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Tumor Inhibition by Chlorambucil Covalently Linked to Antitumor Globulin*

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Abstract—*The alkylating agent chlorambucil has been covalently bound to antitumor globulins directed against cell surface antigen(s) of the mouse EL4 lymphoma. The resulting conjugates retained alkylating and antibody activities and were used to treat C57BL/6J mice after prior inoculation with the syngeneic EL4 lymphoma. Forty per cent of mice treated with the covalent conjugate,* 78% *of mice treated with non-covalent conjugate and* 50% *of mice treated with chlorambucil followed by antitumor globulin survived tumor-free for more than* 100 days. *Untreated mice had a mean survival of* 18 ±1.3 days *and the groups that received chlorambucil or antitumor globulin only, or chlorambucil covalently bound to normal rabbit globulin died within* 30 days *of tumor inoculation.*

INTRODUCTION

IT HAS been postulated that antibodies against tumor-associated cell surface antigens might be used as selective carriers of cancer chemotherapeutic drugs [1]. We have already demonstrated that chlorambucil can be non-covalently linked to anti-tumor associated antigen globulins (ATAG) without impairing the alkylating activity of chlorambucil and without substantially affecting the antibody activity [2]. Chlorambucil so bound was a more effective tumor inhibitor (both *in vivo* and *in vitro*) than chlorambucil bound to normal globulin [3–5]. As chlorambucil non-covalent conjugates dissociate relatively easily under physiological conditions [2], it has been argued that superior tumor inhibition by such non-covalent conjugates might result solely from drug–antibody synergism [6, 7]. We report here that covalently linked chlorambucil–ATAG conjugates retain both drug and antibody activities and are effective tumor inhibitors *in vivo*.

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MATERIALS AND METHODS

Mice and tumors

Twelve to sixteen week-old inbred female C57BL/6J mice purchased from the Jackson Laboratory (Bar Harbour, Maine) were used in all experiments. The EL4 lymphoma was obtained from the Chester Beatty Research Institute (London, England) in 1969 and is maintained by serial intraperitoneal (i.p.) passage in C57BL/6J mice. Recently we described its pattern of growth and lethality [8].

Anti-ovalbumin (OA)

New Zealand white virgin female rabbits (weighing about 3 kg) were immunized with a total of 3 mg OA (five times recrystallized, Pentex Inc., Kankakee, Illinois) in complete Freund's adjuvant (Difco Laboratories Inc., Detroit, Michigan). Each rabbit received 0.1 ml of the emulsion in each of the four foot pads; two sets of injections were given 3 days apart. Five ml of blood was obtained from the ear vein of the rabbits just before immunization and 4 weeks after the second immunizing injection. The anti-OA titers of the resulting sera were determined by double diffusion in agar gel. Rabbits showing precipitins in their serum at antigen concentrations of 1.25 mg/ml or lower were exsanguinated. The resulting sera were pooled and inactivated at 56°C for 30 min.

Antitumor sera

Each member of the groups of 6 rabbits was inoculated intramuscularly (i.m.) in both flanks and shoulders with a total of 4×10^8 freshly obtained EL4 cells, washed three times with 0.01M sodium phosphate buffered saline (PBS) at 4°C (≧98% trypan blue impermeable). For injection the cells were suspended in 4 ml of PBS and mixed with an equal volume of Freund's complete adjuvant. The injections were repeated after 1 week. From 1 week after the second set of injections, the rabbits were inoculated i.m. twice a week with 10^8 cells per rabbit with no adjuvant. Three days after the 10th such injection, 5 ml of blood was obtained from the ear vein of each rabbit (test bleeding). The resulting test sera were separately absorbed with homogenates of normal mouse tissues and assayed thereafter by immunofluorescence for the presence of specific anti-EL4 antibody as described below. Rabbits showing titers of 1/64 or greater were exsanguinated. The pooled sera were inactivated at 56°C for 30 min, and then successively absorbed with washed homogenates of liver, lungs, kidneys and spleen from normal adult C57BL/6J mice. Absorption with each homogenate (homogenate to serum ratio, 1:3 by volume) was carried out at 37°C with gentle shaking for 4 hr, followed by another 8 hr period at 4°C. Sera were separated from homogenates by centrifugation (20,000 ***g***) for 2 hr at 4°C. Absorptions were repeated (usually twice with liver, lung, and kidney and three to four times with spleen homogenate) until the serum reacted on immunofluorescence assay only with EL4 cells and not with either cryostat sections of normal C57BL/6J mouse tissues or suspensions or smears of C57BL/6J lymphoid cells derived from lymph nodes, spleen, and thymus, B16 melanoma cells, two lines of AKR/J lymphoma cells and Ehrlich ascites tumor cells maintained in BALB/cJ mice.

