate (20 mol CBL/mol conjugate) (●) on antibody-reactive cells (E2) and free CBL (■) or conjugate (○) on antibody-nonreactive cells (BW5147) in the 30-min assay.



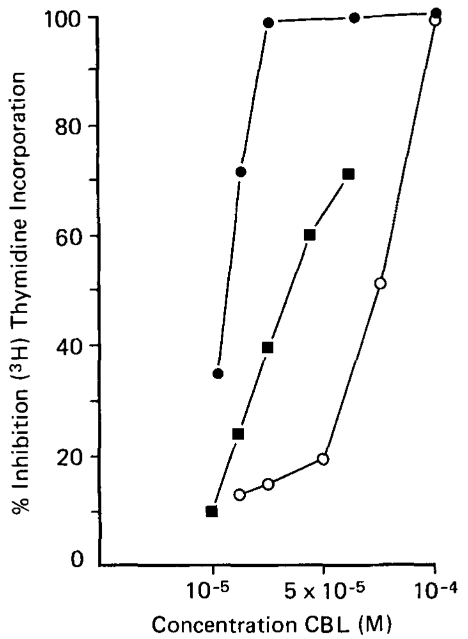
TEXT-FIGURE 6.—Inhibitory effect of free CBL (■), CBL-anti-Ly-2.1 conjugate (20 mol CBL/mol conjugate) (●), or CBL-250-30.6 conjugate (20 mol CBL/mol conjugate) (○) as a control on E2 cells in the 30-min assay.



TEXT-FIGURE 8.—Inhibitory effect of CBL-anti-Ly-2.1 conjugate (30 mol CBL/mol conjugate) (●) and conjugate plus 100 μg neat anti-Ly-2.1 (□) on E2 target cells in the 30-min assay.

was observed in the same molar concentration range.

Procedure 2.—An additional experiment was performed with the use of a CBL-250-30.6 conjugate as a nonspecific negative control—the 30.6 antibody reacting with human but not with murine cells. This conjugate



TEXT-FIGURE 7.—Inhibitory effect of free CBL (■) or CBL-anti-Ly-2.1 conjugate (30 mol CBL/mol conjugate) (●) on E3 cells and free CBL (■) or conjugate (○) on D1 cells in the 30-min assay.

demonstrated no inhibition of the E2 cell line (text-fig. 6).

Procedure 3.—To clarify that the cytotoxicity of the CBL-anti-Ly-2.1 conjugates was primarily dependent on the number of reactive antigen binding sites on the target cells, we used a 30-minute assay involving E3 and D1 cell lines—these cell lines being, respectively, high and low Ly-2^+ variants of the ITT(1)75NS cell line. We presumed that these two cell lines were identical with the exception of their Ly-2.1 antigen density (text-fig. 1); E3 was Ly-2^{HIGH}, whereas D1 was Ly-2^{LOW}. Both the E3 and D1 tumors had an identical response to free CBL; however, the CBL-anti-Ly-2.1 MoAb conjugate was five times more cytotoxic toward E3 compared to the cytotoxicity seen toward the D1 cell line (text-fig. 7).

