Prevention of Growth of Leukemia Cells in Mice by Monoclonal Antibodies Directed against Thy 1.1 Antigen Disulfide Linked to Two Ribosomal Inhibitors: Pokeweed Antiviral Protein or Ricin A Chain

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ABSTRACT

Pokeweed antiviral protein (PAP) and ricin A chain are potent inhibitors of protein synthesis that inactivate eukaryotic 60S ribosomal subunits. Immunotoxins were prepared by linking monoclonal anti-Thy 1.1 antibodies to PAP and ricin A chain through a disulfide bond. Both the conjugates were shown earlier to specifically inhibit protein synthesis of Thy 1.1-positive target leukemic cells (AKR SL3). In the present study, the efficacy of the immunotoxins to prevent the growth of AKR SL3 cell-induced tumor was checked in vivo in a model system. Injection of AKR SL3 cells s.c. into AKR/Cum (Thy 1.2-positive) mice developed into a solid tumor which was fatal. Administration of 31-E6:PAP and 31-E6:ricin A chain suppressed tumor growth. Suppression was specific, as similar treatment could not prevent the growth of a nontarget Thy 1.2-positive leukemia cell line (AKR SL1) derived from a congenic mouse. Unconjugated anti-Thy 1.1 immunoglobulin antibodies also showed significant tumor protection; however, administration of F(ab')2 fragment could not prevent the tumor growth. Injection of F(ab')2:PAP efficiently protected mice from AKR SL3-induced tumor. All the conjugate-treated mice showed antibody response against the toxin polypeptide. Anti-toxin antibody response was found as early as 26 days after the initiation of therapy and lasted as long as 179 days of observation. Further studies indicate that the presence of anti-toxin antibodies blocked completely the inhibitory ability of the respective immunotoxin in vitro. Anti-ricin antibodies neutralized the activity of 31-E6:ricin A chain conjugate but not OX-7:PAP immunotoxin, and similarly, anti-PAP antibodies inhibited the activity of the latter and not the activity of 31-E6:ricin A chain conjugate. These observations indicate that the use of alternate immunotoxins having an immunologically distinct toxin polypeptide may be necessary for tumor therapy during relapse, as exposure to the conjugates results in the formation of specific neutralizing anti-toxin antibodies. The anti-toxin antibodies did not prevent the binding of immunotoxin to target cells. Nevertheless, preincubation of conjugate with anti-toxin antibodies specifically blocked the respective conjugate-induced inhibition of polyuridylic acid translation in a cell-free assay system.

INTRODUCTION

Preferential elimination of tumor cells by antibody-mediated delivery of toxins that inhibit protein synthesis is a possible mode of tumor therapy (31, 38). A highly specific antibody directed against an antigen associated with the tumor cell and covalently bound to a toxin is used to deliver a potentially lethal agent to the tumor cell surface. If the toxin polypeptide can somehow cross the cell membrane, then the resulting inhibition of protein synthesis would selectively kill the targeted cell. The high selectivity of the antibody portion of the immunotoxin would spare normal cells from nonspecific effects of the toxin.

Monoclonal antibodies directed against Thy 1.1 have therapeutic value by themselves (1, 4–6) and are highly selective in the tissues to which they bind (16). In some cases, the antibodies localize preferentially in lymphoid tissues with nearly 500:1 ratios (lymphoid tissue: blood). Immunotoxins made with ricin (15, 35, 39) are probably less specific, especially in the absence of galactose, than are ricin A chain-containing immunotoxins. Immunotoxins made with ricin A chain selectively inhibit protein synthesis (in cell culture) of murine T-cell leukemia cells (7, 40), neoplastic (21) and normal (29) B-cells, colorectal carcinoma cells (14), human breast carcinoma cell line (23), human T-cell leukemia (36), and common acute lymphoblastic leukemia antigen-bearing cells (34). To ensure the stringent specificity of the conjugates, the ricin A chain preparations used should be absolutely free from B chain contamination, a criterion very difficult to meet (32).

