Hybrid Antibodies with Dual Specificity for the Delivery of Ricin to Immunoglobulin-bearing Target Cells¹

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ABSTRACT

Hybrid antibodies possessing one binding site for the toxic lectin ricin and a companion site directed against human immunoglobulin were constructed in vitro. This bifunctional reagent specifically attached to human lymphocyte surface immunoglobulin determinants and, thus situated, could simultaneously capture ricin molecules or its toxic A chain. Attachment of these components to the cell was revealed by specific fluorescein-labeled antibodies. Once concentrated at the target cell membrane, hybrid-bound toxin was subsequently released to function via its normal mechanism of biological action. It gained access to ribosomes, its intracellular target, and curtailed protein synthesis. Toxicity was not augmented for immunoglobulin-negative cells to which hybrid could not bind and free human immunoglobulin G could competitively block the enhanced effects observed for immunoglobulin-bearing cell lines. These results indicate that hybrid antibodies may be utilized to carry active agents within close proximity to the membrane of a specified cell type and thereby selectively enhance their effect.

INTRODUCTION

The potential for using cell-specific antibodies as vehicles for selective delivery of biologically active agents has long been recognized (3). The major thrust of this effort has been directed toward increasing the specificity of cytotoxins for neoplastic cells and to minimize their detrimental effect on normal cells. Attachment of drugs to antibody carriers has been accomplished by several distinct routes of covalent coupling (7). Chemical manipulations, however, can both inactivate antibody-combining sites and cause crucial alterations in the cytotoxic agent itself (11, 32). Further problems may arise if severance of the link between carrier antibody and this agent is essential for full biological action, since covalent bonds are not easily broken. Numerous chemical procedures have been developed to help circumvent some of these difficulties with different degrees of success, and for the most part experimental results have supported the basic correctness of the antibody delivery concept (7).

Bifunctional hybrid antibodies have been designed to achieve both selective delivery and controlled release of biological agents without affecting their structural integrity. Constructed in vitro, these reagents posses one binding site for attachment to surface determinants on a designated cell type and a companion site which can simultaneously sequestor an active agent. Once concentrated at the target cell membrane, such hybrid-bound molecules would subsequently dissociate and function via their usual mechanism of biological action. In addition to avoiding any limitations imposed by covalent linkage, this approach allows hybrid attachment to the cell and "loading" of the second site to proceed as independent steps. The particular hybrids used in this study are composed of defined antibody halves; one which will adhere to cells bearing surface immunoglobulin and its partner which can reversibly bind the toxic lectin ricin. The potent inhibition of protein synthesis produced by ricin (17, 26) serves as a measure of its delivery and entrance into cells. This toxin with the hybrid carrier antibodies was used in a test system utilizing human lymphoid cell lines either with or without immunoglobulin on their surface as antigen-positive and -negative cells.

MATERIALS AND METHODS

Antibodies Used. Rabbit antibodies against human IgG F(ab')₂ fragments were elicited and isolated on an affinity adsorbent as described previously (2). Purified ricin (M.W. 64,000) from Sigma Chemical Co. (St. Louis, Mo.) was resolved into its component A chain (toxic moiety) and B chain (galactose-binding portion) by disulfide cleavage and chromatography (25). The pure A chain (50 μg) was used to immunize rabbits, and the antisera obtained were shown to be reactive with isolated A chain and whole ricin but not with ricin B chain (27). Anti-ricin A chain antibody was purified using an affinity column (27).

Hybrid Formation. The anti-ricin and anti-IgG were used to form hybrid antibodies following the procedures developed by Nisonoff and Rivers (23). Briefly, the 2 types of purified antibodies (40 mg each) were separately digested with pepsin and bivalent F(ab')₂ molecules were obtained after chromatography on Sephadex G-200. Equal amounts (~13 mg) of the 2 F(ab')₂ types were mixed, split by mild reduction into monomeric Fab' molecules, and allowed to randomly reassemble into F(ab')₂ dimers with dual specificity. The progress of each of these steps was verified using a molecular sizing column, and excellent yields of anti-ricin/anti-IgG hybrids resulted.

