Monoclonal Antibody-Ricin A Chain Conjugate Selectively Cytotoxic for Cells Bearing the Common Acute Lymphoblastic Leukemia Antigen

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ABSTRACT

The toxic subunit of ricin has been conjugated by a disulfide bond to a monoclonal murine antibody (J-5) specific for the common acute lymphoblastic leukemia antigen (CALLA) expressed on human lymphoblastic leukemia cells. Both the monoclonal antibody and ricin A chain retained their original biological activity after conjugation. The ricin A chain portion of this conjugate effectively inhibited protein synthesis in a cell-free rabbit reticulocyte lysate system. Its antibody-combining site attached to the membrane of CALLA-bearing cells, whereas upon both components of the molecule, antibody and A chain, could be detected using fluorescein-labeled antibody probes directed against either mouse F(ab')2 or ricin A chain.

This conjugate proved to be a potent cytotoxin for CALLA-positive Nalm-1 cells growing in vitro and produced 50% inhibition of proliferation at levels of 2 x 10^-10 M. In contrast, CALLA-negative cell lines were unaffected until levels of conjugate approached 10^-8 M. When tested for cytotoxic action on Nalm-1 cells, the composite conjugate molecule was at least 2000 times more effective than J-5 antibody alone, ricin A chain alone, or a noncovalent mixture of these components. Cytotoxicity of the conjugate could be blocked completely by addition of antibodies specific for either mouse immunoglobulin or ricin A chain.

Direct comparison of the in vitro cytotoxicity of CALLA-directed univalent Fab versus bivalent F(ab')2 carriers of ricin A chain revealed 70-fold greater efficacy for the latter conjugate. This differential could not be accounted for by differences in A chain activity or binding affinity for the cell membrane but appeared related to the ability of the multivalent agent to induce modulation and internalization of CALLA determinants.

No overt toxic effects were noted when the F(ab')2-A chain conjugate was administered i.v. to rabbits at levels as high as 20 mg/kg single injection or 5 mg/kg for 5 days. The unique specificity characteristics and high potency of this cytotoxic conjugate indicate that it has significant therapeutic potential for the treatment of CALLA-bearing human leukemias and lymphomas.

INTRODUCTION

Cancer chemotherapy, despite recent advances, is still limited by the lack of selectivity of cytotoxic drugs. Agents that are effective in killing neoplastic cells are usually also toxic to normal cells, and any excessive cytotoxicity to vital tissues seriously restricts the pharmacological control of malignant diseases. One method of improving the selectivity of chemotherapeutic agents is to utilize specific delivery agents which will carry lethal drugs to malignant cells but not to normal cells. This concept dates back three-quarters of a century to Paul Ehrlich (6), who proposed that molecules with an affinity for certain tissues might serve as carriers of cytotoxic agents and concentrate them on the appropriate target cells in vivo. Within the past 2 years, the ability to effectively test this concept has been developed. A monoclonal antibody which specifically reacts with leukemic lymphoblasts has been produced, and its reactivity has been defined extensively (30, 31). The groundwork for the construction, characterization, and utilization of unique antibody-ricin A chain cytotoxins has been elucidated in a prototype system which used immunoglobulin present on the membrane of human lymphoid cells as a target antigen. This has allowed for an unequivocal demonstration of highly selective antibody-directed cytotoxicity (28).

Monoclonal J-5 antibody is specific for CALLA which is expressed on leukemic cells from 80% of patients with non-T- or B-cell ALL and approximately 40% of patients with CML in blast crisis (31). In addition, tumor cells from patients with CML nodular lymphoma and Burkitt's lymphoma express CALLA (30). This antigen has also been found on a small number (<2%) of normal bone marrow cells as well as in fetal liver by using the J-5 reagent (30). CALLA has been identified as a M.W. 100,000 cell surface glycoprotein (26, 31, 36), but the precise functional role of this protein is not known. Interestingly, antigenic modulation of CALLA on acute lymphoblastic cells occurs in response to J-5 antibody (32), and this has been shown to be an internalization process (25).