Antithymocyte serum

An adult goat was immunized with fresh (≧95% trypan blue impermeable) thymocytes from BALB/cJ mice following a protocol identical to that for the production of rabbit anti EL4 sera. The reactivity of the immune serum with thymocytes was established by membrane immunofluorescence and complement dependent cytotoxicity assays.

Fractionation of sera

All sera were brought to 33.3% saturation with ammonium sulphate by addition of saturated aqueous ammonium sulphate. The precipitate was dissolved in PBS and the ammonium sulfate removed by repeated dialysis against PBS. For certain experiments, this ammonium sulfate fraction was further purified by DEAE-sephadex column chromatography [9]. Protein was estimated by the method of Lowry *et al.* [10].

Assay of antibody activity

Cytoplasmic and membrane immunofluorescence assays of mouse tumor cells and other control preparations were done by the sandwich method with the use of fluoresceinated goat antiserum against rabbit globulin (Hyland Laboratories, Los Angeles, California) [11]. Anti-OA activity was assessed by the passive hemagglutination of human red cells coated with ovalbumin using a standard procedure [12].

Assay of chlorambucil

Free chlorambucil was estimated by measurement of A_{258} [6] and by the Epstein reaction which measures alkylating activity [13]. Protein bound chlorambucil was estimated by the Epstein reaction [13]. The absorbance at 600 nm was proportional to sodium chlorambucil over the range 10–50 μg/ml. A standard curve using sodium chlorambucil was prepared for each series of measurements. When the Epstein reaction was used to determine alkylating activity in all the various fractions obtained in a coupling experiment, $100 \pm 10\%$ of the alkylating activity of the pure starting material was accounted for. This indicates that there was no loss during these procedures and therefore this method was routinely used to measure the amount of chlorambucil in these fractions. Results obtained with the Epstein reaction were confirmed in several experiments by difference spectrophotometry [14].

Covalent binding of the sodium salt of chlorambucil to immunoglobulin

The sodium salt of chlorambucil (Leukeran, Burroughs Wellcome & Co. Canada Ltd., La Salle, Quebec) was prepared by mixing 12 mg of sodium methoxide (Fisher Scientific, Fair Lawn, N.J.) with 61 mg of chlorambucil dissolved in 2 ml of methanol. The solvent was then removed by placing the solution over sulfuric acid in a desiccator at room temperature.

The conjugation procedure was adapted from that of Ross [15]. In a typical experiment, antitumor globulin (14 mg) and sodium chlorambucil (3 mg) were dissolved in 1.5 ml of PBS. The coupling reaction was initiated by addition of 3.5 mg of 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (ECDI) (Calbiochem., San Diego, California) to the stirred mixture at 4°C, pH 6.9–7.1. Stirring was continued for 2 hr after which the chlorambucil–antibody conjugate was purified by passing the reaction mixture through a Sephadex G-25 (Pharmacia, Dorval, P.Q.) column (1.5. cm diameter, bed volume 40 ml) equilibrated with PBS. Elution in 4 ml fractions was carried out with PBS at a flow rate of 1.6 ml/min. Bound chlorambucil emerged at the void volume while unbound drug and other low molecular weight impurities and side products emerged with the "salt peak" (Fig.1). Reaction conditions which differ from these are specified in the figure legends.

Non-covalent binding of chlorambucil to immunoglobulin

The method of binding chlorambucil non-covalently to immunoglobulins at acid pH has been described [2].