We have evaluated the use of alternative phytotoxins (see Refs. 3 and 30 for review), such as PAP2 that do not naturally contain a B chain counterpart of ricin. PAP-containing immunotoxins were shown to be as effective as ricin A chain-containing immunotoxins in cell culture studies (26, 33), but this could not have been predicted in the light of the studies by Cawley et al. (10). They showed that EGF:ricin A chain and EGF:diphtheria toxin Fragment A conjugates had different biological activities and that transport was apparently influenced by the nature of the toxin coupled to EGF. Binding of the PAP- or ricin A chain-containing immunotoxins to target cells in culture is very fast, and continuous incubation (more than 5 to 10 min) was not necessary for the eventual cytotoxic effect of the immunotoxin (33).

In this study, investigations were carried out to (a) establish the in vivo efficacy of disulfide-linked anti-Thy 1.1 antibody: toxin conjugate in preventing the growth of AKR SL3-induced tumors, and (b) to investigate the possible consequences of conjugate treatment, such as the appearance of antibody directed against the conjugate and the implications of such antibodies on the effectiveness of the conjugate action in vitro.

MATERIALS AND METHODS

Antibody:Toxin Conjugate. Ricin A chain was isolated by the method of Ramakrishnan et al. (32), and PAP was isolated by modification (17)

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2 The abbreviations used are: PAP, pokeweed antiviral protein from spring leaves; PAP-S, pokeweed antiviral protein from seeds; EGF, epidermal growth factor.

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of the method of Irvin et al. (18), Obrig et al. (28), and Barbieri et al. (2). Mouse anti-Thy 1.1 monoclonal antibody (31-E6) was obtained from Dr. Robert Nowinski, and rat Thy 1 monoclonal antibody was from Dr. Alan Williams. Monoclonal antibody (31-E6) directed against mouse Thy 1.1 antigen was linked to either ricin A chain or PAP using N-succinimidyl-3-(2-pyridyldithio)propionate. The detailed method of conjugation and analysis of the conjugates have been described elsewhere (33). F(ab')2 fragment was prepared from the purified antibodies using 2 mg of pepsin (Millipore Co.) for 100 mg of IgG in 0.10 M sodium acetate buffer, pH 4.50, for 16 hr at 37°C. The pH was raised to 8 with Tris, and the undigested IgG and Fc fragments absorbed to protein A-Sepharose. The material that passed through the column was then chromatographed on Sephadex G-100 (2 x 100 cm) in 5 mm sodium phosphate, pH 6.5, containing 200 mm NaCl phosphate-buffered saline. The F(ab')2 fragment was analyzed after reduction with 5 mm dithioerythritol and 1% β-mercaptoethanol and was electrophoretically pure on a 12.5% sodium dodecyl sulfate-containing gel (24). It did not show any complement-mediated cytotoxicity towards cells bearing Thy 1.1 antigen but showed 2-fold reduction (from 1.12 x 10^5 M^-1 to 5.46 x 10^4 M^-1) in affinity (25) as determined by established procedures (25, 37). All the immunotoxins were prepared for their ability to inhibit in vitro protein synthesis using rat liver ribosomes (8, 9, 11). They were checked for their specificity of reaction by assaying their relative cytotoxicities against Thy 1.1-positive and Thy 1.2-positive cells using procedures described previously (15, 33).

Estimation of the Molar Ratio of Toxin to IgG by Radioimmunoassay. PAP (10 μg in 5 μl) was radioiodinated by a solid-phase lactoperoxidase:glucose oxidase:Enzymobeads; Bio-Rad) system. The details of the method have been described elsewhere (32). The iodinated PAP had a specific activity between 12.5 and 54.1 μCi/μg in 3 different iodinations. In a typical assay, 50 μl of the label (approximately 10,000 cpm) were mixed with 50 μl of standard protein or sample protein and 50 μl of appropriately diluted rabbit anti-PAP antibody. The antibody:PAP conjugates were diluted in 0.05 M phosphate buffer containing 0.1% bovine serum albumin and 0.1% sodium azide. Following overnight incubation at room temperature, the antibody-bound label was separated from free label with 50 μl of 1:5 Pansorbin (Calbiochem-Behring). The total protein concentration of the conjugate was determined by using an extinction coefficient at 280 nm of 1.4 for a 1-mg/ml solution. The assay was used to measure PAP concentrations between 0.625 and 10 ng/tube.