Human Cell Lines. Cell lines used in this study which possessed surface immunoglobulin determinants included the Daudi Burkitt lymphoma line (12) and the CCRF-SB human B-lymphoid line (16) as well as 3 Epstein-Barr virus-transformed human B-lymphoblastoid cell lines, LAZ 156 (4), LAZ 007, and LAZ 388. The immunoglobulin-negative human lymphoid cells used were CCRF-CEM (6) and CCRF-HSB2 (16), 2 leukemic T-cell lines, LAZ 221, a null-acute lymphoblastic leukemia cell line (15), and 562, a chronic myelogenous leukemia cell line (19).

Hybrid Specificity. The reactivity of this hybrid preparation...
with immunoglobulin-bearing cell lines was demonstrated by an indirect immunofluorescent procedure using cytofluorographic analysis (18). Cells were incubated with 10 μg of hybrid in 25 μl of PBS for 30 min, washed with PBS, and developed with a fluoresceinated goat anti-rabbit F(ab')2 reagent. The interaction of hybrid molecules with the cell surface was considered specific since blocking occurred using free human IgG but not bovine IgG and because immunoglobulin-negative cell lines displayed no fluorescence labeling after exposure to the hybrid preparation.

Detection of Hybrid-bound Toxin on the Cell Surface. Immunoglobulin-positive Daudi cells were incubated for 30 min with 10 μg of the anti-ricin/anti-IgG hybrid in 25 μl of PBS. These cells were washed with PBS and then exposed to 1 × 10^{-7} M ricin in 0.1 M lactose-PBS for 15 min. They were washed with 3 ml of 0.1 M lactose-PBS and 3 ml of PBS. Surface-localized ricin was detected by treatment with fluoresceinated rabbit anti-ricin A chain and analyzed using a fluorescence-activated cell sorter (FACS I; Becton Dickinson, Mountain View, Calif.). Free A chain was tested on cells without lactose using lactose to block.

Evaluation of Ricin Delivery by Hybrids. Human lymphoblastoid cells (2 × 10^6) were pelleted in tubes and incubated for 30 min in 25 μl of PBS alone or containing 10 μg of hybrid antibody. Free or bovine γ-globulin (500 μg) was included to block binding when indicated. The cells were washed twice with PBS and then exposed to ricin at the designated concentrations in 30 μl of 0.1 M lactose-PBS for 15 min. The incubation was terminated by adding 3 ml of 0.1 M lactose-PBS and pelleting the cells. They were then washed with PBS and resuspended at 1 × 10^6 cells/ml in media lacking L-leucine, and 200-μl aliquots were plated in duplicate in microculture wells for incubation overnight at 37°C. Thereafter, 0.25 μCi [14C]leucine (312 mCi/mmol) was added to each well and after 1 hr at 37°C, and the cells were collected and washed on a glass fiber filter using a Mash II sample harvester. Radioactivity on the dried filter discs was measured by scintillation counting. Isolated A chain was used in the same way, but lactose was excluded.

RESULTS

The upper sequence in Chart 1 depicts the normal mechanism of ricin cytoxicity as elucidated by the work of Olsnes (31), Nicolson (21, 22), and others (26, 31). Ricin B chain binds to galactose determinants on the cell surface and carries the disulfide-linked A chain with it. Shortly thereafter, the intact toxin either enters by endocytotic uptake, or its A chain is transferred directly into the cytoplasm. The released A chain then effects enzymatic inactivation of ribosomes (24) and a shutdown of protein synthesis. The lower series of diagrams outlines how the anti-ricin/anti-IgG hybrids were used for toxicity studies with immunoglobulin-bearing cells. Cells pretreated with hybrid and washed to remove excess reagent were exposed to ricin in the presence of lactose. This allowed the toxin to react with cell-bound hybrid while its normal interaction with membrane glycoproteins was attenuated by the specific sugar. These cells were then washed to remove any unbound ricin and the lactose block. Examination of immunoglobulin-positive cells at this stage with a fluoresceinated anti-ricin A chain reagent clearly revealed the presence of hybrid-bound ricin on their membranes (Chart 2). Subsequent incubation (17 hr) in media gave ample time for the membrane-localized ricin to gain entry and exert its effect. This was evaluated following a 1-hr pulse with [14C]leucine to measure their capacity to synthesize protein.

When untreated immunoglobulin-bearing LAZ-007 cells were exposed to increasing concentrations of ricin following this protocol, they displayed a progressive loss in ability to incorporate leucine (Chart 3a). Significantly improved sensitivity was imparted to identical cells treated with the specific anti-ricin/anti-IgG hybrid. No comparable enhancement was provided by an anti-neocarzinostatin/anti-IgG hybrid (Chart 3a) even though this readily attached to the cell surface. Without the appropriate ricin-binding site, this control hybrid was unable to capture these toxin molecules.