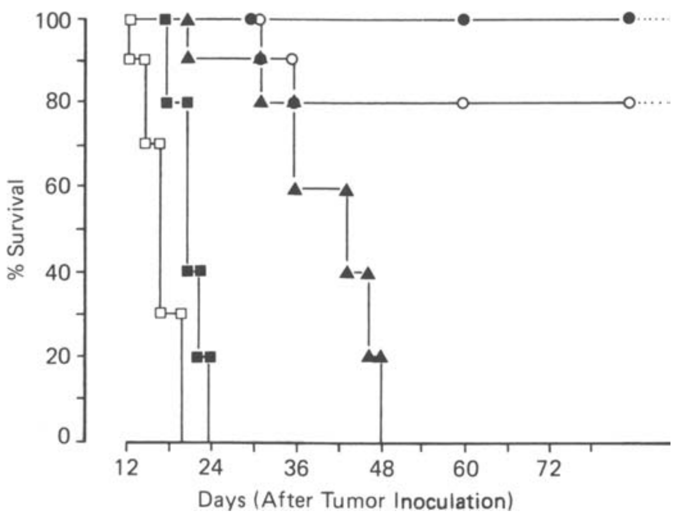
Procedure 4.—To ensure further that the binding of CBL-MoAb conjugate to target cells was specific and occurred at the antibody binding site, we performed studies to inhibit the binding of conjugate by free antibody. At a CBL concentration of 10^{-4} *M* (50 μg anti-Ly-2.1), the cytotoxicity of the anti-Ly-2.1 conjugate on E2 target cells was reduced by 70% upon the addition of 100 μg of anti-Ly-2.1 (text-fig. 8), clearly indicating that the cytotoxicity of a CBL-MoAb conjugate is directly related to its antibody-binding ability. Thus in four different assays the specificity of the MoAb conjugate was demonstrated.

In Vivo Efficacy

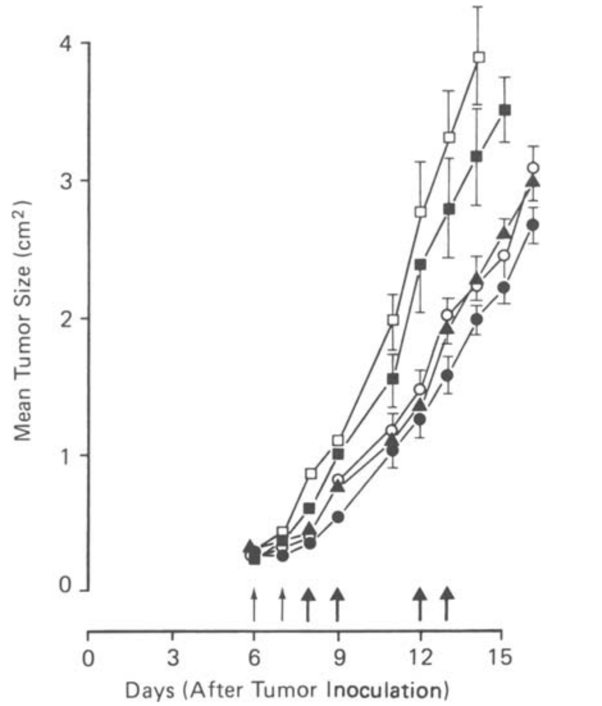
All experiments were performed with the E3 variant of the murine thymoma ITT(1), which in vivo is a rapidly proliferating tumor with a doubling time of less than 24 hours.

Survival study.—Groups of 10 B6CF$_1$ mice were given ip injections of 10^5 tumor cells. Six hours later the mice were given one of the following treatments: 1) PBS, 2) anti-Ly-2.1, 3) free CBL, 4) anti-Ly-2.1 conjugate, or 5) a noncovalent mixture of anti-Ly-2.1 and CBL. Each reagent was administered ip in 200 μl PBS on days 0, 1, 2, 3, 7, and 13 after tumor inoculation. The average amount of CBL and antibody received per injection was 4 and 40 μg, respectively. The PBS-treated mice had a survival time of 20 days, whereas the group that received only CBL died within 22 days of tumor inoculation (text-fig. 9). By contrast, 100% of mice treated with covalently linked CBL-anti-Ly-2.1 MoAb conjugate (and 80% of mice treated with the noncovalent conjugate) survived tumor free for more than 200 days. These groups showed a significant increase in survival compared to the 48-day survival observed with mice receiving anti-Ly-2.1 alone. Thus the CBL-MoAb conjugate effectively "cured" the mice of their tumor, but 80% of the mice treated with CBL-MoAb that was noncovalently linked survived for over 200 days.