Rcin, a potent toxin isolated from castor beans, consists of 2 subunits (A and B chains) connected by a disulfide bond (23). Under normal circumstances, whole ricin adheres to cell surface glycoproteins via a combining site on the B chain. This binding interaction then facilitates entry of ricin or its A chain into cells. Once A chain enters the cytoplasm and is split from the B chain, it acts catalytically on the 60S ribosomal subunit to cause irreversible inactivation and a complete shutdown of de novo protein synthesis (21). Isolated ricin B chain binds to cell membranes but is nontoxic while isolated ricin A chain is a potent toxin if it gains access to ribosomes. The cytotoxicity of free A chain, however, is limited by an inability to attach to the cell surface and traverse its membrane without an associated carrier protein.

Recently, several methods for coupling the isolated ricin A chain to intrinsic or substituent sulfhydryl groups on carrier antibodies have been developed (1, 10, 15, 18, 28). Thus, a disulfide bond which simulates the subunit connection present...
in the native toxin was retained, allowing cleavage and full A chain activity following cellular internalization. In each instance, the new composite molecule acquired the specificity characteristics of the antibody and retained the potent toxic activity of the A chain. These conjugates were thereby cytotoxic exclusively for cells bearing the appropriate antigen. The diffuse toxicity associated with whole toxin was circumvented since only its A chain was used.

Given the previously demonstrated potency and selectivity of antibody-ricin A chain cytotoxins, this present study was undertaken to assess the in vitro activity of such conjugates on cell lines carrying an antigen expressed by human leukemias. Monoclonal J-5 antibody not only reacts specifically with such an antigen (CALLA) but has the additional virtue of being modulated and internalized after binding to the cell membrane. Ricin A chain was therefore coupled to J-5 antibody, which served as a carrier to concentrate it at the surface of CALLA-bearing cells. Expression of the activity of this toxic moiety resulted as conjugate molecules became internalized and gained access to ribosomes within the cytoplasm. Not only was the lethal action focused selectively on CALLA-positive cells but cytotoxicity was further accentuated by the specific modulation phenomena induced by interaction of the J-5 conjugate with this surface antigen.

MATERIALS AND METHODS

Conjugate Preparation. Affinity-purified ricin (M.W. 64,000) from E-Y Laboratories (San Mateo, Calif.) was reduced with 5% mercaptoethanol (22) and applied to a column of lactose-substituted agarose (Selectin 12; Pierce Chemical Co., Rockford, Ill.). Ricin A chain passed directly through upon PBS elution while B chain and intact ricin bound and were subsequently released with 0.1 M lactose in PBS. After removal of mercaptoethanol from the A chain preparation by gel filtration using Sephadex G-25, further traces of whole ricin were eliminated by passage over an acide-treated Sepharose column (9). Monoclonal murine IgG 2a antibody (J-5) directed against human CALLA was isolated from mouse ascites by (NH₄)₂SO₄ fractionation and gel filtration using Sephadex G-200. Whole 7S immunoglobulin was digested with either pepsin or papain (12) to produce bivalent F(ab')₂ and univalent Fab molecules, respectively. These antibody fragments were purified by gel filtration or ion exchange chromatography as described (12). The J-5 fragments were then derivatized using a heterobifunctional SPDP reagent (2) (Pharmacia Fine Chemicals, Piscataway, N. J.) according to the manufacturers specifications so that 3 to 4 mole SPDP per antibody fragment were attached. A 2-fold molar excess of freshly reduced ricin A chain was reacted with the SPDP-substituted J-5 molecule for 6 hr at 23°. The reaction cations so that 3 to 4 mole SPDP per antibody fragment were attached.

