ABSTRACT

In this study, immunotoxins containing monoclonal anti-human T-cell leukemia antibodies are shown to be capable of specific killing of human T-leukemia cells in vitro.

These immunotoxins were prepared by conjugating ricin A chain (RIA) with our recently generated murine monoclonal antibodies, SN1 and SN2, the latter of which was obtained from a hybridoma clone N6/D11 described previously by Negoro and Seon (Cancer Res., 42: 4259-4262, 1982), directed to two unique human T-cell leukemia antigens. We have shown previously that these monoclonal antibodies do not react with non-T-leukemia cells nor with normal cells by using leukemia cell-free bone marrow as targets. In the present study, we selected 2 of these MCAbs, i.e., SN1 and SN2, for preparing immunotoxins [MCAb SN2 was obtained from hybridoma clone N6/D11 described previously (14)]. These MCAbs define unique individual human T-cell leukemia antigens (14, 23) and are directed to different antigen molecules as determined by specific immunoprecipitation followed by SDS-PAGE. Of the toxins, we chose ricin, a plant toxin derived from the castor bean, since the usefulness of the antibody:RIA conjugates for the in vitro killing of tumor cells has been reported already by several groups of investigators (6, 8-11, 15, 18, 28). The ricin molecule consists of 2 subunits, an A and a B chain, which are linked by a disulfide bond (reviewed in Ref. 16). The B chain is a lectin which is specific for galactose residues present on the surface of a wide variety of cells. The binding of the ricin B chain to mammalian cells allows the entry of the entire ricin molecule, and consequently the RIA, into the cytoplasm where the A chain is split from the B chain and acts on the 60S subunit of the ribosome to cause irreversible inhibition of protein synthesis and cell death. Isolated ricin B chain binds to cell surfaces but is nontoxic, while isolated RIA is not significantly toxic because of its inability to efficiently bind to cell surface and to traverse the cell membranes. Thus, conjugates of RIA with appropriate monoclonal antitumor antibodies may provide immunotoxins specifically targeted on the tumor cells that react with the antibodies.

Our present immunotoxins of anti-T-leukemia MCAbs and RIA showed specific killing of T-leukemia cells in vitro. Clinical utilization of such immunotoxins will be important in view of the facts that T-cell leukemia is associated with poor prognosis (1, 2, 7, 17, 20, 22, 29) and that autologous marrow transplantation using leukemia cell-free bone marrow has promising potential in the cure of patients with T-cell leukemia (see “Discussion”).

INTRODUCTION

Antibody carriers of toxins, termed immunotoxins, have drawn considerable attention recently because of their promising potential in tumor therapy. Preparation of useful immunotoxins requires appropriate specific antibodies; thus, monoclonal antitumor antibodies will be very useful. We have generated recently and characterized several MCAbs that are directed to human T-cell leukemia antigens (14, 23). These MCAbs react with T-ALL cells but do not react with various non-T-leukemia cells nor with various normal cells including normal T- and B-cells, thymocytes, and bone marrow cells. In the present study, we selected 2 of these MCAbs, i.e., SN1 and SN2, for preparing immunotoxins [MCAb SN2 was obtained from hybridoma clone N6/D11 described previously (14)]. These MCAbs define unique individual human T-cell leukemia antigens (14, 23) and are directed to different antigen molecules as determined by specific immunoprecipitation followed by SDS-PAGE. Of the toxins, we chose ricin, a plant toxin derived from the castor bean, since the usefulness of the antibody:RIA conjugates for the in vitro killing of tumor cells has been reported already by several groups of investigators (6, 8-11, 15, 18, 28). The ricin molecule consists of 2 subunits, an A and a B chain, which are linked by a disulfide bond (reviewed in Ref. 16). The B chain is a lectin which is specific for galactose residues present on the surface of a wide variety of cells. The binding of the ricin B chain to mammalian cells allows the entry of the entire ricin molecule, and consequently the RIA, into the cytoplasm where the A chain is split from the B chain and acts on the 60S subunit of the ribosome to cause irreversible inhibition of protein synthesis and cell death. Isolated ricin B chain binds to cell surfaces but is nontoxic, while isolated RIA is not significantly toxic because of its inability to efficiently bind to cell surface and to traverse the cell membranes. Thus, conjugates of RIA with appropriate monoclonal antitumor antibodies may provide immunotoxins specifically targeted on the tumor cells that react with the antibodies.