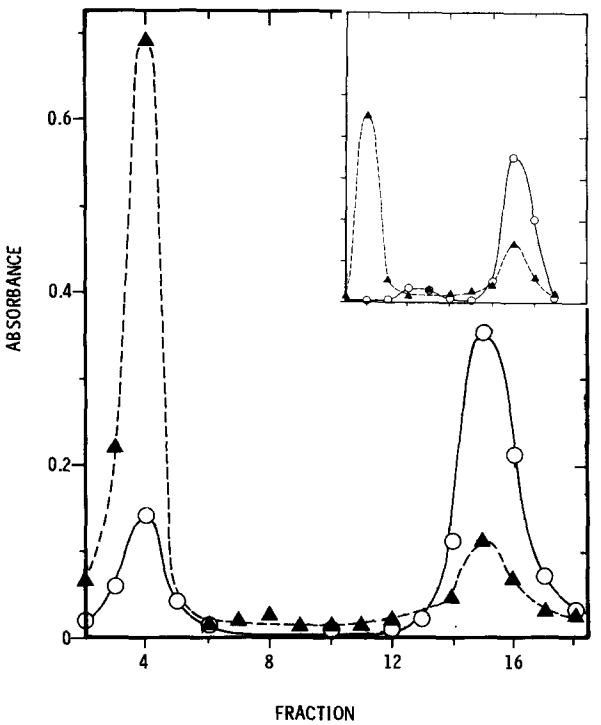

Fig. 1. Purification of covalent chlorambucil–immunoglobulin conjugates by gel filtration through Sephadex G-25. A_{280} (▲) and A_{600} (Epstein reaction) (○) versus column fraction number. The left peak is immunoglobulin with bound chlorambucil and the right peak is unconjugated low mol wt material. Inset: gel filtration of a mixture of sodium chlorambucil and immunoglobulin at pH 6.9, *ECDI omitted, showing absence of bound chlorambucil. The experimental procedure is described in Materials and Methods.*

RESULTS

Effect of covalent binding of chlorambucil to immunoglobulins on antibody activity

Experiments were carried out to determine the number of molecules of chlorambucil that could be linked to rabbit anti-OA globulin using ECDI. A typical experiment is illustrated by the data in Fig. 2. Here, the number of moles of the alkylating form of chlorambucil bound per mole of globulin is plotted as a function of the ratio of sodium chlorambucil to globulin in the reaction mixture. Up to approximately 50 moles of drug per mole of globulin have been covalently bound. Comparable levels of incorporation were obtained with goat antithymocyte globulin and rabbit anti-EL4 globulin.

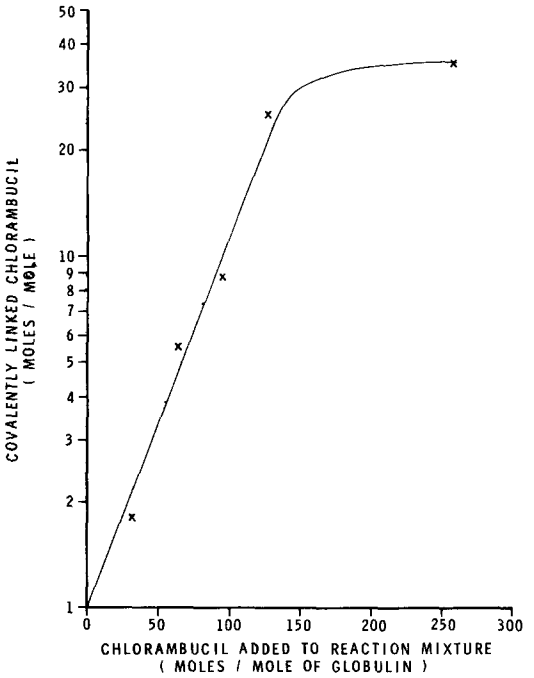

Fig. 2. Covalent binding of chlorambucil to anti-OA globulin. Number of moles of chlorambucil bound per mole of immunoglobulin (as determined by the Epstein reaction) versus the ratio of chlorambucil to immunoglobulin conc. in the reaction mixture. Reaction mixtures contained 20 mg *rabbit anti-OA globulin; sodium chlorambucil at the molar ratios indicated; ECDI (molar conc. equal to* 1.2 *times that of sodium chlorambucil);* 0.3 ml *PBS, in a total volume of* 2.3 ml. *Reaction time* 2 hr.