Tumor growth study.—Groups of 9 B6CF$_1$ mice given an sc injection of 2×10^6 tumor cells developed a solid tumor 0.30 cm^2 in size 7 days after inoculation. These mice with established tumors were then given an injection of one of the five treatment agents described above. The amount of CBL and antibody received per injection was 2 and 20 μg, respectively, on days 7 and 8 after tumor inoculation; it was double this dose—4 μg CBL and 40 μg antibody—on days 9, 10, 13, and 14. Tumor growth was inhibited in mice that had received antibody in their treatments compared to the tumor growth seen in mice that had been given PBS or CBL alone. By day



TEXT-FIGURE 9.—Survival of B6CF$_1$ mice bearing the ITT(1)75NS.E3 tumor. Groups of 10 mice were inoculated with 10^5 cells/mouse; 6 hr later, mice received ip PBS (□), free CBL (■), neat anti-Ly-2.1 (▲), noncovalent anti-Ly-2.1 and CBL (○), or CBL-anti-Ly-2.1 conjugate (●).



TEXT-FIGURE 10.—Growth of the thymoma ITT(1)75NS.E3 in B6CF$_1$ mice given an sc injection of 2×10^6 cells [6 days later (tumor size, $\approx$0.3 cm^2)]. Groups of 10 mice were given treatments ip denoted (↑); PBS (□), free CBL (■), CBL-anti-Ly-2.1 conjugate (●), noncovalently conjugated CBL-anti-Ly-2.1 (○), and anti-Ly-2.1 (▲). *Error bars* represent ± standard error of the mean.

15 the mean tumor size of the group receiving anti-Ly-2.1 MoAb conjugate was only 60% that of PBS-treated, control mice, the conjugate group having significantly smaller tumors than the group given either the noncovalent CBL-anti-Ly-2.1 MoAb conjugate or anti-Ly-2.1 alone (text-fig. 10).

Thus the CBL-MoAb conjugate had some effect on tumor growth in the group with solid tumors, but this effect was not as promising as the 100% survival of mice with tumor in the ascites form.

DISCUSSION

The concept of covalently coupling cytotoxic agents to tumor-specific antibodies has been successfully applied in experimental systems (*1*). Our research represents a model system enabling the investigation of the problems involved in obtaining selective cell killing by covalently linking CBL to MoAb directed against cell surface antigens; to pursue this strategy, our drug-antibody conjugates had to fulfill several criteria: 1) They must exert toxicity specifically on neoplastic cells, 2) the desired biologic properties of the drug and the antibody should not be greatly diminished by the conjugation process, and 3) the toxic moiety of the conjugate must be potent in vitro and in vivo. The CBL-MoAb conjugates appeared to fulfill all of these criteria. With the use of the NHS procedure to produce an active ester derivative of CBL, 15-30 molecules of CBL could be incorporated per molecule of antibody with retention of good antibody activity with reasonable recovery of protein. However, as was found with previous studies (*15*), the solubility and antibody activity of conjugates decreased significantly beyond these levels of incorporation of CBL (text-fig. 2). The CBL-anti-Ly-2.1 conjugate was shown to retain the cytotoxic effect of CBL, increasing the 24-hour antitumor activity of bound CBL to five times that of an equimolar amount of free CBL (text-fig. 4), and with the specific in vitro (and in vivo) binding of CBL (bound to MoAb) to target cells the conjugate exhibited an increased cytotoxicity. These findings are interesting in that virtually all drug-antibody conjugates studied to date (e.g., MTX-DOX) have decreased activity of the drug, which is presumably due to its alteration during the coupling procedure or to the fact that the coupled drug is less active.

The antibody-binding activity of the CBL-MoAb conjugate clearly resulted in selective cytotoxicity. This cytotoxicity was apparent in the specificity assays in which conjugates were only briefly exposed to cells to mimic the in vivo action of a conjugate where there may be only a brief encounter with tumor or normal tissue followed by prolonged association with antigen-positive tumor cells. CBL-anti-Ly-2.1 MoAb conjugates displayed cytotoxicity only to Ly-2^+ cells, their specific recognition and binding to ITT(1)75NS target cells making them more potent than CBL alone.