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Activity Analysis of Conjugates. The ribosome-inactivating capacity of free ricin A chain as well as the A chain conjugates was quantitated as described previously (28) using an in vitro rabbit globin-synthesizing system from Bethesda Research Laboratories (Rockville, Md.). Cell surface reactivity of conjugate molecules was assessed after incubation at 2° for 30 min with CALLA-positive Nalm-1 cells, washing the cells with PBS to remove unbound agent, and development for indirect immunofluorescence using either fluorescein-labeled rabbit anti-ricin A chain or goat anti-mouse F(ab')₂ reagents (28). The washed cells were resuspended in 1 ml of PBS and analyzed with a fluorescence-activated cell sorter (FACS I; Becton Dickinson, Mountain View, Calif.). This technique was used to compare the cellular avidity of J-5 F(ab')₂-A and Fab-A as well as whole J-5 IgG by assaying cell-bound antibody with fluoresceinated anti-mouse F(ab')₂, subsequent to exposure of CALLA-positive cells to varying concentrations of each agent. Levels required to produce maximal and half-maximal cell fluorescence were evaluated, and no differences in immunoreactivity were noted.

Human Cell Lines. CALLA-positive human cell lines examined in this study included Nalm-1, derived from a patient with CML in blast crisis (19), Laz 221, a null ALL line (16), HPB-ALL, a T-cell ALL-derived line (20), as well as 3 Burkitt’s lymphoma cell lines designated Raji (27), Daudi (13), and Ramos (14). The CALLA-negative human cell lines studied were CEM (8) and HSB-2 (17), 2 leukemic T-cell lines, HL-60, a myeloid line (3), and Laz 388, an Epstein-Barr virus-transformed B-lymphoblastoid cell line derived from the same patient as Laz 221.

Cytotoxicity of the Conjugates. Cells growing in suspension with 10% fetal calf serum in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Microbiological Associates, Inc., Walkersville, Md.) were plated into 1.7- x 1.6-cm tissue culture wells at concentrations optimal for growth at 37°. Cytotoxic agents were added to the 1-ml cultures to achieve those levels specified, and aliquots were removed daily to count viable cells using trypan blue (28). When untreated control cultures displayed cell death due to overgrowth, the experiment was terminated. The action of conjugate was blocked in certain time course studies by adding the IgG fraction (50 µg in PBS) of (NH₄)₂SO₄-precipitated rabbit anti-ricin A chain serum, goat anti-mouse F(ab')₂ serum, or control normal rabbit serum. Inhibition of protein synthesis was evaluated after incubating cells in medium with varying concentrations of conjugate or ricin for 24 and 48 hr at 37°. The cells were spun, resuspended at 1 x 10⁶ cells/ml in leucine-free medium containing 1.25 µCi of L-['⁴C]leucine per ml, and incubated for 1 hr at 37°. Incorporation of radioactivity into the cells was measured by scintillation counting after extensive washing on a glass fiber filter (28).

Toxicity in Rabbits. Animals were given the CALLA-directed F(ab')₂-A chain conjugate either as a single i.v. bolus or consecutively for 5 days. Rabbits receiving the highest levels were monitored after treatment for total protein, blood urea nitrogen, serum creatinine, serum glutamic-oxaloacetic transaminase, lactic dehydrogenase, alkaline phosphatase, serum glucose, Na⁺, K⁺, Cl⁻, CO₂, Ca²⁺, serum creatine phosphokinase, WBC, RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and platelets, and their status was compared to control animals.

RESULTS

Monovalent and Bivalent Conjugates Possessing Antibody Plus A Chain Activities. CALLA-directed monoclonal F(ab')₂ and Fab fragments were reacted with the SPDP reagent (2) to introduce 3 to 4 substituent-activated thiol groups. Thereafter, isolated ricin A chain could be efficiently disulfide linked according to the scheme shown in Chart 1. Conjugate species having 1, 2, or 3 A chain moieties/antibody fragment were identified after separation by size using gel filtration and analysis on sodium dodecyl sulfate-polyacrylamide gels. Fractions containing predominantly molecules with antibody and A chain at a ratio of 1:1 were pooled and utilized. A comparison of the ribosome-inactivating capacity of the bivalent J-5 F(ab')₂-A chain and the monovalent J-5 Fab-A chain conjugates demonstrated that they were equipotent and about one-tenth as efficient as free A chain itself (Chart 2). This difference reflects the fact that A chain must be released from the disulfide bond in situ before it becomes active (23). Thus, the disulfide-linked
CALLA-specific Antibody-Ricin A Chain Cytotoxins