Chart 1 shows a typical profile of the competitive inhibition curve observed in the presence of standard PAP and antibody:PAP conjugate. The immunotoxin showed a nearly parallel slope of inhibition when compared to standard PAP. After extrapolation and multiplication with the extinction coefficient at 280 nm of 1.4 for a 1-mg/ml solution. The assay was used to measure PAP concentrations between 0.625 and 10 ng/tube.

Chart 1. Estimation of PAP content in conjugates. Competitive inhibition of 125I-PAP binding to rabbit anti-PAP antibodies by standard PAP (O) and immunotoxin (●). Each value is a mean of triplicate estimations. The slope of the inhibition curve obtained in the presence of standard PAP was -2.40 and nearly parallel to the slope (–2.49) of the antibody:PAP conjugate. Logit = ln(y/100 - y), where y = B/B0. B is the amount of antibody-bound radioactivity in the presence of competing proteins, and B0 is the maximum binding in the absence of competing proteins.

Estimation of Anti-PAP Antibodies in Conjugate-treated Mice. Radioiodinated PAP was used to determine the antibody levels in sera taken at different times after the administration of the conjugate. Tripli- cate 3 of different serial dilutions were checked for each serum, and each time point was represented by the sera from a minimum of 3 mice. The maximum dilution that bound less than 30% of the label was estimated; the antigen-binding capacity was determined by multiplying the actual amount of labeled PAP bound by the dilution factor. In a parallel study, 125I-ricin A chain was also used to establish the specificity of the antibody response in the treated mice.

Neutralization of Conjugate-mediated Cytotoxicity by Specific Antibodies. Antibodies specific for the toxin portions of the conjugates were able to prevent their biological activity. Conjugates containing ricin A chain (31-E6:ricin A chain) and PAP (OX-7:PAP) were preincubated with antiseras raised in rabbits against ricin, PAP, or PAP-S. PAP is immunologically distinct from PAP-S, and they have different, but related amino-terminal primary structures (17). All 3 antiseras had high titers of antibodies. Anti-ricin antibodies were capable of binding 26% of 125I-ricin A chain at a dilution of 9.0 x 10^4, anti-PAP antiserum bound 33% of radioiodelabeled PAP at 2.4 x 10^5 dilution, and anti-PAP-S bound 22% of radioiodinated PAP-S at 5.0 x 10^5 dilution.

Fifty μl of a 1:10 dilution of the antiserum were mixed with 50 μl of a 1:50 dilution of the conjugate and incubated for 1 hr at 37°C. The control set had conjugate plus 1:10 dilution of normal rabbit serum. At the end of incubation, 20 μl of the mixture were added to triplicate cultures of AKR SL3 target cells (1 x 10^5 cells in 0.2 ml). After incubating the cells with the conjugates for 18 hr, the cultures were centrifuged at 1000 rpm for 5 min and resuspended in medium containing 2.0 μCi of [3H]leucine/ml. Following a 1-hr pulse, the amount of [3H]leucine incorporated into protein was estimated by precipitation with 5% trichloroacetic acid. The precipitate was collected on glass fiber filters, washed with cold ethanol, dried, and counted. The percentage of reduction in protein synthesis when compared to control cultures incubated with an equivalent amount of antibody was taken as an index of cytotoxicity.

Protein Synthesis Inhibition In Vitro. Rat liver ribosomes were incubated with immunotoxins and then assayed for their ability to synthesize polyphenylalanine (8).

RESULTS

Rate of Tumor Growth. Twelve to 15 days were required for a palpable nodule to develop after s.c. injection of 1 x 10^6 AKR SL3 cells into AKR/Cum mice. The volume of the solid tumor increased in a typical exponential manner after a lag period of
20 days (Chart 2), reaching a maximum volume of about 22 ml. Ultimately, this resulted in the death of the animals around Day 40, but some mice survived with very large, necrotic tumors for up to 80 days.