The enhanced toxicity could be blocked by free human IgG, included at the time of treatment with hybrid (Chart 3b), while bovine IgG exhibited no reversal effect. These results are compatible with the fluorescence studies which showed that the homologous IgG alone prevents attachment of the hybrid to LAZ-007 cells. Similar tests with immunoglobulin-negative cell lines gave no indication of hybrid-augmented ricin toxicity since comparable dose-response curves were obtained regardless of whether or not these cells were treated with hybrid. Ricin ID_{50} levels were evaluated for several individual immunoglobulin-bearing and immunoglobulin-devoid cell lines both with and without prior exposure to anti-ricin/anti-IgG (Table 1). Protein synthesis in cells to which hybrid bound was inhibited at substantially reduced ricin concentrations compared to identical untreated cells. In contrast, immunoglobulin-negative lines showed very little differential.

Ricin, bound via hybrid to LAZ-007 cells, exerted its toxic effect in a time-dependent manner, since the extent of inhibition of protein synthesis went from 25% when examined after 4 hr

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<sup>1</sup> The abbreviations used are: PBS, 0.14 M NaCl-0.01 M phosphate, pH 7.4; ID_{50}, concentration of ricin needed to produce 50% inhibition of [14C]leucine incorporation.

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![Chart 1. Schematic representation of the pathways followed for ricin toxicity via its normal mechanism and using a hybrid antibody delivery system, adapted in part from Refsnes et al. (31). A and B, toxic and galactose-binding subunits, respectively, of ricin.](chart1.png)
of incubation in media to 80% after 24 hr. This is consistent
with the scheme shown in Chart 1 wherein ricin toxicity is
preceded by multiple time-dependent steps including disso-
ciation from antibody, binding to galactose sites, and entry into
the cell. The sequence of steps was examined further to see if
penetration of hybrid-delivered lectin could be prevented by
including lactose throughout this prolonged incubation period.
Indeed hybrid-treated LAZ-156 cells displayed increased sus-
ceptibility to ricin compared to untreated cells if plated in
normal media, but this toxicity was largely averted by incuba-
tion in media containing 25 mM lactose (Chart 4). Thus, hybrid
delivery did not interfere with the normal mechanism of trans-
port of ricin into the cell. The small component of toxicity which
was refractory to lactose blockage may be significant since the
sugar produced complete reversal for cells which were ex-
posed to ricin without prior treatment using the hybrid (Chart
4). Perhaps a highly stabilized mode of cell attachment occurs
involving simultaneous interactions between the hybrid-ricin
complex and both surface immunoglobulin and membrane gly-
coprotein receptors (Chart 1). Alternatively, a fraction of the
whole toxin or its A chain may be transported into the cell
without prior binding via the B chain.

To further resolve this aspect, preliminary hybrid antibody
experiments designed to deliver active isolated A chain alone
were undertaken. Fluorescence studies paralleling those for
whole ricin (Chart 2) showed that A chain was specifically
bound via hybrid to the surface of immunoglobulin-positive
Daudi cells. The capacity of these cells to synthesize protein
was found to be substantially reduced upon overnight incuba-
tion (Table 2) while controls which were treated with hybrid

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**Chart 2.** Demonstration of hybrid-bound cell surface-localized ricin mole-
cules. — — — , immunofluorescence profile of hybrid-treated immunoglobulin-
positive Daudi cells exposed to $1 \times 10^{-7}$ M ricin in the presence of lactose;
– – – , background fluorescence of untreated cells or cells exposed to the ricin
plus lactose but not pretreated with hybrid. Fluorescein-conjugated rabbit anti-
ricin A chain was used for staining.

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**Chart 3.** Dose-response curves for ricin inhibition of $[^{14}C]$leucine incorpora-
tion. Immunoglobulin-bearing LAZ-007 cells were treated with PBS alone (○), 10
μg anti-ricin/anti-human IgG (△), 10 μg anti-neocarzinostatin/anti-human IgG
(▲), 10 μg anti-ricin/anti-human IgG plus 500 μg human IgG (□), or 10 μg anti-
ricin/anti-human IgG plus 500 μg bovine IgG (■). A and B represent separate
experiments performed with different batches of cultured LAZ-007 cells.