J5 ANTIBODY

RICIN

Pepsin Digestion

2-Mercaptoethanol

J5 (Fab’2)

2-Mercaptoethanol

SPDP

Affinity Chromatography (Lactose)

A

B

A

B

A

B

A

B

J5-A Chain Conjugate

Chart 1. Conjugation of isolated ricin A chain to the F(ab’)2 fragment of monoclonal anti-CALLA antibody (J-5) using SPDP reagent.

Chart 2. Comparison of the ribosome-inactivating capacity of free ricin A chain, the bivalent J-5 F(ab’)2-A chain conjugate, and the monovalent J-5 Fab-A chain conjugate. A rabbit reticulocyte lysate system for 14C-protein synthesis was used.

A chain of the conjugate retained its ability to catalytically inactivate ribosomes after reductive cleavage in the proteinsynthesizing system.

Effective binding of intact conjugate to the surface of CALLA-positive Nalm-1 cells was revealed after exposure to the agent at 2°, washing to remove unbound molecules, and developing with either fluoresceinated anti-ricin A chain or anti-mouse F(ab’)2 reagents. These 2 indirect immunofluorescent probes demonstrated the simultaneous presence of both monoclonal mouse F(ab’)2 and ricin A chain on the cell membrane as shown by the FACS II analysis in Chart 3. Cells treated with the appropriate immunofluorescent reagent, without prior exposure to conjugate, served as controls. Fluorescence patterns similar to those shown in Chart 3 were obtained with the Fab-A chain conjugate. In accordance with its stoichiometry, the fluorescence intensity observed with this monovalent agent was one-half of that seen with the F(ab’)2-A chain molecule which has twice as many sites for reaction with the anti-mouse F(ab’)2 probe. Conjugate-treated CALLA-negative cells were completely unreactive with both fluorescence probes, showing that neither portion of the agent could attach in the absence of a specific interaction with the CALLA determinant.

Evaluation of Specific Cytotoxicity of Ricin A Chain Antibody Conjugates. The cell growth curves shown in Chart 4 demonstrate the in vitro cytotoxic activity of the J-5 F(ab’)2-A chain conjugate on CALLA-positive Nalm-1 cells. Control Nalm-1 cells incubated without conjugate proliferated in culture for 7 days while addition of increasing doses of the specific A chain conjugate to these cells produced progressive cytotoxic effects. Levels of 10^-9 M were completely cytostatic, and higher concentrations produced extensive cell death and lysis over several days. The growth and viability of CALLA-negative cell lines in contrast were not adversely affected by these concentrations of conjugate. In vitro studies also demonstrated that the toxic effects of this conjugate were truly a property of the intact molecule. Neither antibody alone nor A chain alone had equivalent cytotoxicity (Chart 5). Comparison of the concentrations required to produce 50% inhibition of the proliferation of Nalm-1 cells in culture indicated that the monoclonal anti-CALLA F(ab’)2-A chain conjugate was 2000 times more potent than either component by itself. A noncovalent mixture of the 2 parts did not augment their individual toxicities, demonstrating that the covalent disulfide linkage was indeed required for the potent cytotoxicity displayed by conjugate.

Selectivity of the Conjugate for CALLA-bearing Cell Lines. The selectivity of this conjugate became manifest when its...
cytotoxic effect was evaluated on a panel of actively dividing CALLA-positive and -negative cell lines (Tables 1 and 2). No significant growth differences were noted when CALLA-negative cells were cultured in the presence of high levels of the specific F(ab')2-A chain while all CALLA-bearing cells were affected by this selective cytotoxin. It is apparent that the effectiveness of its action on susceptible cells varied, and some conjugate-treated CALLA-bearing lines continued to grow, albeit at a reduced rate.