Our present immunotoxins of anti-T-leukemia MCAbs and RIA showed specific killing of T-leukemia cells in vitro. Clinical utilization of such immunotoxins will be important in view of the facts that T-cell leukemia is associated with poor prognosis (1, 2, 7, 17, 20, 22, 29) and that autologous marrow transplantation using leukemia cell-free bone marrow has promising potential in the cure of patients with T-cell leukemia (see “Discussion”).

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amino acid mixture (15 different 3H-amino acids) were purchased from S. K. Seon.

In the present study, 3 leukemic T-cell lines, i.e., MOLT-4, CCRF-CEM, and CCRF-SB, were used. All of the 3 leukemic T-cell lines were derived from individual patients with ALL (5, 13, 21).

Inhibition Test of Protein Synthesis by Immunotoxins and Control Conjugate. Leukemia or normal cell lines were suspended in RPMI Tissue Culture Medium 1640 containing FCS (5%), penicillin (100 units/ml), streptomycin (100 μg/ml), and gentamicin (50 μg/ml) to give 1 × 10⁶ cells/ml. For each cell suspension, 0.5 ml was placed in sterilized 1.5-ml polypropylene tubes and incubated with varying concentrations of the conjugates for 20 or 42 hr at 37° in a humidified 5% CO₂ incubator. The incubated cells were centrifuged, washed, and resuspended in amino acid-free (except for glutamine) medium containing 1.5 μCi of [³H]threonine or ³H-labeled amino acid mixture (see above).

The cell suspensions were incubated for 2 hr at 37° and centrifuged, and the resulting cell pellets were washed extensively. The washed cell pellets were treated with 5% TCA, and the insoluble material was washed 3 times with 5% TCA. The resultant 5% TCA-insoluble material was dissolved in Protosol (New England Nuclear, Boston, Mass.) and mixed subsequently with Scintisol (Isolab, Inc., Akron, Ohio). The ³H radioactivity was determined in a liquid scintillation counter.

Preparation of Rabbit Antiserum to RIA Chain. Two New Zealand White rabbits (Nos. 2010 and 2011) were immunized s.c. at multiple sites over the back with approximately 0.2 mg of the reduced RIA in 2 ml of PBS mixed with an equal volume of Freund’s complete adjuvant. Two booster immunizations were carried out in 5 and 7 weeks after the first immunization by injecting s.c. and i.m. with approximately 0.2 mg of the reduced RIA in 1 ml of PBS mixed with an equal volume of Freund’s incomplete adjuvant.

RESULTS

Analyses of SN1 IgG:RIA (Preparation 1) and Control IgG:RIA. The RIA conjugates prepared in the initial conjugation experiment, i.e., SN1 IgG:RIA and control mouse IgG:RIA (see “Materials and Methods”), were separated from unbound RIA by gel filtration on a calibrated Sephacryl S-200 column and analyzed by SDS:PAGE using a discontinuous buffer (Fig. 1). In the case of both SN1 IgG (Fig. 1, Gel A) and control IgG (Gel B), the major conjugate components were RIA, IgG (one RIA molecule per IgG molecule), RIA₁₂, IgG, and RIA₂, IgG. When the gels were scanned by a densitometer, the approximate ratios of these 3 components were 1.0:1.4:0.65 for Gel A (SN1 IgG) and 1:0:1:1:0 for Gel B (control IgG). In both cases, the remaining intact IgG was detected as a minor component (<10% of total proteins detected).

The SN1 IgG:RIA and control IgG:RIA conjugates were analyzed further by a double-gel diffusion test using Rabbit No. 2011 anti-RIA antiserum (Fig. 2). Both of these conjugates reacted with the anti-RIA antiserum, and a complete coalescence was observed between immunoprecipitin lines formed by free RIA and these conjugates. Essentially the same result was obtained using anti-RIA antiserum of another rabbit (Rabbit 2010). These results indicate that the conjugate was not undetected.

Cytotoxicity of SN1 IgG:RIA (Preparation 1). The in vitro cytotoxicity of the SN1 IgG:RIA conjugate and the control IgG:RIA conjugate was evaluated by 2 different methods. In the first method, leukemia or normal cell lines were cultured in individual wells of a tissue culture plate, in triplicate, in the absence (control) or in the presence of various concentrations of the conjugates; the viability of the cultured cells was then determined (Chart 1).

