Selective killing of normal or neoplastic B cells by antibodies coupled to the A chain of ricin
(leukemia/immunotherapy/anti-tumor antibody/ricin)

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ABSTRACT Highly specific antibodies (affinity-purified or hybridoma) directed against cell surface immunoglobulins on normal or neoplastic murine B lymphocytes were covalently coupled to the A chain of the plant toxin ricin. Such conjugates containing antibodies specific for IgM, for either of the two allotypes of IgD, or for the idiotype of the B cell tumor BCL1 rapidly bound in vitro to cells expressing the corresponding surface antigen and inhibited protein synthesis in such cells. The results demonstrate that A chain-coupled anti-idiotype antibody may be useful as a tumor-specific cytotoxic agent.

The possibility of utilizing the exquisite specificity of antibodies to direct cytotoxic agents to tumor cells has been considered since the studies of Ehrlich (1). Studies using drug-antibody conjugates for this purpose have been hampered by the difficulty of raising antisera specific for tumor cells, the inability in preparing purified antibody for drug conjugation, and the problems of preserving both pharmacologic and antibody activity after production of hybrid molecules (2). Several recent developments suggest that these obstacles can be surmounted and that the time is ripe for a reinvestigation of the above approach. First, the development of techniques for the generation of somatic cell hybrids secreting monoclonal antibody (3) and successful application of affinity chromatography of conventional antisera (4) make it possible to prepare highly purified antibody. Second, it has been demonstrated that the idiotype of the cell surface Ig of a B cell tumor represents a clonally expressed tumor specific marker (5). Finally, many potent protein toxins such as ricin, abrin, and diphtheria toxin have been shown to consist of a toxic portion (A chain) covalently bound to a portion (B chain) that can bind to surface moieties on cells and thereby facilitate entry of the toxic peptide into the cell (6, 7). The internalized toxic peptide kills the cells by catalytic inhibition of protein synthesis. By substituting specific antibody for the B chain, it should be possible to direct the peptide to cells for which the antibody is specific.

In the present study, affinity-purified or hybridoma antibodies directed against immunoglobulin isotypes, allotypes, or idiotypes expressed on the surface of normal or malignant murine B lymphocytes were conjugated to the A chain of ricin. Such antibody conjugates were incubated with cells cultured in vitro and their cytotoxic effects were evaluated. The results indicate that the hybrid molecules maintain both their antigen-binding capacity and their toxic properties and that minute amounts of the conjugates are effective in specifically killing target cells in vitro.

MATERIALS AND METHODS

Preparation of Antibody-Ricin A Chain Conjugates. The ricin molecule is composed of a cytotoxic (A) chain and a binding (B) chain associated by a disulfide bond. The A chain from Ricinus communis agglutinin II (Vector Labs, Burlingame, CA) was isolated after reduction of the disulfide bonds by ion-exchange chromatography following the procedures of Olsnes and Pihl (8). Ricin A chain was concentrated by vacuum dialysis against 10 mM phosphate-buffered saline (pH 7.0), containing 0.05% 2-mercaptoethanol. Thiols were introduced into affinity-purified or monoclonal antibodies (9) by using a 30-fold excess of N-succinimidyl-3-(3-mercaptopropionate (Pharmacia). After introduction of the 3-mercaptopropionate group, free sulfhydryl groups were generated by reduction in 5 mM dithiothreitol at room temperature for 90 min.

A 5-fold molar excess of ricin A chain was then mixed with the thiolted antibodies and the mixture was dialyzed extensively against P1/NaCl, pH 7.0, thus allowing oxidation-induced disulfide coupling of ricin A chain to antibody.