Hemagglutination assays of anti-OA globulin–chlorambucil covalent conjugates were performed by double dilution starting with an anti-OA globulin protein concentration of 1.0 mg/ml. At chlorambucil in-

corporation levels of 0, 2, 5, 9 and 40 mole per mole the titers were 2048, 1024, 512, 256 and 128, respectively. The retention of activity of the goat anti-thymocyte globulin–chlorambucil conjugates was assayed by immunofluorescence. Titers of 128, 32 and 24 (1 mg/ml starting concentration) were obtained at chlorambucil incorporation levels of 0, 20 and 52 moles per mole, respectively.

Anti-EL4 antibody–chlorambucil conjugates retained reactivity with EL4 cells at an incorporation level of 40 moles per mole, e.g. the titer by membrane immunofluorescence decreased to 16 from an original value of 32 (1 mg/ml starting protein concentration). This pattern of loss of antibody activity is similar to that observed with non-covalent conjugates [2].

Retention of alkylating activity of conjugates

Covalent conjugates incorporating widely varying amounts of chlorambucil (molar ratios between (17:1 and 42:1) showed no decrease in alkylating activity for at least 24 hr at 4°C in PBS. In contrast, about half of the alkylating activity was lost in 45 min at 37°C (Fig.3). Addition of normal human sera (NHS), normal mouse sera (NMS) or bovine serum albumin (BSA) decreased the rate of loss of alkylating activity at 37°C, whereas bovine gamma globulin (BGG), normal goat globulin (NGG) and OA had no effect (Fig. 3). Chlorambucil non-covalently bound exhibited similar stability characteristics.

A number of covalent and non-covalent conjugates of chlorambucil with normal rabbit globulin (NRG) or NGG (molar ratios ranging from 10:1 to 41:1) were dialyzed against PBS at 4°C. After 24 hr, covalent conjugates retained virtually all of their alkylating activity, whereas, non-covalent conjugates retained $\leqq 1\%$ initial activity. This latter result is in agreement with the findings of Davis and O'Neill using non-covalent conjugates of chlorambucil with goat serum globulin [6]. However, after one and 4 hr dialysis, we found that non-covalent conjugates retained approximately 50 and 25% of the protein bound chlorambucil, as determined by alkylating activity, i.e., in the fraction retained on ultrafiltration with an Amicon centriflo CF50A membrane [2].

The persistence of alkylating activity in the serum of groups of mice was investigated by injecting intravenously (i.v.) free and immunoglobulin-bound chlorambucil at 3.3 mg per kg body weight. When a covalent conjugate with 28 moles of drug per mole of