**In Vivo Efficacy of PAP-containing Immunotoxins.** Protection against tumor-induced death was afforded by 31-E6:ricin A chain (none of the 12 mice developed a tumor) and 31-E6:PAP (one of 6 mice developed a tumor and died). However, mice given an equivalent amount of 31-E6 antibody were also protected against tumor growth; one of 12 animals developed a tumor and died. All unprotected control mice died by Day 50. Because 31-E6 antibody prevented tumor growth, it could not be concluded that the immunotoxin had any additional effect over that afforded by the antibody alone. It is clear, however, that coupling the toxin polypeptides to the immunoglobulin had no deleterious effect on the activity of the antibody insofar as the ultimate protection was concerned. Furthermore, it was apparent that no nonspecific effects of such severity as to result in the death of the mouse were present at the doses used of PAP- or ricin A chain-containing conjugates.

In contrast to the protective effect of intact antibodies against Thy 1.1, the administration of F(ab')2 fragment of 31-E6 did not prevent the growth of AKR SL3 cells in vivo. All 6 animals treated with 31-E6-F(ab')2 (10 µg/injection) developed tumors. By Day 60, 83% of the animals died, and the remaining mouse survived to Day 90 despite the burden of a very large tumor (Chart 3). Therefore, to better investigate the efficacy of immunotoxin-mediated protection, we treated mice with 31-E6-F(ab')2:PAP conjugate. Mice treated with 31-E6-F(ab')2:PAP showed protection in 83% of the animals over the period of 87 days. There was a delayed appearance of tumor in one of the animals on Day 64. Additional control groups of 5 mice were given injections of either PAP (2 µg/injection) or PAP mixed with 31-E6-F(ab')2 (20 µg/injection) in a separate experiment. There was 100% incidence of tumor, and all the animals died within 35 days after the injection of leukemia cells. Thus, the F(ab')2:PAP immunotoxin was able to prevent the growth of target tumor cells in vivo, where F(ab')2 and/or PAP treatment could not.

**Specificity of the Antitumor Effect of 31-E6:PAP Immunotoxin in Vivo.** Earlier studies (33) with cells grown in culture indicated that the 31-E6:PAP conjugates were highly specific in inhibiting protein synthesis of the target cells bearing Thy 1.1 antigen but not Thy 1.2 antigen. To extrapolate this strict specificity, experiments were carried out with AKR SL1 cells in a tumor model system (Chart 4). AKR SL1 cells (1 × 10^6 cells in 0.1 ml) were injected s.c. in the flank region of AKR/Cum mice and treated with the 31-E6:PAP conjugate on Day 0, 2, 4, 8, and 16. A control group of mice treated with 31-E6:PAP conjugate on Day 0, 2, 4, 8, and 16. A control group of mice received 0.9% NaCl solution injections. AKR SL1 cells induced palpable tumor nodules by Day 8, and 100% of the control mice developed tumor. By Day 31, all the control mice given injections of AKR SL1 cells died.

The administration of 31-E6:PAP did not prevent the development of the Thy 1.2-positive tumor or its growth in any of the treated mice, all the animals receiving the conjugate also developed tumors, and the survival curve was quite similar to that of untreated control mice. All 5 control animals were dead by Day 32.