Inhibition of Cellular Protein Synthesis. Inactivation of ribosomal function in intact cells becomes evident following the passage of ricin A chain through the plasma membrane into the cytosol, and this event can be quantified as a diminished capacity for protein synthesis (23). Levels of [14C]leucine incorporation were measured after treatment of Nalm-1 cells for 24 hr in culture with either CALLA-specific F(ab')2-A chain or intact ricin which interacts strongly with membrane glycoproteins (34) (Table 3). In contrast to the action of whole ricin which provided increasing effects with dose, the extent of inhibition exhibited by conjugate plateaued at approximately 30% beyond concentrations of \(10^{-9}\) M. This threshold level for inhibition of protein synthesis corresponds to the concentration of conjugate required to produce substantial cytotoxicity as seen in the growth curves (Charts 4 and 5). Inclusion of a 100-fold molar excess of unmodified J-5 antibody effectively blocked any action of F(ab')2-A chain on Nalm-1 cells, demonstrating that inhibition of protein synthesis was mediated by the CALLA-directed combining site of the conjugate. The test cultures described in Table 3 were also monitored for growth and viability during 6 days. While comparable cytotoxicity was observed for ricin at \(1 \times 10^{-11}\) M and each of the conjugate levels tested, the \(5 \times 10^{-11}\) M level of ricin showed more rapid and complete cell destruction.

Role of Antibody Valency on Cytotoxic Effectiveness. Since the univalent Fab-A chain and bivalent F(ab')2-A chain conjugates possessed equivalent inhibitory activity in the cell-
free protein synthesis assay (Chart 2), it was of interest to determine if they were similarly effective in producing cytotoxicity for intact CALLA-bearing cells. A comparison of the molar concentration of conjugate required to effect a 50% reduction of cell growth for Nalm-1 cells showed clearly that the univalent carrier was 70-fold less potent than its bivalent counterpart (Chart 6). The Fab-A chain cytotoxin did provide complete inhibition at levels of $5 \times 10^{-8}$ M but apparently traversed into the cell less efficiently than did the F(ab')$_2$-A chain molecule. It seemed possible that the difference might occur at the level of cell surface binding. However, titrations conducted on Nalm-1 cells at $2^\circ$ by indirect immunofluorescence and FACS analysis showed that maximal and half-maximal saturation of CALLA determinants with either univalent or bivalent conjugates was achieved at identical levels on the basis of antibody-combining sites added (maximal at $4 \times 10^{-7}$ M; half-maximal at $8 \times 10^{-8}$ M).

**Modulation of CALLA Determinants.** Nalm-1 cells incubated for 24 hr at $37^\circ$ with excess (1 $\times 10^{-6}$ M antibody sites) F(ab')$_2$-A chain, Fab-A chain, or in medium alone were examined for the presence of cell-bound conjugate molecules as well as for any free CALLA sites on the membrane. Direct treatment of washed cells with either the anti-A chain or anti-mouse F(ab')$_2$ fluorescent probes was used to reveal conjugate remaining on the surface while reexposure of a second portion of the washed cells to additional conjugate at $2^\circ$ before developing with the fluorescent reagents served to detect any unoccupied CALLA sites. Comparison of the fluorescence intensity to 0-time controls showed that a simultaneous loss of 85% of F(ab')$_2$-A chain conjugate molecules and CALLA determinants had occurred over this period. Within this same 24-hr time span, monovalent Fab-A chain produced only a 30% drop in the level of conjugate and CALLA found on the surface membrane of Nalm-1 cells. These results are in accord with the previously described internalization of CALLA induced by whole J-5 antibody (25, 32) and is consistent with the differential toxicity displayed by the monovalent and bivalent J-5 carriers of ricin A chain.