The SN1 IgG:RIA conjugate showed cytotoxicity against JM cells, leukemia T-cells, at the conjugate concentrations of 10⁻⁸...
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Chart 1. Specific cytotoxic activity of conjugate of RIA and MCab SN1. JM, a human leukemic T-cell line, and RPMI 8057, a human nonmalignant B-cell line, at initial cell densities of 5.5 x 10^5 cells/ml, were grown individually in medium alone or medium containing the designated concentrations of SN1 lgG:RIA conjugate (Preparation 1) or control mouse lgG:RIA conjugate. •, medium alone; O, 10^-11 M conjugate; A, 10^-10 M conjugate; A, 10^-9 M conjugate; x, 10^-8 M conjugate. See "Materials and Methods" for the experimental details. Portions of the cells were removed on 1 and 2 days after the start of the culture, and viability was determined using trypan blue.

Fig. 1. SDS-PAGE (7.5% gels) of unreduced SN1 lgG:RIA (Preparation 1) (Gel A) and control IgG:RIA (Gel B). The proteins in the gels were stained with Coomassie Brilliant Blue R250. The reference marker proteins used are: myosin (M, 200,000); murine lgG (M, 150,000); β-galactosidase (M, 116,000); F(ab')2 (M, 106,000); bovine serum albumin (M, 68,000); and ovalbumin (M, 43,000). Arrow, position of the reference dye marker, bromophenol blue. The sample protein bands indicated by the horizontal lines are: 0, intact IgG; 1, RIA1:lgG (one RIA molecule per lgG molecule); 2, RIA2:lgG; 3, RIA3:lgG.

and 10^-8 M. After 2 days of culture at 10^-8 M concentration, over 90% of JM cells were killed (Chart 1a). This SN1 lgG:RIA conjugate did not show any significant cytotoxicity against RPMI 8057 cells (normal B-cells) at any concentrations tested including 10^-9 and 10^-8 M (Chart 1b). Furthermore, the control lgG:RIA conjugate did not significantly kill either leukemic T-cells (Chart 1c) or normal B-cells (Chart 1d) at any concentrations tested. Only a slight cytotoxic effect was observed at the highest concentration, i.e., 10^-8 M, of the conjugate against leukemic T-cells after 2 days of culture (Chart 1c).

In the second method, the inhibitory activity of the SN1 IgG:RIA and the control lgG:RIA conjugates on protein synthesis in leukemia and control cell lines was determined (Chart 2). SN1 lgG:RIA conjugate showed significant inhibitory activity against MOLT-4 cells (leukemic T-cells) at concentrations higher than 5 x 10^-9 M (Chart 2a), whereas the same conjugate did not significantly inhibit protein synthesis in RPMI 1788 cells (normal B-cells) (Chart 2b). Moreover, the control lgG:RIA conjugate showed only slight inhibitory activity against both MOLT-4 and RPMI 1788 cells (Chart 2). These results are in agreement with the results shown in Chart 1, where direct cytolytic activity of these conjugates was tested against JM cells, a leukemic T-cell line, and RPMI 8057, a normal B-cell line.

Analyses of SN1 IgG:RIA (Preparation 2) and SN2 lgG:RIA.
The RIA conjugates prepared in the second conjugation experiment, i.e., SN1 IgG:RIA (Preparation 2) and SN2 lgG:RIA, were separated from unbound RIA (see "Materials and Methods") and analyzed by SDS-PAGE (Fig. 3). In the cases of both SN1 (Fig.

Fig. 2. Double-gel diffusion test using rabbit anti-RIA chain antisera. The antisera (Rabbit 2011) was placed in the center well, and the test samples were placed in each of the surrounding wells. The samples used are: 1, RIA; 2, SN1 lgG:RIA (Preparation 1); 3, control lgG:RIA; 4, SN1 lgG; 5, SN2 lgG:RIA; 6, SN1 lgG:RIA (Preparation 2).
B. K. Seon

3, Gel A) and SN2 (Fig. 3, Gel B), 3 components, i.e., intact IgG, RIA1:lgG (one RIA molecule per IgG molecule), and RIA2:lgG, were detected. The approximate ratios of these components as determined from densitometer scans were 1.0:0.93:0.18 for SN1 and 1.0:0.86:0.15 for SN2.

It should be noted that the yield of RIA incorporation in the present conjugation is significantly lower than that in the earlier conjugation (see Fig. 1). This is apparently due to the fact that the SPDP-treated IgG proteins used for preparing the present conjugates contained significantly less 2-pyridyl disulfide groups than those used for preparing the earlier conjugates (see "Materials and Methods"). This lower degree of RIA incorporation affected significantly the cytotoxic activity of the conjugation products (see below). This is probably because the intact MCAb remaining in the conjugation product inhibits the cytotoxic activity of the MCAb:RIA conjugate by competing with the conjugated MCAb in binding to the antigens on the cells.

