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SPECIFIC CYTOTOXICITY OF A HUMAN IMMUNOGLOBULIN-DIRECTED FAB'-RICIN A CHAIN CONJUGATE[1]

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The toxic A chain of ricin and the Fab' fragment of antibody directed against human immunoglobulin (Ig) have been disulfide linked via their intrinsic sulfhydryl groups. This Fab'-A chain conjugate retained the activities of its component parts. It produced effective inhibition of protein synthesis in a cellfree rabbit reticulocyte lysate system and bound tightly to Ig determinants on human cells. Attachment of the intact molecule to the cell surface was revealed by using fluorescein-labeled antibodies directed against both its Fab' and A chain halves.

The Fab'-A chain conjugate was evaluated for cytotoxicity by using human, surface Ig positive target cells and surface Ig negative control cells. *In vitro* toxicity was entirely selective since the conjugate produced inhibition of *de novo* protein synthesis, impedence of growth, as well as death and lysis, only for cells possessing surface Ig determinants. Moreover, the effects on Ig positive cells were abrogated by the addition of free human IgG that competitively blocked the Fab' combining site of this conjugate and prevented cell surface binding. Addition of lactose, which inhibits binding of whole ricin, did not influence the action of the Fab'-A chain conjugate.

These results verified that the specific binding site on the antibody half of the molecule could function as a directional carrier that facilitates A chain entry into the cytoplasm. Expression of cytotoxic effects was thereby restricted exclusively to cells bearing the appropriate target site. This new conjugate molecule thus possessed both the absolute specificity of the antibody and the potent lethality of its parent toxin.

Effective erradication of malignant cells often requires the use of chemotherapeutic agents at their maximally tolerated levels where adverse cytotoxic effects on normal cells become a major limitation to the pharmacologic control of neoplastic disease. Over the past two decades a substantial effort has been directed toward developing a method for combining the determinant specificity of antibody with the lethal effects of toxic agents (1). The impetus toward treating malignant disease with tumor-directed cytotoxic conjugates has become even more pronounced with the prospective availability of unlimited supplies of hybridoma-derived homogeneous antibodies, with defined specificity (2). In conjunction with these developments has come the important realization that an antibody delivery system, in order to be successful, would have to carry an extremely toxic substance (3, 4). Molecules such as diphtheria toxin as well as the poisonous lectins, abrin, ricin, and modeccin satisfy such a requirement in that small amounts of their effector moiety, once within the cytoplasm, act enzymatically to shut down protein synthesis (5–7). Initial attempts to covalently couple whole toxins to antibodies by using cross-linking agents gave marginal success (1, 3, 4); whereas more recent approaches have shown more promise (8). Utilization of the complete toxin, however, incurs the risk of nonspecific toxicity.

Structural segregation of the cell-binding and toxic activities into separate regions or subunits of the molecule is a feature common to many bacterial and plant toxins. For example, cytoplasmic entry of toxic ricin A chain is mediated by the disulfide linked B chain that adhers to the galactose residues of receptors on cell membranes (6). These subunits can be split at the disulfide bond and the isolated chains retain their individual biologic activities (6). Although free A chain is fully capable of inactivating ribosomes, it cannot bind to or cross the cell membrane and is therefore not cytotoxic.

During the course of a study with hybrid antibodies and ricin (9, 10), we recognized the unique opportunity for combining the Fab' fragment of an antibody with isolated ricin A chain, utilizing in each case, their single intrinsic sulfhydryl group (Fig. 1). This mode of attachment mimics the linkage of each half with its natural partner and retains a cleavable disulfide bond. Monovalent antibody bound to surface Ig determinants enters cells by an endocytotic uptake into vesicles (11, 12), which closely parallels that seen for toxic lectins (6, 13–15). Therefore, it was anticipated that ricin A chain linked to an anti-Ig Fab' carrier would attach to the cell surface, where internalization and cellular damage could ensue.

We now report that incorporation of Fab' with cell surface reactivity into such a conjugate renders A chain cytotoxic. Antibody thus not only fulfills the function of ricin B chain but its sharply defined specificity circumvents the problems associated with the diffuse toxicity of whole ricin.