**Immune Response against PAP in Conjugate-treated Animals.** AKR/Cum mice treated with 31-E6:PAP and 31-E6:F(ab')2:PAP were checked for antibody response against PAP at different times. Immunotoxins were inoculated into mice on Days 0, 2, 4, 8, and 16, and blood samples were collected on Days 26, 36, 52, 105, 21, and 179. These sera were assayed for their ability to bind radiiodinated PAP in a radioimmunoassay system. Appreciable levels of anti-PAP antibodies (1 µg PAP bound/ml of serum) were observed as early as Day 26. Peak titers (12.71 µg of PAP bound/ml of serum) were reached in the group of mice bled on Day 36, and significantly high titers were still present 170 days after the first injection of the immunotoxin.
with anti-toxin antibodies on the ability of conjugates to inhibit protein synthesis. This set of cultures showed only 6.2 and 3.4% protein synthesis, respectively. Under similar conditions, antibodies raised against PAP and PAP-S did not interfere with 31-E6:ricin A chain action. In the presence of anti-PAP and anti-PAP-S antibodies, 31-E6:ricin A chain inhibited protein synthesis 88 and 83%, respectively. In a similar manner, the cytotoxic action of OX-7:PAP was specifically neutralized with antibodies against PAP, but not with anti-ricin or with anti-PAP-S antibodies. OX-7:PAP reduced the protein synthesis of AKR SL3 target cells to a level of about 1.6% of the control in the presence of normal rabbit serum. Addition of anti-PAP antibodies relieved this inhibition, and the cells retained about 81% of protein synthesis in spite of the presence of conjugate in the medium. Preincubation with anti-ricin antibodies or anti-PAP-S antibodies could not neutralize the inhibitory ability of OX-7:PAP conjugate. This set of cultures showed only 6.2 and 3.4% protein synthesis, respectively.

Effect of Anti-Toxin Antibodies on Immunotoxin-dependent Inhibition of Cell-free Translation. Immunotoxins made from both ricin A chain (31-E6:ricin A chain) and PAP (OX-7:PAP) were potent inhibitors of polyuridylic acid translation using rat liver ribosomes (33). Data in Table 1 show the effect of preincubation with anti-toxin antibodies on the ability of conjugates to inhibit polyphenylalanine synthesis. 31-E6:ricin A chain was specifically blocked by anti-ricin antibodies. Addition of anti-PAP or anti-PAP-S did not influence the conjugate action. Similarly, OX-7:PAP was inhibited by only anti-PAP antibodies and not by anti-ricin or by anti-PAP-S antibodies.

Effect of Anti-Toxin Antibodies on the Binding of Radioiodinated Conjugate to Target Cells. OX-7:PAP was radioiodinated using Enzymobeads. The labeled conjugate was used to study the ability of immunotoxins to bind glutaraldehyde-fixed AKR SL3 target cells. Conjugate preincubated with normal rabbit serum (1:400) bound 6.23 × 10^6 cpm/10^6 cells. An equivalent amount of the immunotoxin preincubated with either anti-ricin, anti-PAP, or anti-PAP-S antiserum (1:400) at 37°C for 2 hr. All sera were diluted 1:1000. Triplicate 10-μl samples were assayed in a cell-free translation system (9) and compared to a control without antiserum. The control tubes contained 86,495 cpm in the trichloroacetic acid precipitate.

### Table 1

<table>
<thead>
<tr>
<th>Conjugate</th>
<th>31-E6:ricin A chain</th>
<th>OX-7:PAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conjugate preincubation</td>
<td>Mean cpm</td>
<td>% of protein synthesis</td>
</tr>
<tr>
<td>Normal rabbit serum</td>
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<td>67</td>
</tr>
<tr>
<td>Anti-ricin</td>
<td>83,956</td>
<td>97</td>
</tr>
<tr>
<td>Anti-PAP</td>
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<td>69</td>
</tr>
<tr>
<td>Anti-PAP-S</td>
<td>49,586</td>
<td>57</td>
</tr>
</tbody>
</table>

DISCUSSION

Our earlier studies (33) have shown that immunotoxins prepared with either ricin A chain or with PAP were equally cytotoxic.
The excess reactive groups were blocked with 3% bovine serum albumin in 0.1 M borate-buffered 0.9% NaCl solution (pH 8.3). Cells (1 x 10^6) in a volume of 0.1 ml were washed in phosphate-buffered saline 3 times and fixed with 0.25% glutaraldehyde. OX-7:PAP was iodinated using Enzymobeads. Target cells (AKR SL3) were used are: 7, normal rabbit serum (1:400); 2, rabbit anti-ricin antiserum (1:400); 3, anti-rabbit-F(ab')2 linked to PAP was added. A control without the addition of antibody was used for comparison. Following incubation at 4°C overnight, the cells were washed in phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% azide, and the radioactivity bound to the cells was determined. The antibodies and the dilutions used are: 1, normal rabbit serum (1:400); 2, rabbit anti-ricin antiserum (1:400); 3, rabbit anti-PAP antiserum (1:400); and 4, rabbit anti-PAP-S (1:400) antiserum. Purified antibodies and the amounts used are: 5, 10 μg mouse monoclonal antibodies against human transferrin receptor as control; 6, 10 μg 31-E6 IgG; and 7, 10 μg OX-7 IgG.