**Time Course of Toxicity for Actively Dividing Nalm-1 Cells.** The growth inhibition curves shown in Chart 4 represent the action of conjugate present continually throughout the incubation period. While this delineates levels needed to achieve in vitro cytotoxicity, it is equally important to determine toxicity requirements in terms of length of exposure to this agent. Data presented in Chart 7 illustrate that the simultaneous inclusion of excess antibodies specific for either half of the conjugate molecule effectively blocks its cytotoxic effect on Nalm-1 cells. This feature provides a highly discriminating and convenient method for sequestering free conjugate at any point in the growth curve. Thus, rabbit anti-ricin A chain was used to arrest further action of the CALLA-specific F(ab')$_2$-A chain conjugate ($5 \times 10^{-6}$ M) on identical cultures of Nalm-1 cells growing in parallel after either 0, 4, 24, or 48 hr of treatment. The extent of inhibition of growth was compared to conjugate-treated cells which received normal rabbit IgG in place of the specific rescue antibody (Chart 8). Slight effects on subsequent cell growth were noted even when conjugate treatment was terminated as soon as 4 hr after commencement. Exposure times of 24 and 48 hr, however, produced progressively greater inhibitory effects on the initiation and rate of cell replication. The inhibition curve in which no specific blocking antibody was present

<table>
<thead>
<tr>
<th>Addition</th>
<th>cpm</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>3400 ± 476$^a$</td>
<td></td>
</tr>
<tr>
<td>J-5 F(ab')$_2$-A chain-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$1 \times 10^{-9}$</td>
<td>2888 ± 102</td>
<td>15</td>
</tr>
<tr>
<td>$1 \times 10^{-8}$</td>
<td>2392 ± 202</td>
<td>30</td>
</tr>
<tr>
<td>$1 \times 10^{-7}$</td>
<td>2300 ± 194</td>
<td>32</td>
</tr>
<tr>
<td>$1 \times 10^{-6}$</td>
<td>2406 ± 277</td>
<td>29</td>
</tr>
<tr>
<td>Ricin-treated cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$2 \times 10^{-12}$</td>
<td>3636 ± 110</td>
<td>0</td>
</tr>
<tr>
<td>$1 \times 10^{-11}$</td>
<td>2394 ± 16</td>
<td>30</td>
</tr>
<tr>
<td>$5 \times 10^{-11}$</td>
<td>1190 ± 161</td>
<td>65</td>
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$^a$ Mean ± S.D.
dose for whole ricin (7), and the lack of toxicity indicates little
tology tests revealed no differences between controls and
these animals all appeared unaffected
as an i.v. injection for 5 consecutive days at levels of 0.1, 1,
the cells died.
completely suppressed growth in the culture and eventually all of
shows that continual pressure provided by the conjugate com-
pletely suppressed growth in the culture and eventually all of
the cells died.
Toxicity Trials in Animals. The J-5 F(ab')2-A chain prepa-
ration was administered to rabbits at 0.1, 1, 10, and 20 mg/kg
as a single i.v. bolus. Additional rabbits received the conjugate
as an i.v. injection for 5 consecutive days at levels of 0.1, 1,
and 5 mg/kg each day. These animals all appeared unaffected
by the agent, and standard blood chemistry as well as hema-
tology tests revealed no differences between controls and
rabbits treated at the highest levels of conjugate. These doses
were approximately 10,000-fold higher than the minimum lethal
dose for whole ricin (7), and the lack of toxicity indicates little
or no contamination with native toxin. It further suggests that
these animals can catabolize the conjugates without toxic side
effects.

DISCUSSION

The disulfide-linked conjugate formed using a monoclonal
antibody directed against CALLA and purified ricin A chain
retained its ability to inactivate ribosomes in a cell-free system
as well as to specifically bind to antigen-bearing cells. Intact
conjugate molecules were identified on the surface of CALLA-
positive cells using fluorescent antibody reagents. The inhibi-
tion of protein synthesis and selective cytotoxicity of this con-
jugate indicate that membrane-bound A chain entered
the cytoplasm. These toxic effects were antibody mediated since
complete blockage of conjugate action was observed if its
binding was obstructed by anti-mouse F(ab')2 antibodies or if
excess unmodified J-5 antibody was present to compete for
CALLA receptor sites on the cell. This was further inferred by
the lack of any potent cytotoxic effects of conjugate on CALLA-
negative cell lines. Lastly, the individual component halves of
the conjugate molecule failed to display strong cytotoxicity on
their own or as a noncovalent mixture when tested with CALLA-
positive cells.