The cytotoxicity that our CBL-anti-Ly-2.1 MoAb conjugate exhibited on E2 target cells could be inhibited in a competitive binding assay in which conjugate was incubated with cells in the presence of anti-Ly-2.1 antibody, demonstrating that antibody binding is essential for the cytotoxic action of the conjugate. Similarly, the degree of antibody binding is partially dependent on the antigen density of the target cells; hence the Ly-2^{HIGH} E3 cell line is five times more susceptible to the cytotoxic activity of the CBL-anti-Ly-2.1 MoAb conjugate than is the Ly-2^{LOW} D1 cell line.

Once we had firmly established the specificity and efficacy of CBL-anti-Ly-2.1 in vitro, the in vivo activity of the CBL-anti-Ly-2.1 MoAb conjugate was investigated in both survival and tumor growth experiments. The survival times of mice inoculated with the murine E3 tumor clearly demonstrate that CBL covalently bound to MoAb is a more effective tumor inhibitor than free CBL or antibody alone, as is CBL noncovalently bound to antibody; thus further experiments are being undertaken to determine correctly any difference in the efficacy of covalent and noncovalent CBL-MoAb conjugates against ascites tumors. If the number of tumor cells injected and the treatment dosage are increased, a greater difference in the efficacy of the various treatments may be observed. In tumor growth experiments, therapy did not commence until tumors were well established ($\approx$0.3 cm^2 in size). Of the treatments administered, the covalent CBL-anti-Ly-2.1 MoAb conjugate was again the most effective tumor inhibitor. The monoclonal anti-Ly-2.1 alone (i.e., in the absence of CBL) displayed significant antitumor activity toward the murine thymoma E3, both in survival and tumor growth experiments, inasmuch as there was no in vitro cytotoxic action—presumably, this involving humoral or cellular mechanisms that affect the graft. One must also take into account that the activity of unconjugated anti-Ly-2.1 was three to five times greater than that of the same amount of anti-Ly-2.1 conjugated to CBL.

CBL-bound antibody has previously been reported to cause greater tumor inhibition than antibody or CBL alone. In particular, success has been reported with CBL noncovalently bound to antitumor globulins in a polyclonal antiserum (*16*). If the activity of CBL requires dissociation from the antibody, binding noncovalently would be a useful method, providing the bond is stable in blood and body fluids. The survival experiment performed (text-fig. 9) indicated that CBL noncovalently bound to anti-Ly-2.1 is a more effective tumor inhibitor than is CBL or anti-Ly-2.1 alone; however, this type of in vivo situation requires minimal targeting of the drug. By comparison, inhibition of a well-established subcutaneous tumor requires localization of the drug-antibody conjugate, and as shown (text-fig. 10) CBL noncovalently bound to anti-Ly-2.1 is not more effective than is anti-Ly-2.1 alone. This finding suggests that the noncovalent association between CBL and antibody is insufficiently stable for specific targeting of CBL to tumors. Specific targeting of CBL by the covalent attachment to MoAb may reduce any instability in vivo; however, several other problems in this type of chemotherapy exist. Selective proliferation of antigen-negative and/or drug-resistant tumor cells could hinder the effective eradication of tumor cells by immunotherapy—a problem possibly overcome by attacking the tumor cell at two or more antigenic sites with a multi-drug-antibody conjugate treatment schedule. However, in vitro studies have yet to be performed to define the efficacy of this approach. In addition, the development of an immune response against foreign globulins constitutes another limitation. Furthermore, although 30 mol of CBL is bound, it would be desirable to bind even more drug to antibody, and the use of Fab fragments and human MoAb for targeting or the use of more toxic agents could well improve results. Greater attention needs to be directed toward the difficulty in treating established solid tumors where therapeutically effective localization of conjugates is critical.