Binding Specificity of the Antibody-A Chain Conjugates. Antibody-A chain conjugates were prepared with $^{125}$I-labeled A chain. R. communis agglutinin (50 μg) was labeled with Na$^{125}$I by lactoperoxidase-catalyzed iodination (10). The reaction was terminated by the addition of 1 ml of a 1 mg/ml solution of unlabeled ricin, followed by extensive dialysis against P1/NaCl. The A chain was then isolated and antibody conjugates were prepared as described above. A solid-phase radiolimunoassay was used to demonstrate the binding specificity of antibody-A chain conjugates.

Preparation of a Hybridoma Secreting the IgM$\alpha$ of the BCL1 Tumor Cells. Because the BCL1 tumor cells do not secrete IgM, it was necessary to hybridize these cells with myeloma cells in order to generate a cell line secreting the IgM. The secreted IgM possessing the BCL1 idiotype could then be used to affinity purify the anti-idiotypic antibody. P3/X63-Ag8 myeloma cells and BCL1 cells previously activated by treatment with lipopolysaccharide (LPS) at 50 μg/ml for 24 hr were mixed and hybridized, using the procedure described by Kennett (11) with modifications. After the addition of poly-

Abbreviations: RAMlg, rabbit anti-mouse immunoglobulin; RAM$\alpha$, rabbit anti-mouse $\alpha$ heavy chain; NRlg, normal rabbit immunoglobulin; anti-id, anti-idiotype (otypic); anti-$\delta$, anti-$\delta$ heavy chain antibody specific for the Ig$\delta$ allotype; anti-$\delta$, anti-$\delta$ heavy chain antibody specific for the Ig$\delta$ allotype; GAILg, goat anti-rabbit immunoglobulin; P1/NaCl, phosphate-buffered saline; LPS, lipopolysaccharide.

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ethylene glycol, the cells were centrifuged for 4.5 min at 400 × g and the hybridization was terminated at 6 min. The cells were suspended in media containing 5 × 10^6 fresh BALB/c splenocytes per ml. Cells were distributed into 96-well microculture plates (Falcon) and incubated at 37°C in a 5% CO2 atmosphere. Medium containing aminopterin was first added 24 hr after the hybridization. Colonies of hybrid cells were visible microscopically by 8 days after hybridization.

In order to determine if a particular clone had secreted IgMλ, culture supernatant was transferred in duplicate to the wells of an assay plate coated with affinity-purified rabbit anti-mouse μ heavy chain (RAMμ) (10). This was followed by exposing the wells to 125I-rabbit anti-mouse λ light chain. Radioactivity bound to the wells was then compared to that of wells in which standard control proteins were substituted for culture fluid. IgMλ-positive clones were transferred to larger vessels and were adapted to growth in culture on Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. To demonstrate that the IgMλ secreted by the hybrids carried the BCL1 idiotype, cells were then incubated with [3H]leucine and the radioactive secreted Igs were analyzed by immunoprecipitation and polyacrylamide gel electrophoresis (Fig. 1). The hybrids that secreted an idiotype-positive IgMλ were maintained in continuous culture and also adapted to grow as an ascites in the peritoneal cavity of mineral oil-primed BALB/c mice.

Antibodies. Preliminary experiments indicated that the conjugates formed from the Ig fractions of antisera and A chain did not kill cells bearing the corresponding antigen specifically. It was considered essential, therefore, to prepare affinity-purified antibodies. RAMμ and hybridoma antibodies directed against the a or b allotypes of mouse IgD (anti-β or anti-δ) (12) were prepared as described (13). A rabbit serum directed against the IgMλ molecule of the BCL1 tumor cells (14) was purified by affinity chromatography on Sepharose bound to the BCL1-IgMλ isolated from the ascites of BALB/c mice inoculated with BCL1-P3/X63-Ag8 hybridoma cells. Antibody was eluted in 3.5 M MgCl2, dialyzed, concentrated, and absorbed sequentially with Sepharose bound to mouse IgG, MOPC-21 protein (γ, κ), TEPC-183 protein (μ, κ), MOPC-315 protein (α, λ), and paraformaldehyde-fixed BALB/c spleen cells. The resulting anti-idiotypic (anti-Id) antibody was judged to be idotype-specific by the criteria described previously (14). NaDodSO4/polyacrylamide gels of molecules precipitated from lysates of radiiodinated normal or BCL1 spleen cells bound by the affinity-purified anti-Id antibody are shown in Fig. 2. In addition, analysis of the fluorescent staining on the fluorescence-activated cell sorter (14) indicated that there was no binding of anti-Id to normal spleen cells but there was intense staining of tumor cells.