Examination of the activity of the CALLA-directed ricin A
chain delivery system disclosed several unique features. Inhi-
bition of cellular protein synthesis reached a maximum level of
30% at concentrations of conjugate between 10^{-9} and 10^{-8}
m, well below the level required to saturate cell surface CALLA
determinants. No further inhibition was observed at higher
concentrations even though potentially more conjugate had
bound to the cells. Similarly, effects on cell growth and viability
reached a maximum within this same concentration range, and
cytotoxicity was neither greatly increased nor accelerated by
increasing the dose of conjugate beyond that level. These
results suggest that some factor other than the amount of
conjugate attached to membrane may influence toxicity in this
system.

Even though inhibition of protein synthesis was not complete
for Nalm-1 cells, exposure to the conjugate effectively pre-
vented growth and produced a continual decline in cell number
over 7 days. Treatment of these cells with a concentration of
whole ricin adjusted to also produce approximately 30% inhibi-
tion of protein synthesis likewise displayed a similar pattern
of growth inhibition and cell death. In contrast to the activity of
specific F(ab')2-A chain, however, these events were greatly
accelerated at higher doses of ricin which also produced
increasing inhibition of protein synthesis. The number of recep-
tors for ricin on a cell (34) vastly exceeds the number of CALLA
determinants making it difficult to judge if distinctions in toxicity
are based upon qualitative or quantitative differences between
the 2 delivery systems. Interestingly, ricin A chain conjugates
directed against human immunoglobulin on lymphoblastic cell
lines produced 80% inhibition of protein synthesis yet still
required 4 to 5 days to effect extensive cell lysis (28). Recent
studies have lent support to the idea that ricin B chain may
fulfill a role in addition to cell surface binding which augments
toxicity (5, 35, 37). This phenomenon might also be reflected in
the contrasting activity of whole ricin and isolated A chain
when delivered by hybrid antibodies which noncovalently bind
and deliver these toxin moieties to the cell membrane (29).

A further indication of the complexity of action of this con-
jugate on CALLA-bearing cells became apparent from the
differential sensitivity of the 3 Burkitt's lymphoma cell lines in
comparison to the CML and ALL lines tested. Growth of theBurkitt's cells was not fully curtailed by the conjugate. Since
these cell lines each express similar amounts of CALLA on
their surface, it is likely that the biology of the individual cell
type can influence susceptibility to the conjugate. Factors such
as cell cycle kinetics as well as the mode of internalization and
intracellular processing of conjugate (24) should be considered as
probable points of departure.

Previous studies (32) have demonstrated that binding of J-5
antibody to the cell surface induces the specific modulation of
CALLA. This process is temperature dependent and results in
the loss of both antigen and antibody from the cell membrane.
Experiments using radiolabeled surface antigen have shown that
CALLA is internalized and degraded in the process (25). Furthermore, antigenic modulation occurs both more rapidly

Chart 8. The time course of conjugate action. Individual cultures of Nalm-1
cells were plated at Time 0 using 80 x 10^6 cells/ml in medium containing 5 x
10^{-8} M Flab').-A chain. Rabbit anti-ricin A chain was added either immediately
(0) or after 4 (A), 24 (B), or 48 (C) hr of incubation with conjugate at 37°. A
control culture (O) received nonspecific rabbit IgG in place of the blocking
antibodies.
and extensively with whole J-5 or F(ab')2 fragments than with univalent Fab. The fact that J-5 antibody-A chain conjugates are cytotoxic suggests that the intact molecule penetrates the membrane bound to CALLA and is then degraded. Moreover, finding that bivalent F(ab')2-A chain was a significantly more potent cytotoxin than the univalent Fab-A conjugate supports the notion that modulation and toxin entry are intertwined. This is especially pertinent since both preparations displayed equivalent A chain activity and binding to CALLA-positive cells. The premise was further substantiated by demonstrating that bivalent A chain conjugate was effective in modulating CALLA from the surface of cells while the monovalent agent was not.