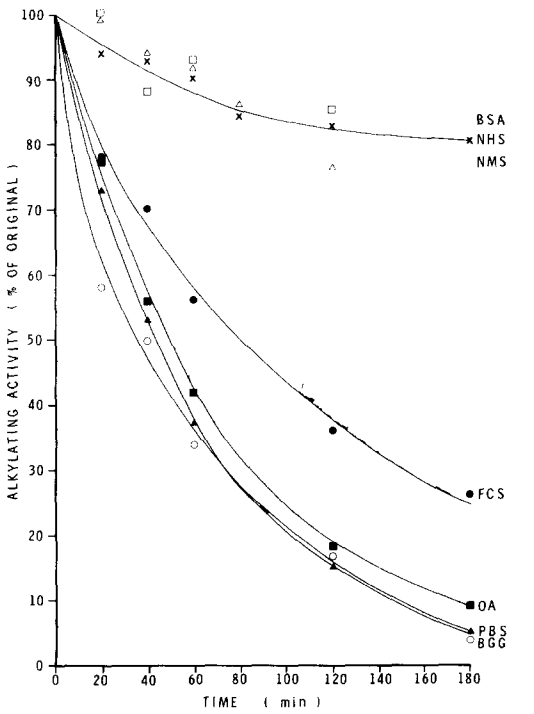

Fig. 3. Stability of chlorambucil covalently bound to immunoglobulin. Alkylating activity versus time at 37°C. The conjugate was prepared by reacting 100 mg normal goat globulin with 12 mg sodium chlorambucil in the presence of 10 mg ECDI. After Sephadex G-25 gel filtration, 2 ml aliquots of conjugate solution were mixed with an equal volume of one of the following solutions prepared in PBS buffer: bovine serum albumin (BSA) 20 mg/ml (□), 50% normal human serum (NHS) (X), 50% normal mouse serum (NMS) (△), 10% fetal calf serum, (FCS) (●), ovalbumin (OA) (20 mg/ml) (■), PBS itself (▲) and bovine gamma globulin (BCG) (20 mg/ml) (○). The solutions were incubated at 37°C and 0.6 ml aliquots were removed at the times indicated for analysis by the Epstein reaction.

globulin was injected, the plasma level of "active" chlorambucil was 40 μg/ml at 5 min, 46 μg/ml at 15 min, 28μg/ml at 30 min and 20 μg/ml at 90 min. Injection of the same amount of non-covalently bound (20 moles per mole) or free chlorambucil gave a plasma level of only about 20 μg/ml at 5 min. Thereafter, the alkylating activity decreased very slowly, reaching 10 μg/ml after 90 min.

Tumor inhibition in vivo *by chlorambucil—ATAG covalent conjugates*

Groups of mice containing at least 9 individuals each were inoculated i.p. with 10^4 EL4 cells per mouse. Starting 2 hr later, different groups received i.p. daily for 5 consecutive days one of the following (per mouse per day): (a) chlorambucil alone; (b) ATAG alone; (c) chlorambucil followed by ATAG; (d) chlorambucil covalently linked to NRG; (e) chlorambucil covalently linked to ATAG; (f) chlorambucil non-covalently linked to

ATAG; or (g) chlorambucil non-covalently linked to NRG (Fig. 4). The total doses of chlorambucil (free or bound) and globulin (ATAG or NRG) were 400 μg and approximately 10 mg, respectively, divided into 5 doses spaced at 24 hr intervals. The ratio of chlorambucil:globulin was 17–21 moles per mole.

The untreated mice had a mean survival of 18 ± 1.3 days and the groups that received

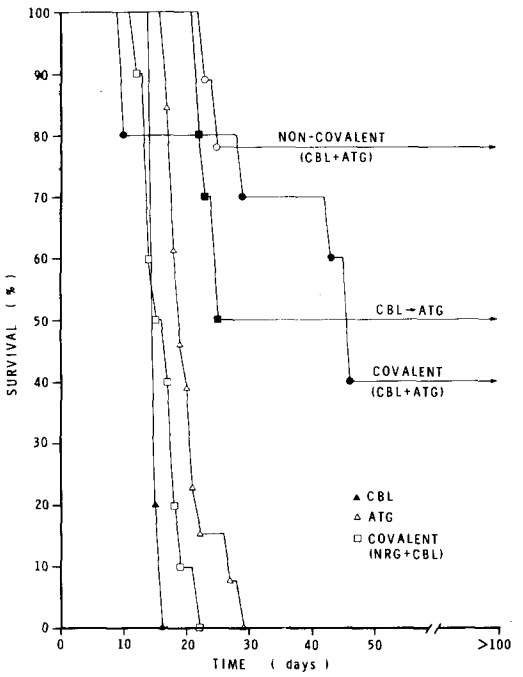

Fig. 4. Survival curves for EL4 tumor bearing C57BL/6J mice. Groups, each containing at least 9 individuals, were inoculated i.p. with 10^4 EL4 cells per mouse. Starting 2 hr later, different groups received i.p. daily for 5 consecutive days *one of the following (per mouse per day): (a) chlorambucil (CBL) alone (▲); (b) anti-EL4 globulin (ATG) alone (△); (c) CBL followed by ATG (■); (d) CBL covalently linked to NRG (□); (e) CBL covalently linked to ATG (●) or (f) CBL non-covalently linked to ATG (○). The total doses of CBL (free or bound) and globulin (ATG or NRG) were* 400 μg *and approximately* 10 mg, *respectively, divided into* 5 *doses spaced at* 24 hr *intervals. Control untreated mice and mice treated with chlorambucil non-covalently linked to NRG died at* 18 ± 1.3 *and* 17.5 ± 0.58 days, *respectively. Conjugates were prepared fresh daily and the ratio chlorambucil:globulin was maintained between* 17 *and* 21 moles *per* mole.