Inhibition of Protein Synthesis by Antibody-A Chain Conjugates. Previous studies on the mechanisms of action of ricin have shown that protein synthesis is irreversibly and cat-

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**FIG. 1.** Characterization of the IgMλ secreted by the BCL1-P3/X63-Ag8 hybridoma. Hybridoma cells were labeled with [3H]-leucine and the secreted Igs were precipitated with the antiserum indicated and *Staphylococcus aureus*. The radioactivity bound to *S. aureus* pellets was eluted with NaDodSO4 and 2-mercaptoethanol, and the eluates were electrophoresed on 7.5% NaDodSO4 gels. μ chains, γ chains, and light chains migrate to positions 16-18, 27-29, and 46-48, respectively. Anti-Id, anti-idiotypic; NRIg, normal rabbit immunoglobulin.

**FIG. 2.** Specificity of affinity-purified anti-Id. Cell surface Ig was immunoprecipitated from lysates of radiiodinated spleen cells from BCL1-bearing (Upper) or normal (Lower) BALB/c mice. Immune complexes were bound to *S. aureus* and eluted in NaDodSO4/urea containing 2-mercaptoethanol. Samples were electrophoresed on 7.5% NaDodSO4/polyacrylamide gels. μ and light chains are at fractions 13-15 and 41-43, respectively. δ chains are at fraction 17. ●, Anti-λ in Upper and anti-Ig in Lower; ○, anti-Id.
alytically inhibited in treated cells and thereby leads to cell death (reviewed in ref. 6). Therefore, in all the studies described in this report, inhibition of protein synthesis was used as an indicator of cell death. Spleens from normal 2- to 4-month-old BALB/c or C57BL/6 mice (Cumberland Farms, Clinton, TN) or from BALB/c mice inoculated previously with BCL1 tumor cells were teased into single-cell suspensions in balanced salt solution containing 0.05% fetal calf serum. After two washes, 2 × 10^6 cells were added to the wells of Micro Test II culture plates (Falcon) containing 10 μl per well of antibody-A chain conjugates at various concentrations. After 14–30 min at 4°C, the cells were spun down in a centrifuge adapted for microculture plates, followed by two subsequent washes. Each well received 200 μl of Hepes-buffered RPMI 1640 medium (GIBCO) supplemented with gentamycin at 10 μg/ml, 2 mM glutamine, 5% fetal calf serum (GIBCO), and 50 μM 2-mercaptoethanol. In addition, the B lymphocyte mitogen LPS (Salmonella typhosa 0901) (Difco), was added to a final concentration of 50 μg/ml. The cells were cultured at 37°C in a 10% CO₂ atmosphere. After 48 hr, each well received 1 μCi (3.7 × 10^4 becquerels) of [³H]leucine (25 Ci/mmol; New England Nuclear) and the incubation was continued for an additional 16 hr. At this time the cells were harvested from the wells with a multiple automated cell harvester (MASH II; Microbiological Associates, Bethesda, MD) and incorporation of [³H]leucine into protein was determined in a Beckman LS-330 scintillation counter. Untreated cultures incorporated 4000–15,000 cpm per 2 × 10^6 cells.

RESULTS

Inhibition of Protein Synthesis in Normal B Lymphocytes by an Anti-μ-A Chain Conjugate. Cell surface IgM is expressed on the majority of murine B lymphocytes and, therefore, represents a suitable target for investigating the specificity of inhibition of protein synthesis by antibody-A chain conjugates.