MATERIALS AND METHODS

Preparation of the Fab'-A chain conjugate. Ricin obtained from Sigma Chemical Co. (St. Louis, MO) was chromatographically separated into its component A chain (toxic portion) and B-chain (galactose specific moiety) after cleavage of the disulfide bond with mercaptoethanol (16). The A chain was concentrated by ultrafiltration, freed of mercaptan with Sephadex G-

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25, and passed through an 80 x 1 cm column of Sepharose 4B to eliminate any contaminating whole ricin or B chain.

Rabbit antibodies against human IgG $F(ab')_2$ fragments were produced by immunization with antigen in complete Freunds adjuvant and then isolated from serum by using an affinity adsorbant (17). The purified antibody was digested with pepsin (18), and $F(ab')_2$ molecules were obtained by gel filtration on Sephadex G-200. The bivalent antibody (36 mg) was split into monovalent Fab' fragments by mild reduction with 0.01 M mercaptoethylamine, and then an ion exchance column was used to remove the reducing agent (18). Immediately thereafter, the Fab' was reacted with 2 mM Ellman's reagent (5,5'-dithio-*bis*-(2-nitrobenzoic acid)) in 200 mM Na phosphate buffer, 3 mM ethylenediaminetetraacetic acid, pH 8.0 (19). After 2 hr at 20°C, excess reagent was removed by dialysis against buffer. The U.V. spectrum of the product showed a new absorption at 330 nm, and size analysis on a Sephadex G-100 column revealed a single homogenous peak corresponding to Ellman's blocked Fab'-E[4] molecules (m.w. = 50,000). Pure A chain (32,000 m.w.), 1.28 mg, was added to 2 mg of the Fab'-E in equimolar amounts at 2×10^{-5} M final concentration. The progress of the coupling reaction was followed by the increase in absorbance of free Ellman's reagent (λmax 412 nm) as it was displaced by A chain. This reaction was 86% complete after 2.5 hr at 20°C and the product was dialyzed against PBS. Production of the Fab'-A chain conjugate was verified by its elution profile on a G-100 molecular sizing column and its reactivity with both anti-rabbit $F(ab')_2$ and anti-ricin A chain antisera. A small amount of unreacted Fab'-E was present but no free A chain was detected with these reagents.

Inhibition of protein synthesis in vitro. A rabbit reticulocyte lysate protein synthesizing system from Bethesda Research Labs, Inc. was used to assay the inhibitory activity of the conjugate. All of the necessary components for protein synthesis plus ^{14}C-L-leucine (0.25 μCi) and globin mRNA (0.2 μg) as well as any inhibitor to be tested were included in a final 30 μl reaction volume. Incubation proceeded for 45 min at 30°C before assaying for incorporation of radioactive leucine into protein.

Human cell lines. Cell lines used in this study that possessed surface Ig determinants included the Daudi Burkitt lymphoma line (20), the CCRF-SB human B-lymphoid line (21), as well as three Epstein-Barr virus-transformed human B lymphoblastoid cell lines, LAZ 156 (22), LAZ 007, and LAZ 444. The Ig negative human lymphoid cells used were CCRF-CEM (23) and CCRF-HSB2 (21), two leukemic T cell lines, and LAZ 221, a null-acute lymphoblastic leukemia cell line (24).

Surface analysis of cells. Binding of the Fab'-A chain conjugate to cell surface determinants was evaluated by an indirect immunofluorescent procedure utilizing a fluorescence activated cell sorter (Becton Dickinson, Mountain View, CA). Cells were incubated with 10 μg conjugate in 25 μl of PBS for 30 min at room temperature. After washing with PBS, the treated cells were exposed to the appropriate fluorescein-labeled second antibody for 30 min at 4°C. These cells were washed again, suspended in 1 ml of PBS, and analyzed. The developing second antibodies utilized were anti-ricin A chain produced in rabbits and anti-rabbit $F(ab')_2$ produced in a goat. The γ-globulin fraction from the immune sera of these animals was fluoresceinated by standard procedures. Surface immunoglobulin μ-chain determinants were detected following the same protocol but by using murine monoclonal anti-human μ-chain antibody (provided by Dr. Lee Nadler) and a fluorescein-labeled anti-mouse IgG developing reagent (Meloy Labs, Springfield, VA).