It is difficult to reconcile the observation that these bivalent molecules stimulated modulation, presumably via cross-linking CALLA on the surface yet bound no more strongly to the cell than univalent antibody (4, 11) as judged by fluorescence titrations. This discrepancy might be explained by the fact that antibody binding had to be measured on cells at a low temperature to suppress modulation and thus prevent loss of CALLA from their surface. Since expression of CALLA on these cells is not very dense, the higher temperature at which toxicity and modulation occur may also be necessary for mobility of CALLA determinants within the membrane and to achieve the resulting proximity required for divalent interactions to occur (4).

The impetus for pursuing a study of CALLA-specific J-5 antibody as a carrier or ricin A chain stems from the clinical relevance of this biological model. In a recent trial of serotherapy of ALL, CALLA-specific monoclonal J-5 antibody was given to patients by i.v. infusion (33). Although J-5 antibody rapidly bound to leukemic cells in peripheral blood and bone marrow, activated complement at the cell surface, and initiated the clearance of circulating blasts, it was also noted that leukemic cells rapidly became resistant to serotherapy. This recovery of circulating lymphoblasts was due in part to antigenic modulation of CALLA from their surface. The results of this study indicated that serotherapy with antibody alone would not be sufficient to eradicate large numbers of leukemic cells and emphasized the point that antibodies are not inherently cytotoxic and are, therefore, dependent on the activation of natural effector systems for cell lysis. It is unlikely that these systems alone would be capable of effectively killing all tumor cells especially in situations where patients have relatively large numbers of leukemic cells. In this regard, conjugates of J-5 antibody and ricin A chain may be particularly suited for circumventing the inadequacy of natural effectors. As this present work has demonstrated, antigenic modulation of CALLA potentiates the toxic activity of this agent on tumor cells instead of providing an escape mechanism. This specific cell membrane phenomenon may thereby be utilized for therapeutic advantage, allowing greater potency and improved selectivity of action for the conjugate. Indeed, the prerequisite binding, internalization, and disulfide cleavage steps all provide safeguards to insure against leakage of toxic effects to neighboring cells which lack CALLA. In a pharmacological setting, this implies that systemic side effects would be minimal while specific therapeutic action upon neoplastic cells would be greatly enhanced. Since the ricin A chain conjugate shuts down cellular protein production rather than acting directly on nucleic acid synthesis, it might be useful in combination therapy or after failure with conventional anticancer drugs. Its effectiveness would not be dependent upon the fraction of cycling cells, and it could thus be useful for killing slowly dividing or quiescent malignant cells which still require protein synthesis to survive.

The in vitro results presented in this paper have shown F(ab')2-A chain conjugates did attain the selectivity characteristics aimed for and demonstrated little nonspecific toxicity. While the agent produced highly specific toxic effects at relatively low concentrations, it did not possess the potency of whole ricin and was not equally effective on all CALLA-bearing cells. Furthermore, its presence in culture for 2 days or more was required to completely suppress actively proliferating cell lines. Such considerations would have to be taken into account for the design of in vivo studies. Moreover, the possible existence of CALLA or cross-reacting antigens on normal tissues and their accessibility to antibody-delivered ricin A chain must be evaluated further.

REFERENCES

19. Minowada, J., Tsubota, T., Greaves, M. F., and Walters, T. R. A non-T, non-B human leukemia cell line (NALM-1): establishment of the cell line and

CALLA-specific Antibody-Ricin A Chain Cytotoxins
Monoclonal Antibody-Ricin A Chain Conjugate Selectively Cytotoxic for Cells Bearing the Common Acute Lymphoblastic Leukemia Antigen

Vic Raso, Jerome Ritz, Marylu Basala, et al.


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