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**Table 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Alone</th>
<th>Hybrid</th>
<th>Enhancement</th>
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<tr>
<td>Immunoglobulin-positive cells</td>
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<td>156*</td>
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<td>33</td>
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<tr>
<td>156</td>
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<td>25</td>
<td>16</td>
</tr>
<tr>
<td>Daudi</td>
<td>240</td>
<td>12</td>
<td>20</td>
</tr>
<tr>
<td>38B</td>
<td>13.5</td>
<td>1.4</td>
<td>10</td>
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<tr>
<td>SB</td>
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*Each line represents ID₅₀ levels calculated from a semilog plot of dose-
response data obtained from separate experiments with a different batch of the
designated cell type.

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alone or A chain alone were absolutely unaffected. These results indicate that active A chain can gain access to ribosomes in the absence of the B chain (10). It remains to be shown whether A chain penetrates the cell membrane after dissociation from the hybrid antibody-combining site or if the undissociated complex becomes internalized as a whole unit.

DISCUSSION

The use of antibodies directed against cell surface determinants has long been an attractive method for increasing the selective toxicity of antineoplastic agents. The ready availability of antibodies of sharply defined specificity produced by hybridoma technology has recently renewed interest in this form of therapy (13). Moreover, successful in vivo localization of antibodies directed against important membrane antigens (1, 9) has further supported the reasonableness of this approach.

At the outset of this work (28, 29), only chemical cross-linking or nonspecific adsorption methods had been used previously to couple drugs and toxins to antibody carriers. As an alternative approach, the procedures developed by Nisonoff and Rivers (23) were adapted to design bifunctional hybrid antibodies for the express purpose of bringing biologically active agents in close proximity to cell membranes. Several aspects of this mode of delivery were examined in a test system which utilized surface immunoglobulin, present on human lymphoid cell lines, as a target antigen and ricin as a toxic agent with a readily detectable intracellular activity.

Indirect immunofluorescence studies revealed anti-IgG/anti-ricin hybrid molecules bound to the surface of immunoglobulin-positive but not immunoglobulin-negative cell lines. Similarly, an immunofluorescent probe showed that ricin was localized on the surface of hybrid-coated cells after exposure to toxin in the presence of lactose. Thus, the dual specificity of these cell-bound hybrid antibodies facilitated attachment of ricin to the membrane under conditions where the saccharide-binding site of the toxin was blocked.

Hybrid-bound whole ricin or its active A chain gained entry into the cytoplasm of the cells to which it adhered, since protein synthesis was sharply curtailed. Indeed, access of active toxin to cells was substantially augmented in a selective manner by hybrid delivery. This was evident from the 4- to 30-fold reduction of its ID50 for leucine incorporation following hybrid treatment of immunoglobulin-bearing lines. Identical treatment exhibited little influence on the toxicity found for immunoglobulin-negative cells. Moreover, the enhanced effects on immunoglobulin-positive cells were abrogated by the addition of free human IgG that competitively blocked the hybrid-combining site and prevented cell surface binding. These results verify that the amplified toxicity was mediated by hybrid antibodies attached to the cell surface. The requirement of an appropriate toxin-combining site on the hybrid was established by the inactivity of control hybrids which bound to the cell but could not subsequently bind ricin. Evidence was obtained for the participation of the sugar-binding site of ricin after primary attachment of toxin to the cell via hybrid. Thus, the cytotoxicity of hybrid-bound ricin was almost completely blocked when lactose was continually present during the incubation. Lastly, it was shown that the anti-lgG/anti-ricin A chain hybrid antibodies could mediate binding of free A chain to the surface of immunoglobulin-positive cells, thereby rendering it toxic to these cells.

During development of this hybrid delivery system, description of a novel approach for covalent linkage of toxin A chains or fragments to cell reactive antibody carriers has come from this laboratory (30) and others (8, 14, 20). These all use a cleavable disulphide bond between the A chain and antibody, which mimics the connection to its normal cell surface binding moiety. Thus, the undesirable toxicity associated with whole toxin is avoided yet a high degree of antibody-directed cytotoxicity can be retained. While this is very encouraging, depending on the system, toxicity can be 2 to 3 orders of magnitude less potent than observed for the natural parent toxin.

It appears that successful covalent conjugation will be limited to this class of plant and bacterial toxins. Since the various factors which determine the effectiveness of such conjugates are unknown, general applicability to all surface antigen systems and cell types may not be attainable.