Inhibition of cellular protein synthesis. Ig bearing Daudi cells (2×10^6) were pelleted in tubes and incubated for 30 min in 25 μl PBS alone (control) or containing 1.3×10^{-10} mole Fab'-A chain or Fab'-E. Human or bovine γ-globulin (500 μg) was included where indicated. The cells were then washed once with 3 ml PBS, resuspended in serum-free media lacking L-leucine, and 200-μl aliquots were plated in duplicate into microculture wells for 20 hr incubation at 37°C. Thereafter, 0.25 μCi ^{14}C-L-leucine was added to each well, and after 1 hr at 37°C the cells were collected and washed on a glass fiber filter by using a Mash II sample harvester (Microbiological Associates, Walkersville, MD). Radioactivity on the dried filter discs was measured by scintillation counting. In the lactose blocking experiments all operations were performed as described above except that 5×10^{-11} mole ricin or Fab'-A chain was added. When indicated, 100 mM lactose in PBS was used to block during the initial incubation as well as the wash step and 10 mM lactose was included in the serum-free media during the second incubation.

In vitro cytotoxicity studies. Cells in RPMI 1640 media (Microbiological Associates) containing antibiotics plus 10% FCS were plated in 1.7 x 1.6 cm tissue culture wells and incubated at 37°C. As designated, Fab'-A chain, A chain, and Fab'-E were added to the final concentration specified. Where noted, human or bovine γ-globulin was included at 2 mg/ml. Aliquots were removed, trypan blue added, and cell counts were performed at the indicated intervals.

RESULTS

Retention of A chain activity and antibody specificity. Pursuit of this study required both unequivocal production of active conjugate and the demonstration of selective cytotoxicity in an appropriate cellular test system. Initial attempts to directly react freshly reduced Fab' with isolated A chain resulted in Fab' combining with itself to form $F(ab')_2$. More recently, other investigators have produced hybrid toxin molecules by disulfide linkage methods that prevent homodimer formation (19, 25–29). One such blocking procedure (19) was adopted for the exclusive production of a Fab'-A chain heterodimer with specificity for human Ig (Fig. 1).

This purified Fab'-A chain produced potent inhibition of protein synthesis ($I.D._{50} = 4 \times 10^{-16}$ moles added) in a cellfree rabbit reticulocyte lysate system (Fig. 2). The fact that the conjugate was approximately 20-fold less effective than free A chain is consistent with the finding that the subunits of whole ricin must first be reductively cleaved before A chain can catalytically inactive ribosomes (6).

In order to test the site-directed killing of this agent, various human lymphoid cell lines either with or without Ig on their surface were utilized as target cells. The interaction of Fab'-A chain with Ig-bearing human lymphoblastoid cell lines was demonstrated by indirect immunofluorescence with cytofluorographic analysis (30). Surface binding was revealed after incubation of cells with excess conjugate for 30 min, washing, and development either with a fluoresceinated goat anti-rabbit $F(ab')_2$ reagent, or a fluoresceinated rabbit anti-ricin A chain reagent. Fluorescence profiles obtained in this manner (Fig. 3)

[4] Abbreviations used in this paper: Fab'-E, monovalent antibody fragment produced by pepsin digestion, with its sulfhydryl group blocked by Ellman's reagent; Fab'-A chain, disulfide-linked conjugate between antibody Fab' fragment and the A chain of ricin; HGG, human γ-globulin; BGG, bovine γ-globulin; $I.D._{50}$, the concentration required to produce 50% inhibition.

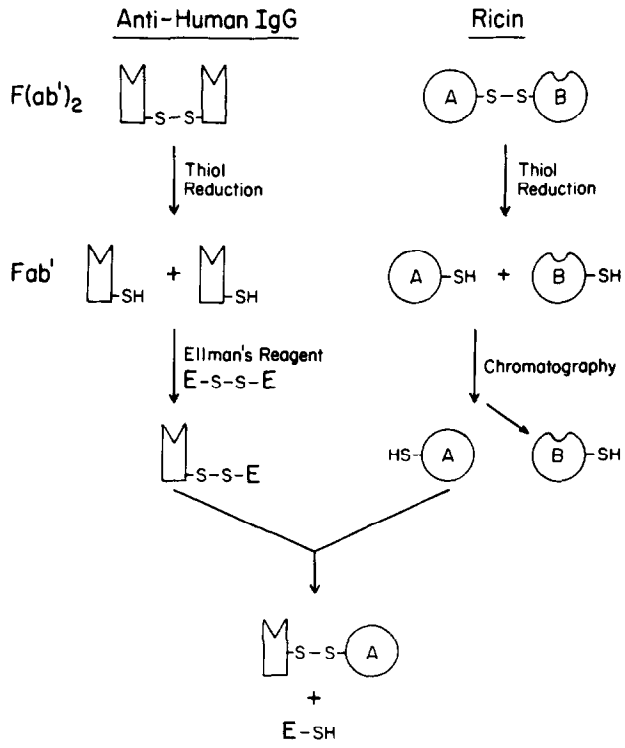

Figure 1. Scheme for the construction of antibody Fab'-ricin A chain conjugate.