At this time we have shown that CBL-MoAb conjugates are more toxic than is free CBL, and this phenomenon also implies greater specificity of drug action. The conjugate is effective in vivo and indeed will cure a mouse bearing a tumor in the ascites form—somewhat analogous to the in vitro treatment of tumor cells. Although the attack on established solid tumors is far from satisfactory, one must take into account the following facts: *a*) The E3 tumor divides extremely rapidly; *b*) small doses of CBL have been given; *c*) treatment agents were administered ip; and *d*) the antibody that conjugate-treated mice received is three to five times less active than the antibody received by anti-Ly-2.1-treated mice (as determined by rosetting, text-fig. 3). All of these experimental parameters detract from the efficacy of covalent and noncovalent CBL-MoAb conjugates, and thus further attention to the tumor's growth, the dose, and relative antibody activity of the treatment agent and its route of administration may improve results.

REFERENCES

(1) GHOSE T, BLAIR AH, KULKARNI P. Preparation of antibody linked cytotoxic agents. Methods Enzymol 1983; 93:280-333.

(2) LARSON SM. Radiolabeled monoclonal anti-tumor antibodies in diagnosis and therapy. J Nucl Med 1985; 26:538-545.

(3) VITETTA ES, KROLICK KA, MIYAMA-INABA M, et al. Immunotoxins: A new approach to cancer therapy. Science 1983; 219: 644-650.

(4) EMBLETON MJ, ROWLAND GF, SIMMONDS RG, et al. Selective cytotoxicity against human tumor cells by a vindesine-monoclonal antibody conjugate. Br J Cancer 1983; 47:43-49.

(5) HURWITZ E, LEVY R, MARON R, et al. The covalent binding of daunomycin and adriamycin to antibodies with retention of both drug and antibody activities. Cancer Res 1975; 35: 1175-1178.

(6) PIETERSZ GA, ZALCBERG JR, MCKENZIE IF. The use of adriamycin-monoclonal antibody complexes for specific anti-tumor antibody. Unpublished data.

(7) KANELLOS J, PIETERSZ GA, MCKENZIE IF. Studies of methotrexate-monoclonal antibody conjugates for immunotherapy. JNCI 1985; 75:319-332.

(8) HYMAN R, STALLINGS V. Complementation patterns of Thy-1 variants and evidence that antigen loss variants "pre-exist" in the parental population. J Natl Cancer Inst 1974; 52:429-436.

(9) HOGARTH PM, HENNING MM, MCKENZIE IF. Alloantigenic phenotype of radiation-induced thymomas in the mouse. JNCI 1982; 69:619-626.

(10) HOGARTH PM, EDWARDS J, MCKENZIE IF, et al. Monoclonal antibodies to murine Ly-2.1 cell surface antigen. Immunology 1982; 46:135-144.

(11) THOMPSON CH, JONES SL, PIHL E, et al. Monoclonal antibodies to human colon and colorectal carcinoma. Br J Cancer 1983; 47:595-605.

(12) PARISH CR, MCKENZIE IF. A sensitive rosetting method for detecting subpopulations of lymphocytes which react with alloantisera. J Immunol Methods 1978; 20:173-183.

(13) BRADFORD MM. A rapid and sensitive method of quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Anal Biochem 1976; 72:248-253.

(14) EPSTEIN J, ROSENTHAL RW, ESS RJ. Use of α-(4-nitro benzyl)-pyridine as analytical reagent for ethylene imines and alkylating agents. Anal Chem 1955; 27:1435-1439.

(15) ROSS WC. The conjugation of chlorambucil with human γ-globulin. Chem Biol Interact 1975; 10:169-172.

(16) O'NEILL GJ, PEARSON BA, DAVIES DA. In vitro cytotoxicity of anti-θ (Thy-1) antibodies combined with chlorambucil. Immunology 1975; 28:323-329.