These conjugates, i.e., SN2 lgG:RIA and SN1 lgG:RIA (Preparation 2), were further analyzed by double-gel immunodiffusion using rabbit anti-RIA antiserum (Fig. 2, Wells 5 and 6). As with SN1 lgG:RIA (Preparation 1) and control lgG:RIA (Fig. 2, Wells 2 and 3), complete coalescence was observed between immunoprecipitin lines formed by free RIA and these conjugates.

**Cytotoxicity of SN1 lgG:RIA (Preparation 2) and SN2 lgG:RIA.** The inhibitory activity of SN1 lgG:RIA (Preparation 2) and SN2 lgG:RIA on protein synthesis was determined using CCRF-CEM, a leukemic T-cell line. Both SN1 lgG:RIA and SN2 lgG:RIA inhibited protein synthesis in CCRF-CEM cells with the latter conjugate showing higher activity (Chart 3). Consistent with the author's earlier finding (see Chart 2b), these conjugates inhibited protein synthesis only marginally in CCRF-SB cells, a normal B-cell line (data not shown). The addition of both conjugates showed increased inhibitory activity against leukemia cells compared with that of individual conjugate (Chart 3). Further, 3 different human leukemic T-cell lines, i.e., JM, MOLT-4, and CCRF-CEM, were compared with respect to their susceptibility to the inhibition of protein synthesis by the combined use of these immunotoxins (Chart 4). All 3 were inhibited strongly, with CCRF-CEM being the most susceptible and with JM and MOLT-4 being inhibited to similar degrees.

**DISCUSSION**

The induction rate of the initial remission of overall ALL is high, especially in children. However, many ALL patients, especially T-cell ALL patients, who have been in remission suffer a relapse, and at present, it is very difficult to cure ALL patients with relapsed disease (4, 7, 12, 20). One promising approach to solving this problem is bone marrow transplantation of patients who have received aggressive high-dose chemotherapy and radiotherapy (26, 27). The applicability of this approach, however, has been limited, because the majority of the patients do not have suitable donors of normal bone marrow. One means of overcoming this limitation is to use autologous bone marrow transplantation if bone marrow from the cancer patient can be made tumor cell free in vitro (25, 26). For this, specific anti-ALL MC Abs will be very useful in the in vitro eradication of tumor cells in the bone marrow from ALL patients (19), and since our anti-T-leukemia MC Abs do not react with normal bone marrow cells (14, 23), immunotoxins prepared using RIA (see "Introduction") and our anti-T-leukemia MC Abs have good potential for such use.
In the present preclinical experimental study, RIA was conjugated with 2 MC Abs, SN1 and SN2, and with control mouse IgG. The cytotoxic activity of these conjugates was tested in vitro against cultured T-leukemia cells and control cultured normal B-cells (Charts 1 to 4). Both conjugates containing antileukemia MA b SN1 or SN2 showed specific cytotoxic activity against leukemia T-cells but not against normal B-cells, whereas the control conjugate was not significantly cytotoxic against either cell type. In the clinical utilization of antileukemia MA bs, the simultaneous use of multiple MA bs targeted on different leukemia antigen molecules will be more effective in complete eradication of leukemia cells than the use of a single antileukemia MA b. This is especially so in view of the fact that tumor cells, in general, display heterogeneity with respect to the expression of tumor-associated antigens. Therefore, in the present study, 2 MA bs, SN1 and SN2, that react with different leukemia antigen molecules (Refs. 14 and 23; Footnote 4) were used with established cell lines. Although the increase in cytotoxicity by the combined use of SN1 IgG:RIA and SN2 IgG:RIA conjugates compared to their individual use was not impressive against homogeneous leukemic cell lines (Charts 3 and 4), such a combined use would be important against heterogeneous uncultured leukemia cells freshly derived from patients.

As a next step to clinical utilization, tests against uncultured normal bone marrow cells and uncultured bone marrow cells from T-cell ALL patients will be carried out, and the present results will provide information important in designing such experiments.

Recently, we have obtained preliminary results using the granulocyte, erythroid, macrophage, and megakaryocyte colony-forming unit assay which indicate that neither SN1 IgG:RIA nor SN2 IgG:RIA is cytotoxic against hemopoietic stem cells.\(^5\)

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Specific Killing of Human T-Leukemia Cells by Immunotoxins Prepared with Ricin A Chain and Monoclonal Anti-Human T-Cell Leukemia Antibodies

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