clearly demonstrated the presence of both the Fab' and A chain moieties on the surface of Ig positive Burkitt lymphoma (Daudi) cells. The reaction was considered specific since identically treated Ig negative cell lines displayed no fluorescence labeling compared to their untreated controls.

A human B cell-derived line (LAZ 444) gave superimposable fluorescence profiles when treated with stoichiometric amounts of Fab'-A chain, Fab'-E, or $F(ab')_2$ followed by incubation with fluoresceinated goat anti-rabbit $F(ab')_2$ to quantitate bound Fab' (Fig. 4). Conjugated Fab'-A chain thus retained unimpaired surface binding, indicating that the chemical manipulations produced no adverse effect upon its combining site and that coupled A chain did not sterically hinder attachment. Furthermore, no differences were noted in the extent of binding to surface Ig for the bivalent *versus* monovalent species under these conditions.

Inhibition of cellular protein synthesis. Having demonstrated the selective binding of the conjugate to Ig-bearing cells as well as its potency in a cellfree system, we next tested the ability of Fab'-A chain to inhibit *de novo* protein synthesis of intact cells. Daudi cells were treated with excess conjugate for 30 min to ensure maximal binding to their surface as determined by fluorescence analysis. The cells were then washed to remove unbound reagent and incubated at 37°C for 20 hr before pulse labeling with ^{14}C-leucine. Data summarized in Table I indicate that membrane bound Fab'-A chain entered these cells in an active form that produced substantial inhibition of protein synthesis as measured by ^{14}C-leucine incorporation. The effect was specific since it was blocked by free human γ-globulin but not by bovine γ-globulin, included during exposure of cells to the conjugate. Tests with Fab'-E, which bound equally well to Daudi cells but caused no inhibition, confirmed that A-chain was the active moiety.

Since binding of ricin B chain to cells can be specifically blocked by lactose (6), surface Ig positive Daudi cells were exposed to equivalent amounts of either Fab'-A chain or whole ricin in the presence of 0.1 M lactose. Protein synthesis of these cells was compared to cells treated identically except that no lactose was included. As shown in Table II; lactose reversed the effect of a high level of ricin but produced no significant attenuation for the Fab'-A chain. This ensures that the toxicity of the Fab'-A chain conjugate was not due to whole ricin contamination in the conjugate preparation. It could thus be concluded that the sugar specificity of the original toxic lectin had indeed been exchanged for that defined by antibody.

In vitro cytotoxicity. The influence of the Fab'-A chain conjugate on cell growth and viability was assessed on actively dividing cells during extended culture. Ig-bearing Daudi cells increased 4-fold in number and reached a plateau phase by 72 hr in medium alone. Inclusion of the Ig directed Fab'-A chain

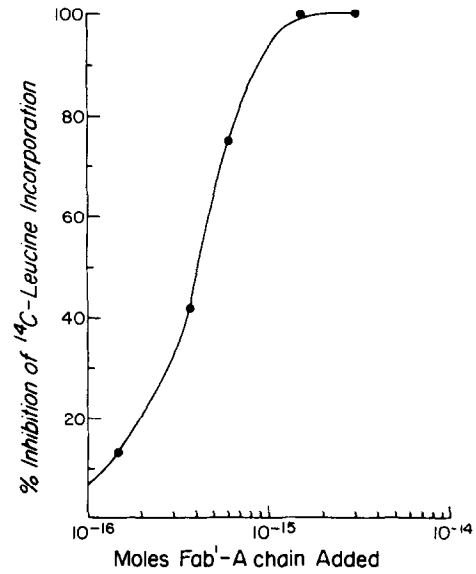

Figure 2. Ribosome inactivating capacity of the Fab'-A chain conjugate. A rabbit reticulocyte lysate system containing all of the components to synthesize ^{14}C-labeled protein was used to measure the inhibitory activity of the Fab'-A chain molecule.