chlorambucil or ATAG only, or chlorambucil bound covalently or non-covalently to NRG died within 30 days of tumor inoculation. In contrast, 40% of mice treated with the covalent ATAG conjugate, 78% of mice treated with the non-covalent conjugate, and 50% of mice treated with chlorambucil followed by ATAG survived tumor-free for more than 100 days. The group treated with the non-covalent ATAG conjugate showed significant increase in survival compared either to the group treated with the covalent conjugate ($P = 0.05$) or to the group treated with chlorambucil followed by ATAG ($P = 0.04$), using Fisher's exact test. Autopsy of the mice that died after treatment revealed intraperitoneal as well as disseminated tumor in all the internal organs examined. However, the mice that died after treatment with the non-covalent conjugate had no gross tumor and histological examination did not reveal any tumor in the sections of the internal organs and lymph nodes examined. These mice had pneumonic consolidation of the lungs. In a separate experiment, all 12 mice treated with ATAG chlorambucil non-covalent conjugates 2 hr after i.p. inoculation of 10^4 EL4 cells survived tumor-free for at least 200 days.

DISCUSSION

Cytotoxic agents such as diphtheria toxin [16], *N*, *N*-bis (2-chlorethyl)-*p*-phenylenediamine [17] and daunomycin [18], when covalently linked to ATAG, cause more inhibition of a number of experimental tumors than drug or ATAG alone. We have demonstrated that antibodies to tumor cell surface antigens, including anti-EL4 globulin, localize specifically in tumor tissue when administered intravenously [19–21]. Such localization of antibodies is consistent with their specific carrier role [1, 5]. Although chlorambucil retains alkylating activity after both non-covalent and covalent linkage to immunoglobulin [2, 5], it is not known whether intact conjugates are sterically suitable for intracellular transport and subsequent interaction with DNA molecules in living cells. If chlorambucil–antibody conjugates cannot use specific drug receptors for intracellular transport [22, 23], internalization of intact conjugates may be brought about either by events following binding of carrier immunoglobulin to its cell surface receptors (i.e., capping) or by non-specific pinocytosis and phagocytosis [1]. On the other hand, if free drug is essential for transport and/or target alkylation, chlorambucil may be released from covalent conjugates by cell surface or lysosomal enzymes and from non-covalent conjugates by simple dissociation. Other factors that may contribute to tumor inhibition by drug–antibody conjugates include: (i) drug–antibody synergism, (ii) a depot effect arising from protracted drug release from the conjugate [5]. The present results confirm synergism between chlorambucil and anti-EL4 globulin unlinked

(Fig. 4) [6]. If synergism at the cell surface is dependent on individual exposures to drug and antibody [24], ready dissociation of non-covalently linked chlorambucil might contribute to the marginally superior inhibition by the non-covalent chlorambucil-anti-EL4 globulin conjugate (Fig. 4). Furthermore, both chlorambucil [25] and antibodies against cell surface antigens, including anti-EL4 globulins, have been shown to damage cell membrane [6, 26, 27]. Even if independent or synergistic action by conjugate components at the cell surface is not itself lethal, permeability changes may occur which in turn facilitate transport of active conjugates or free drugs to intracellular targets. There was no evidence of a depot effect since mice treated with chlorambucil covalently or non-covalently linked to NRG did not survive longer than control untreated mice (Fig. 4). This finding is consistent with our previous report on lack of tumor inhibition by chlorambucil–NRG conjugates [4, 11].

The mechanistic events leading to cell kill are likely to differ depending upon whether chlorambucil and antibody are linked and, if linked, upon the nature of that linkage. Each modality will be characterized by a distinctive pattern of injury to cell surface and/or intracellular targets.

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