against target cells when coupled through a disulfide bond (a cleavable linkage). Nevertheless, when conjugates were prepared by linking the toxin to the antibody via a thioether bond (a noncleavable linkage), only the PAP-containing immunotoxin was cytotoxic. Ricin A chain linked to antibody through the noncleavable bond was not cytotoxic to target cells, although it was a potent inhibitor of polyuridylic acid transcription in vitro in support of other observations (27). Although no specific internalization mechanism is known for these conjugates, they are transported into cultured cells with an efficiency that approaches that of ricin (33), a toxin designed for highly efficient transport (13).

By using AKR/J-derived Thy 1.1-positive leukemia cells (AKR SL3) transplanted into Thy 1.2-positive AKR/Cum mice, we can eliminate problems associated with nonspecific adsorption of the immunotoxins in tissues other than the tumor target, since anti-Thy 1.1 antibodies are not tumor specific per se. This serves to keep the level of the immunotoxins in circulation as high as possible, subject only to the natural clearance mechanisms that may operate. The half-life of anti-Thy 1.1 antibody (IgG2a) in AKR/Cum mico is about 5.5 days, and the antibody is eliminated in a first-order process. In contrast, about 90% of the Thy 1.1 antibody is eliminated from the blood of AKR/J mice in the first few hr, but after about 2 days, the rate of clearance is equal in AKR/Cum and AKR/J mice (16).

The better homing efficiency of F(ab')2 fragment compared to IgG may be more beneficial, because toxins coupled to the former would be more rapidly cleared from the system. This may serve to minimize any possible nonspecific effects that the presence of the toxins may bring about. In addition, the removal of the Fc fragment may further reduce nonspecific effects. Normal T-cells of AKR/Cum mice should not be affected by the Thy 1.1-directed immunotoxins; in addition, the studies of Bernstein and coworkers (1, 4–6) have shown no deleterious effects on the normal immunological system in either AKR/J or AKR/Cum mice as the result of the treatment with anti-Thy 1.1 monoclonal antibodies.

Administration of disulfide-linked 31-E6:PAP or 31-E6:ricin A chain resulted in the specific suppression of AKR SL3 (Thy 1.1 positive)-induced tumors in AKR/Cum mice. In a parallel study, similar treatment could not prevent the growth of AKR SL1 (Thy 1.2 positive)-induced tumors in AKR/Cum mice. This observation confirms our previous results, showing stringent specificity of the immunotoxins in vitro in selectively inhibiting protein synthesis in AKR SL3 but not AKR SL1 cells (33). In the present investigation, 90% of a control group of mice treated with antibody alone were also protected, making it difficult to conclude from the first set of experiments that the immunotoxins played a positive role in suppressing the growth of AKR SL3-induced tumors. In in vitro studies, antibody alone did not inhibit the protein synthesis of the target cells, but it was cytotoxic in the presence of complement. Other recent studies have illustrated the protective effects of antibody alone directed against Thy 1.1 (1) or anti-transferrin receptor antibodies against human melanoma cells (36).

Removal of the Fc portion of the antibody abolished the protective ability of the antibody. If F(ab')2 linked to PAP was capable of preventing the growth of leukemia cells in vivo, this would be a clear demonstration of the effect of the immunotoxin. While PAP alone or PAP mixed with 31-E6-F(ab')2 failed to protect mice from tumor growth, 31-E6-F(ab')2 linked to PAP was highly protective, demonstrating that the antibody portion of the immunotoxin is actually responsible for localizing and internalizing the toxin moiety specifically into the target tumor cells.