The specificity of an anti-μ-A chain conjugate was investigated. Intact ricin was radiolabeled with ¹²⁵I. Its A chain was isolated and coupled to affinity-purified RAMμ, and the capacity of this conjugate to bind specifically to IgM on immunoadsorbants was studied. Radioactivity that bound to the immunoadsorbants represented binding of ricin A chain through the antibody portion of the conjugate. As shown in Table 1, covalent binding of A chain and antibody was required for significant specific binding of the antibody conjugate. A mixture of anti-μ and radiolabeled A chain resulted in only modest binding to Sepharose-IgM. The partial reduction of antibody activity (relative to the input) may be caused by A chain conjugation to the Fab portion of the anti-μ with resultant steric hindrance of the antigen combining site. In similar experiments, unlabeled conjugates were tested for their ability to bind to IgM in a solid-phase radioimmunoassay. Bound conjugates were detected with an ¹²⁵I-labeled goat anti-rabbit Ig (GARlg). As can be seen in Table 2, the anti-μ-A chain conjugates bound to MOPC-104E protein but the NRlg-A chain conjugate did not. Hence, the anti-μ conjugates retained antibody activity.

Table 1. Binding of antibody-A chain conjugates to Sepharose immunoadsorbants

<table>
<thead>
<tr>
<th>Radioactive material added*</th>
<th>cpm bound to Sepharose-IgM</th>
<th>cpm bound to Sepharose-ovalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-μ, ¹²⁵I-A chain (conjugate)</td>
<td>48,900</td>
<td>5800</td>
</tr>
<tr>
<td>Anti-μ + ¹²⁵I-A chain (mixture)</td>
<td>8,200</td>
<td>2000</td>
</tr>
</tbody>
</table>

* Input cpm = 10⁵.

Table 2. Binding of antibody-A chain conjugates to a solid-phase immunoadsorbant

<table>
<thead>
<tr>
<th>Material added</th>
<th>cpm bound† to MOPC-104E (μ, λ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-μ</td>
<td>49,500</td>
</tr>
<tr>
<td>NRlg</td>
<td>550</td>
</tr>
<tr>
<td>Anti-μ-A chain</td>
<td>30,300</td>
</tr>
<tr>
<td>NRlg-A chain</td>
<td>890</td>
</tr>
</tbody>
</table>

† Wells of a microtiter plate were coated with MOPC-104E protein (μ, λ) and treated with the indicated and ¹²⁵I-labeled GARlg.

The next step was to determine the effect of anti-μ-A chain on protein synthesis of LPS-stimulated BALB/c spleen cells. LPS stimulates B lymphocytes, resulting in maintenance of viability, increased macromolecular synthesis, and, eventually, cell proliferation. As shown in Fig. 3, protein synthesis was inhibited more than 80% by the conjugate at concentrations of 5 or 10 μg/ml. Treatment with unconjugated antibody alone had no effect. Additional experiments indicated a similar inhibition of [³H]thymidine incorporation (data not shown). Ricin A chains conjugated to NRlg did not inhibit either protein or DNA synthesis. Hence, inhibition of protein and DNA synthesis by the anti-μ-A chain conjugate appeared to be antigen specific.

Inhibition of Protein Synthesis in Normal B Lymphocytes by Allotype-Specific Anti-μ-A Chain Conjugates. The specificity of antibody-ricin A chain conjugates was investigated further by using ricin A chain conjugated to monoclonal antibodies directed against either the a or b allotype of IgD on B cells. These conjugates with allotype specificity should recognize only B cells from mouse strains expressing the corresponding allotype.