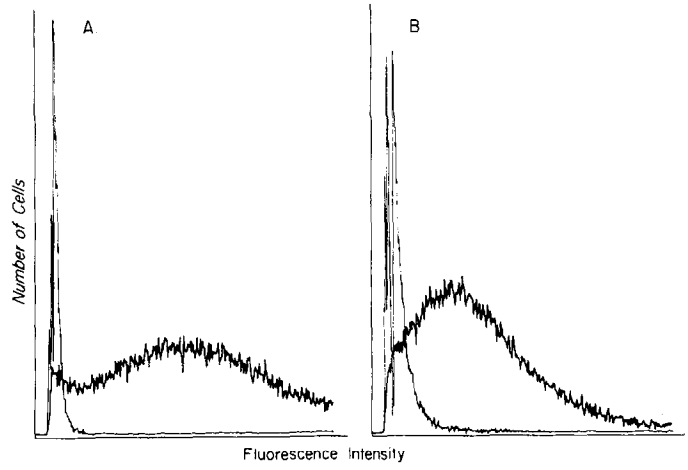

Figure 3. Attachment of intact Fab'-A chain molecules to membrane Ig determinants on Daudi cells. *A*, fluoresceinated rabbit anti-ricin A chain used to identify A-chain on the surface of cells that had been exposed either to PBS alone, ——, or PBS containing 10 μg Fab'-A chain, ——. *B*, fluoresceinated goat anti-rabbit $F(ab')_2$ used to reveal the Fab' moiety on the surface of cells that had been exposed either to PBS alone, ——, or PBS containing 10 μg Fab'-A chain, ——. Surface fluorescence quantified by using a fluorescence-activated cell sorter that evaluated 40,000 cells.

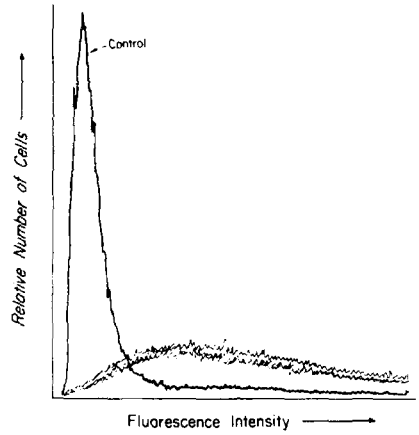

Figure 4. Comparative binding of anti-Ig directed Fab'-A chain, Fab'-E, and $F(ab')_2$ to an Ig positive human lymphoblastoid cell line (LAZ 444). Aliquots of cells were treated with either PBS alone (control) or PBS containing each of the antibody species adjusted to a final concentration of 5×10^{-6} M with respect to the number of antibody binding sites. The extent of cell surface binding was quantified by using a fluorescence-activated cell sorter after treatment of these cells with a fluoresceinated goat anti-rabbit $F(ab')_2$ reagent.

TABLE I

Inhibition of ^{14}C-L-leucine incorporation in Daudi cells by using the Fab'-A chain conjugate[a]

Addition	cpm	Inhibition
		%
Cells alone	5501 ± 411	
+ Fab'-A chain	1182 ± 52	79
+ Fab'-A chain + HGG	4919 ± 394	11
+ Fab'-A chain + BGG	1473 ± 69	73
+ Fab'-E	5043 ± 955	8

[a] Daudi cells were exposed to the Fab'-A chain conjugate or Fab'-E for 30 min and human or bovine γ-globulin was present as indicated. The cells were washed to remove any unbound agent and then were incubated at 37°C for 20 hr before adding ^{14}C-leucine.

produced cytotoxicity initially detected as impeded cell growth but which ultimately culminated in cell death and lysis (Table III). Neither A chain alone, Fab'-E alone, nor a mixture of the two produced such a dramatic effect. As was the case for experiments that measured inhibition of protein synthesis, toxicity was entirely averted by homologous human γ-globulin whereas bovine γ-globulin provided no blockage. A dose response curve for growth of Daudi cells in the presence of Fab'-A chain during a 96-hr time period gave an $I.D._{50}$ of 2.5×10^{-9} M for the agent (Fig. 5). Ricin displayed growth inhibition of Daudi cells at a 50-fold lower concentration range under these culture conditions.