Ricin A chain-based immunotoxins have been used successfully in treating mice bearing BCL1 tumors (22). Polyclonal antibodies against surface IgD or anti-idiotypic antibodies against IgM coupled to ricin A chain were effective in prolonging remission in mice after extensive cytoreduction using conventional means, such as total lymphoid irradiation and splenectomy. The total effectiveness of the passive therapy with immunotoxin alone in the absence of total lymphoid irradiation and splenectomy is yet to be demonstrated. The effectiveness of therapy may depend on the entire tumor load and promptness of treatment. For example, immunotherapy with antibody was not effective if initiated 24 hr after the transplantation of ASL-1 T-cell tumor (20). Immunotoxin therapy using anti-Thy 1.2 (IgM isotype) antibody disulfide linked to ricin A chain only delayed the eventual appearance of leukemia in treated mice (7). This low efficiency in vivo may in part depend on the differences in isotype and avidity of the antibody, affinity and the density of antigen on the surface of the tumor cells, and stability in vivo of the immunotoxin and its disulfide linkage.

A potential problem in the therapeutic use of immunotoxins is the appearance of antibodies to the foreign protein directed against both the murine IgG (19) and the toxin moiety. These antibodies could jeopardize the subsequent use of the immunotoxins by neutralizing their action and could lead to problems, such as anaphylactic shock and immune complex-related pathological situations. The production of anti-murine IgG antibodies could possibly be circumvented by using monoclonal antibodies produced by human x human hybridomas (12) or by the induction of specific tolerance against mouse IgG. It has been possible in vitro to eliminate antigen-reactive B-cells (and thus specific antibody production) by exposing B-cell preparations to antigen linked to ricin A chain (29, 38).

The procedures for inducing specific suppression of immune response against selective determinants are yet to become a...
realilty in vivo. During immunotoxin therapy, it was proposed that the B-cells reactive with the determinants present on the immu-
notoxin (through the receptors on antigen-specific B-cells) would be killed upon exposure to the conjugate (22). This could result in the abrogation of antibody response against the immunotoxin. However, our data indicate that specific suppression against immunotoxin did not result in conjugate-treated animals; i.e., the antigen-reactive B-cells having receptors for the toxin polypep-
tide were not eliminated from the system, and in fact, all the treated mice showed a fairly rapid response of anti-PAP antibodies irrespective of the carrier (lgG). The in vitro studies further show that the anti-toxin antibodies can interfere with the action of immunotoxin and prevent its inhibition of protein synthesis in target cells. Preincubation of OX-7-PAP with anti-PAP antibodies completely blocked the ability of the conjugate to prevent protein synthesis in the target SL3 cells. Similarly, the effect of 31-
E6:ricin A chain conjugate was neutralized specifically by anti-
ricin antibodies. However, antibodies directed against PAP-S or PAP did not neutralize ricin A chain-containing conjugates, and likewise, anti-ricin antibodies did not block the action of immu-
notoxins prepared from PAP.

The anti-toxin antibodies could either (a) interfere with the binding of immunotoxin to the target cell, (b) block the internalization process, or (c) prevent the toxin moiety from acting on 60S ribosomal subunits. To evaluate these possibilities, we investigated the effect of anti-toxin antibodies on the binding of radioiodinated OX-7-PAP conjugate to target AKR SL3 cells. The results indicate that the binding is not affected by either antici-
ricin, anti-PAP, or anti-PAP-S antibodies. However, inclusion of 10 µg of free IgG (31E6 or OX-7) competitively inhibited the binding of the immunotoxin (OX-7-PAP) to target cells. Further studies reveal that specific anti-toxin antibodies could differentially block the inhibitory action of immunotoxin in a cell-free translation system. These experiments, however, do not rule out the possibility of blockade at the internalization process by anti-
toxin antibodies.

We believe the evaluation of alternate immunotoxins based on non-cross-reactive toxin polypeptides is essential in developing protocols for immunotoxin therapy in vivo. Because PAP, PAP-
S, and ricin A chain are immunologically distinct, and because the immunotoxins prepared with these toxins are almost equally potent, it may be possible to treat a patient with relapse, who had been exposed to one type of conjugate and developed antibodies against that particular toxin, with another conjugate containing the same antibody, or its F(ab')2 fragment, but cou-
pled to a different toxin polypeptide chain.

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