![Graph](image-url)

**Fig. 3.** Inhibition of protein synthesis in B lymphocytes by affinity-purified RAMμ-A chain. BALB/c spleen cells (2 × 10⁶) were pulsed for 15–30 min with various concentrations of RAMμ-A chain at 4°C, washed, and then cultured in triplicate for 2 days in the presence of LPS at 50 μg/ml. Cultures received 1 μCi of [³H]leucine, were incubated an additional 14–16 hr, and were then harvested and analyzed for incorporation of leucine into protein. Hatched bars, anti-μ-A; empty bars, NRlg-A. In this and subsequent figures, SEMs are indicated by error bars.
Immunology: Krollick et al.

Inhibition of protein synthesis in B lymphocytes by monoclonal allotype-specific anti-\(\delta^b\)-A chain conjugates. Culture conditions were the same as described for Fig. 3. Antibodies coupled to ricin A chain were specific for either the \(\delta^b\) allotype expressed on B cells of C57BL/6 mice (empty bars) or the \(\delta^a\) allotype expressed on B cells from BALB/c mice (hatched bars).

As shown in Fig. 4, ricin A chain conjugates prepared with anti-\(\delta^b\) antibody inhibited 95% of protein synthesis in LPS-stimulated spleen cells obtained from C57BL/6 mice, which express the \(\delta^b\) (Ig\(5^b\)) allotype. LPS-stimulated BALB/c spleen cells, which express the \(\delta^a\) (Ig\(5^a\)) allotype, were inhibited less than 10%. A high degree of specificity was also demonstrated by using an anti-\(\delta^a\) antibody conjugate on the same two populations of B lymphocytes. Neither anti-\(\delta^a\) nor anti-\(\delta^b\) alone had any effect on protein synthesis.

Inhibition of Protein Synthesis in BCL\(_1\) Tumor Cells by Anti-Id Coupled to A Chain. To evaluate the immunotherapeutic potential of a tumor-specific antibody conjugate, an affinity-purified rabbit anti-Id specific for the IgM on BCL\(_1\) tumor cells was coupled to A chain and cultured with normal B cells, BCL\(_1\) tumor cells, or two unrelated tumors. One of these tumors, CH1, also expresses IgM\(_b\) on its surface (15), but the IgM\(_a\) does not express the BCL\(_1\) idiotypic. As documented in previous experiments (16), and as shown in Table 3, an average of 77% of the splenic cells from mice injected with the BCL\(_1\) tumor 7–10 weeks previously express the BCL\(_1\) idiotypic.

The results of treating BCL\(_1\) cells and normal cells with an anti-Id-ricin A chain conjugate are shown in Fig. 5. The anti-Id conjugate caused 70% inhibition of protein synthesis in LPS-stimulated spleen cells from mice bearing the BCL\(_1\) tumor, suggesting that virtually all BCL\(_1\) cells were killed. The same conjugate caused only 5% inhibition of normal BALB/c splenocytes. Neither NRIg coupled to A chain (Fig. 5) nor antibody alone (Fig. 6) had any effect on either normal or tumor cells. Furthermore, as shown in Fig. 7, the anti-Id-A chain conjugate did not inhibit protein synthesis in a T cell tumor (ASL-1) and had a marginal effect on a B cell tumor (CH1) that lacks the idiotypic marker. The modest effect on the CH1 cells might be due to a minute amount of anti-\(\lambda\) activity in the anti-Id antibody preparation. This effect was not seen in two other experiments using CH1 cells. These results demonstrate that A chain coupled to anti-Id antibody decreases protein synthesis in spleens from BCL\(_1\)-bearing mice to an extent consistent with the number of tumor cells present. These experiments further demonstrate the potential usefulness of anti-Id conjugates as B cell tumor-specific toxins.