In this current hybrid antibody study, lactose was used to

<table>
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<th>Addition</th>
<th>cpm</th>
<th>% of inhibition</th>
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<tr>
<td>Untreated cells</td>
<td>7922 ± 668</td>
<td>0</td>
</tr>
<tr>
<td>Hybrid alone</td>
<td>8273 ± 665</td>
<td>0</td>
</tr>
<tr>
<td>A chain (10^{-7} M) alone</td>
<td>8279 ± 38</td>
<td>0</td>
</tr>
<tr>
<td>A chain (10^{-5} M) alone</td>
<td>6073 ± 152</td>
<td>0</td>
</tr>
<tr>
<td>Hybrid + A chain (10^{-7} M)</td>
<td>4730 ± 61</td>
<td>40</td>
</tr>
<tr>
<td>Hybrid + A chain (10^{-5} M)</td>
<td>4269 ± 63</td>
<td>46</td>
</tr>
</tbody>
</table>

- \(^{4}\)[^1]Cleucine incorporation (1-hr pulse label) was evaluated after 17 hr of incubation at 37°C.
- \(^{5}\) Anti-lgG/anti-ricin A chain antibody treatment, 10 μg in 25 μl of PBS, lasted 30 min, and then the cells were washed with PBS.
- \(^{6}\) Cells were exposed to the specified concentration of purified ricin A chain in 30 μl of PBS for 15 min and then were washed with PBS.
- \(^{7}\) Cells were consecutively treated as described in Footnotes b and c.
suppress binding of whole ricin to glycoprotein receptors while “loading” the hybrid sites. A balance had to be struck between attaining a concentration of ricin sufficient to fill the combining site of cell-bound hybrid antibody and keeping it low enough to prevent ricin binding to cells via its B subunit. This was not required in the case of free A chain delivery since it showed no inherent affinity for the cell membrane and binding was solely through its interaction with hybrid. A chain alone, however, produced only approximately 50% inhibition of protein synthesis for hybrid-treated Daudi cells even when used at concentrations much higher than whole ricin (Tables 1 and 2). The more complete inhibition achieved with hybrid-delivered intact toxin, especially at lower concentrations, reflects the subsequent participation of B chain in cell binding. Successful application of ricin A chain may also be more dependent on the amount of hybrid attached to the cell and hence membrane antigen density.

The 2 types of purified antibodies used for this work were isolated from conventional heteroantisera. Thus, a complicated array of affinity and specificity combinations must arise upon annealing these 2 populations. The advent of homogeneous hybridoma-derived antibodies will afford absolute control over the binding affinities of the constituent halves of a hybrid antibody, and this uniformity should greatly boost their ultimate effectiveness as delivery vehicles.

For the ricin system, such an increase in affinity of the hybrid reagent and greater enhancement effects would be desirable before initiating in vivo studies. In this regard, it is important to note that subtoxic doses of i.v. administered abrin and ricin have already been shown to inhibit the growth of human tumors in nude mice (5). Even though there are a number of potential limitations to in vivo utilization of a hybrid antibody delivery system, it is interesting to speculate as to how it might be used to focus the action of such toxins. Using a 2-step protocol, an innocuous tumor-directed ricin-binding hybrid preparation could be infused until neoplastic cells were maximally “sensitized.” Thereafter, an appropriate interval would be allowed to permit clearance of unbound hybrid molecules. Pursuant treatment with ricin, ricin plus lactose, or free A chain should be designed so that hybrid-coated tumor cells would preferentially bind and be destroyed by toxin while normal cells remain unaffected. Alternatively, toxin and hybrid could be premixed in vitro, and the complex subsequently could be isolated from unbound molecules by size. This preloaded hybrid-toxin complex could then be injected so that its free cell-specific binding site would home in on target cells and deliver the toxin.

The hybrid antibody approach described in this paper represents a significant departure from most covalent coupling procedures in that neither the antibody-binding site nor the active agent is altered. It shows promise for delivery of biologically important molecules to designated cell types, and the reversible nature of antibody binding ensures full potency upon their release in the vicinity of the cell surface. The basic idea is flexible and can potentially be applied to alternative active agent-surface determinant combinations. This could be important in situations where covalent linkage of an effector molecule is not possible or results in its inactivation.

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REFERENCES

V. Raso and T. Griffin

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