The precise selectivity of antibody-delivered A chain was further illustrated when its effect on a panel of Ig positive and Ig negative cell lines was compared (Table IV). Fab'-A chain was either cytostatic or cytotoxic for all Ig positive lines examined, whereas it was totally innocuous for Ig negative lines, despite the prolonged incubation period. Indeed this distinction was found to persist when conjugate sensitive Daudi cells were co-cultivated with Ig negative CEM cells in the presence of the anti-Ig Fab'-A chain (Table V). The populations in mixed culture were resolved by fluorescence labeling with antibody against μ-chain, a phenotypic marker present on Daudi (20) but not CEM cells. Only the antigen positive Daudi cells were susceptible to the toxic action of conjugate.

In vivo toxicity of the Fab'-A chain conjugate. In accord with its controlled specificity and in extreme contradiction to the parent compound ricin, the Fab'-A chain conjugate was completely nontoxic in BALB/c as well as nude mice. Although ricin is lethal at 0.1 μg (6), mice given as much as 600 μg of the conjugate showed no ill effects. This feature will allow for testing of conjugate action *in vivo* on heterotransplanted Ig-bearing human cell lines and tumors (31). Immunofluorescence

TABLE II

Effect of lactose blockage[a]

Addition	cpm	Inhibition
		%
Cells alone	5773 ± 985	
+ Lactose	4040 ± 45	
+ Ricin	175 ± 11	97
+ Ricin + lactose	3130 ± 880	23
+ Fab'-A chain	1245 ± 379	78
+ Fab'-A chain + lactose	1187 ± 258	71

[a] Where designated 100 mM lactose in PBS was used to block during the 30 min exposure of Daudi cells to ricin or Fab'-A chain and 10 mM lactose was included in the incubation media.

TABLE III

Specificity of the toxicity of Fab'-A chain for Ig on Daudi cells[a]

Addition	Cells/ml × 10^{-4}			
	0 hr	72 hr	96 hr	120 hr
Cells alone	38	162	165	141
+ Fab'-A chain	38	52	24	4
+ A chain	38	166	152	150
+ Fab'-E	38	120	144	143
+ A chain + Fab'-E	38	179	145	145
+ Fab'-A chain + HGG	38	183	180	167
+ Fab'-A chain + BGG	38	45	27	6

[a] Daudi cells were initially plated at 38×10^4 cells/ml and as designated, Fab'-A chain, A chain, or Fab'-E were added to a final concentration of 5×10^{-8} M. Human and bovine γ-globulin were used at 2 mg/ml to block binding.

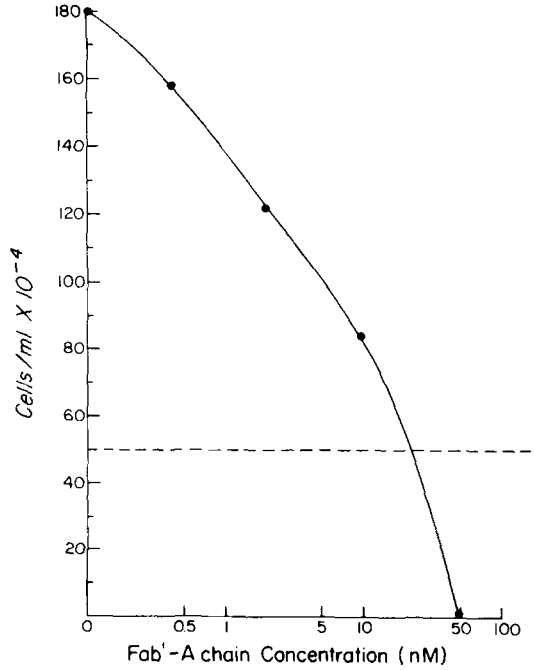

Figure 5. Growth inhibition of Daudi cells after 96 hr exposure to the Fab'-A chain conjugate. The dashed line indicates the initial starting concentration of 50×10^4 cells/ml.

TABLE IV

Comparison of toxicity of the Fab'-A chain conjugate on Ig-bearing and Ig-devoid cell lines[a]

Cell Line	Cells/ml × 10^{-4}		
	0 hr	72 hr	120 hr
Ig Positive Cells			
156	40	93	173
156 + Fab'-A chain	40	30	33
444	40	88	87
444 + Fab'-A chain	40	35	1
007	40	236	142
007 + Fab'-A chain	40	52	69
SB	40	260	172
SB + Fab'-A chain	40	66	125
Ig Negative Cells			
CEM	40	165	376
CEM + Fab'-A chain	40	128	423
HSB2	40	132	265
HSB2 + Fab'-A chain	40	149	272
221	40	78	143
221 + Fab'-A chain	40	101	153

[a] The various cell lines were adjusted to 40 × 10^4 cells/ml and Fab'-A chain was included at 8.6 × 10^{-8} M in the test cultures.