DISCUSSION

The results presented in this study indicate that ricin A chain coupled to antibodies directed against either immunoglobulin isotypes or allotypes on normal B cells or to an idiotype on a B cell tumor are specifically cytotoxic in vitro as judged by inhibition of protein synthesis. The specificity of the cytotoxicity

![Fig. 4](image)

![Fig. 5](image)

![Fig. 6](image)

**Table 3.** Percent idiotype-positive cells in the spleens of mice injected with BCL\(_1\) tumor cells

<table>
<thead>
<tr>
<th>Time after injection, weeks</th>
<th>% idiotype-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>62–79 (7)</td>
</tr>
<tr>
<td>8</td>
<td>62–85 (9)</td>
</tr>
<tr>
<td>10</td>
<td>75–78 (2)</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td><strong>77.2 (18)</strong></td>
</tr>
</tbody>
</table>

Each mouse received 1 × 10\(^6\) BCL\(_1\) tumor cells. In parentheses are numbers of mice analyzed, using rabbit anti-Id and fluorescent GARIg (13, 14).
was demonstrated both by the inability of nonimmune Ig-A chain conjugates to kill any cells and by the inability of the anti-Ig conjugates to kill cells lacking the corresponding Ig antigen. The specific binding of the antibody-A chain conjugates to their target cells was rapid, requiring only a 15-min treatment to produce the subsequent inhibitory effect on protein synthesis. Moreover, the conjugates were effective at doses as low as 0.25 – 1 μg/ml. Unconjugated antibody was not cytotoxic under these conditions. The effectiveness of the antibody-A chain conjugates was a result of both the use of purified antibody and the coupling of the A chain to the antibody by a crosslinker (9) that did not significantly denature either the toxic polypeptide or the antibody.

The immediate importance of these observations is that an affinity-purified antibody conjugate specific for the idiotype of the surface immunoglobulin on a particular B cell tumor represents a specific toxin for that tumor. Thus, because a B cell tumor expressing a particular idiotype is of monoclonal origin (5), all the tumor cells expressing that idiotype are potential targets for the cytotoxic agent. In contrast, only a negligible number of normal B cells express the same or crossreacting idiotype and therefore the overwhelming majority escape the cytotoxic effect of the anti-Id conjugate. Moreover, the above approach is theoretically applicable to any tumor to which a specific antibody can be generated. Therefore, with the development of the hybridoma antibody technology, it is likely that antibodies that are highly specific to tumor-associated surface antigens can be generated to other types of tumors and used in a similar manner.

There are a number of reports of the coupling of toxic polypeptides to lectins, hormones, and antibodies. Gilliland et al. (17) coupled the A fragment of diphtheria toxin to concanavalin A. This conjugate was toxic but displayed the expected broad binding specificity. Uchida et al. (18) have found similar results, using Wistaria floribunda lectin. Oeltmann and Heath (19) produced a conjugate between the A chain of ricin and human chorionic gonadotropin that showed selective killing of cells bearing receptors for the hormone. Similarly, Chang and Neville (20) have coupled the A fragment of diphtheria toxin to human placental lactogen. Conjugates have also been prepared between whole diphtheria toxin and antibodies directed against human lymphoblastoid cell lines (21) and virus-transformed hamster fibroblasts (22). These conjugates were also cytotoxic for the appropriate cell types. Most recently, Masuho et al. (23) demonstrated that the Fab' fragments of an antibody directed against a murine leukemia (L1210) were cytotoxic for tumor cells when covalently associated with the A fragment of diphtheria toxin. In contrast, intact diphtheria toxin was not cytotoxic for the same cells, which lack either the toxin receptor or the ability to transmit the A fragment across the membrane.

The present experiments are distinguished from the above studies by the exploitation of an idiotype to prepare an antibody that is highly specific for a nonsecreting B cell tumor. Non-specific binding of the drug-antibody conjugate to tumor cells was diminished by affinity purification of the specific antibody prior to conjugation. The latter was achieved by preparing the antigen from a somatic cell hybrid between the nonsecreting tumor and a myeloma cell line that secreted the idiotype-positive molecule. This approach is potentially applicable to the therapy of any B cell tumor.

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