TABLE V

Selective toxicity of Fab'-A chain conjugate in mixed cell culture[a]

Addition	Cells/ml × 10^{-4}	Bearing μ-Chain	Cells/ml × 10^{-4} Calculated Daudi:CEM
		%	
Daudi	164	96	
Daudi + Fab'-A chain	21	79	
CEM	123	0.3	
CEM + Fab'-A chain	91	0.3	
Daudi + CEM	216	49.5	107:109
Daudi + CEM + Fab'-A chain	115	19.6	22:93

[a] Each cell type was started at 30 × 10^4 cells/ml and contained 2 × 10^{-7} M Fab'-A chain as noted. Aliquots were removed at 72 hr for cell counts and to process for anti-human μ-chain fluorescence surface labeling. Subsequent quantification was achieved by cytofluorographic analysis.

examination of human Daudi lymphoma cells removed from a large subcutaneous tumor growing in a C3H nude mouse revealed the presence of both surface Fab' and ricin A chain, after an i.v. injection of 500 μg of the conjugate. These preliminary results indicate that the intact molecule can reach and bind to tumor target cells *in vivo*.

DISCUSSION

Fab' specific for surface Ig can now be added to the list of lectins, hormones, and other molecules with cell surface affinity that facilitate membrane penetration of the toxic moiety of plant or bacterial toxins (32). It is likely that antibodies with specificity for many diverse membrane components will serve a similar function. Indeed, during completion of this study, a communication describing the cytotoxicity of diphtheria toxin fragment A linked to Fab' directed against undefined antigens on mouse L1210 cells was reported (33). The fact that membrane reactive antibody can deliver such toxic moieties to the cytoplasm in an active form is of interest from a mechanistic standpoint. There remains some controversy as to whether those toxin molecules that ultimately act on the ribosomes escape from endocytotic vesicles or arrive via direct transfer through the plasma membrane (32). Fab' might facilitate the traverse of A chain into the cytoplasm by holding it in close proximity to the membrane. It is unlikely, however, that it could play an active role in this penetration, a function that has been postulated for the B subunit of ricin. In the anti-Ig system, there is ample evidence for endocytotic uptake of both surface bound monovalent and bivalent antibodies (11, 12), so covalently linked A chain could gain access by this route. If such a mechanism is in fact operative, increased toxicity would be expected for a bivalent *versus* monovalent antibody carrier, since capping and endocytosis are stimulated by cross-linking of surface Ig determinants (11).

The relative potency of native ricin and the anti-Ig Fab'-A chain conjugate is an important consideration for judging if they act in a strictly parallel manner. A rigorous comparison is difficult to obtain for two reasons. Designed as a general reagent for reaction with all Ig-bearing cells, the conjugate preparation used in this study represents a collection of site-directed agents, recognizing γ-heavy chain, as well as λ and κ-light chain determinants. Thus, the $I.D._{50}$ level derived for Daudi cells is an underestimate of conjugate potency because binding to these cells occurs strictly by virtue of its anti-κ chain components (20). Similarly, inclusion of the required serum in cell culture medium reduces the full effectiveness of whole ricin (34). Nevertheless, the finding that ricin is 50 times more potent than Fab'-A chain for Daudi cells must reflect a real difference at some point in the intoxication process.

Ricin and Fab'-A chain are capable of monovalent binding interactions with membrane glycoprotein and κ-light chain determinants, respectively, on the Daudi cell surface. Toxicity is determined by the number of toxin molecules attached to the cell membrane (35), and this level is a function of the inherent binding affinity as well as the number of surface receptor sites. Studies that have examined these parameters (35, 36) indicate that although comparable binding affinities of interaction would be expected, the number of sites available for ricin attachment on a Daudi cell should exceed those accessible to the Ig directed Fab'-A chain by 100-fold or more. This discrepancy could account for the higher potency of the natural toxin.

Elevated levels of toxicity might well be achieved by using conjugates formed with antibodies directed against multiple membrane components or to alternate target molecules that are more abundant than surface Ig. Such a system, however, would less likely parallel the situation expected for tumor specific antigens or determinants, that could be weakly expressed or sparsely distributed on the membrane.

The selective cytotoxic action demonstrated for this Ig-directed Fab'-A chain conjugate makes speculation on the use of such delivery systems for practical application to therapeutic problems appear plausible. Isolated ricin A chain alone is nontoxic because it lacks the ability to bind to and penetrate the cell membrane. This feature precludes serious problems associated with the nonspecific toxicity that is prevalent when using whole toxins. The inherent potency of A chain is retained, however, so that its toxicity may be appended to any antibody that can transport it into the cytoplasm. Intracytoplasmic severance of its disulfide connection to Fab' most likely precedes A chain's catalytic action on ribosomes. The resulting cytotoxicity exhibited for Ig-bearing Daudi cells followed a sequence of

events that began with the shutdown of protein synthesis, proceeded with a subsequent impairment of replicative ability, and culminated in cell lysis.

In vivo tests with mice have shown that the conjugate is nontoxic even at levels that are 6000-fold higher than the lethal dose of the parent toxin, ricin. This finding is in accord with the agent's strict specificity for human Ig determinants and reaffirms its innocuous properties toward cells to which it cannot bind. Catabolism of the A chain conjugate apparently proceeds without any detrimental effects to these animals.

The utilization of an Fab' fragment as a carrier of A chain entails some added advantages or immunotherapy studies. The monovalent conjugate cannot form precipitates with antigen and possesses no Fc region. Thus, dangers of renal complications due to immune complex deposits are remote, and nonspecific effects caused by Fc binding, agglutination reactions, or C fixation are reduced. Removal of the Fc region also renders the molecule less antigenic, a plus when they are to be used repeatedly in experimental animals.

Whole 7S immunoglobulin and antibody fragments display different plasma half-lives and distribution characteristics when passively administered to animals (37). Ricin A chain conjugates formed with these carriers should also show different pharmacologic properties. Increased cell binding affinity would be expected with a bivalent antibody carrier in surface antigen systems where determinant density is high enough to permit polyvalent interactions. Cross-linking could also stimulate internalization, but whether this will result in more productive processing and greater A chain toxicity remains to be seen.

If eventual clinical application is projected for an antibody-delivered conjugate, the source of toxic A fragment should also be carefully considered. For example, since the general population has been actively immunized with diphtheria toxoid, the effectiveness of an agent constructed with this toxin could be rapidly neutralized by circulating antibodies.

Surface Ig is a potential target that is prominent on human chronic lymphatic leukemias and lymphomas of B cell origin. *In vivo* killing of such cells in the nude mouse model might be accomplished with an anti-Ig conjugate but the presence of serum IgG in humans would obviously preclude the effectiveness of this particular delivery system in a clinical setting. Antibodies directed against alternate Ig determinants as well as other distinguishing cell surface markers that will not be blocked are being examined as carriers of ricin A chain. In particular, adaptation of the coupling procedure to homogeneous hybridoma antibodies reactive with pertinent tumor specific or associated antigen targets is being explored. Conjugates formed with appropriate antibodies could also allow for the depletion of phenotypically distinct, functional subsets of normal cell populations.

In summary, the disulfide-linked anti-human Ig Fab'-ricin A chain conjugate retained both its antigen binding capacity, as well as the ribosome inactivating function of the A chain. It was strongly reactive with surface Ig determinants on human lymphocyte cell lines, and both of the constitutive halves of the conjugate could be detected on the membrane by fluorescent antibody reagents.

Surface localized A chain gained access to ribosomes within the cytoplasm and produced inhibition of protein synthesis, as well as eventual cell death. The action of the conjugate was restricted to cells bearing Ig determinants and could be blocked by competition with free human IgG. The separate component halves of the molecule exhibited no cytotoxicity. Furthermore, it should be stressed that none of these toxic effects was attributable to contaminating whole ricin since conjugate action was not influenced by the presence of lactose, and the preparation was completely inert on Ig negative cell lines that are acutely sensitive to native ricin.

Apart from tailoring the specificity of A chain's toxic potential, the strong point of the Fab' conjugate resides in its benign nature until it enters the designated target cell. This salient feature precludes leakage of its cytotoxic effect to neighboring cells lacking the precise antigenic determinant. Thus, new therapeutic strategies may be designed in the future by using high affinity antibody carriers of ricin A chain where specific surface binding and internalization are obligatory for cell death.

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