

CYTOTOXICITY OF A CELL-REACTIVE  
IMMUNOTOXIN CONTAINING RICIN A CHAIN  
IS POTENTIATED BY AN ANTI-IMMUNOTOXIN  
CONTAINING RICIN B CHAIN

BY ELLEN S. VITETTA, R. JERROLD FULTON, AND JONATHAN W. UHR

*From the Department of Microbiology, University of Texas Health Science Center, Dallas, Texas  
75235*

The plant toxin, ricin (1, 2), is composed of an A chain that enzymatically inactivates 60s ribosomes and a B chain that is a galactose-specific lectin (1). The A chain can be coupled to a cell-reactive antibody creating a toxic hybrid (immunotoxin). Many ricin A chain-containing immunotoxins (IT) are not as toxic as IT containing intact ricin (3-8), possibly due to the capacity of B chain to facilitate the translocation of the A chain into the cytoplasm. We have recently shown that a B chain-containing IT (B-IT) can markedly potentiate the specific toxicity of an A chain-containing IT (A-IT) when both IT are directed against the same molecule on the target cells (9). An important variation of this approach with potential advantages for in vivo application would be to attach a univalent fragment of the cell-binding antibody to the A chain and an anti-antibody to the B chain. The univalent A-IT should remain on the target cell for an extended period of time and the secondary B-IT could be administered several hours later. Only cells coated with the A-IT that bind the B-IT should be killed. The present studies represent in vitro experiments to test the feasibility of this approach.

**Materials and Methods**

*Cells.* Daudi cells were maintained and used as described previously (10, 11).

*Antibodies.* Affinity-purified rabbit anti-human Ig (RAHIg), goat anti-rabbit Ig (GARIG), goat anti-ovalbumin (GA-OVA), and rabbit anti-ricin (RAR) were prepared as described (9-11).

*Ricin A and B Chains.* The A and B chain subunits of ricin were purchased from Worthington Biochemical Corp. and evaluated for purity and activity as described previously (9-12).

*Preparation of F(ab')<sub>2</sub> Fragments of RAHIg.* F(ab')<sub>2</sub> fragments of affinity-purified RAHIg antibodies were prepared by pepsin digestion (13). Undigested antibody was removed on protein A-Sepharose. F(ab')<sub>2</sub> fragments were affinity-purified on Sepharose-human Ig (9).

F(ab') fragments were prepared from F(ab')<sub>2</sub> fragments by reduction with 10 mM dithiothreitol (DTT) and alkylation with 22 mM iodoacetamide.

*Preparation and Affinity Purification of IT.* IT containing intact Ig or alkylated F(ab') fragments were prepared, separated from free ricin chains and were affinity purified as described previously (9). The recovery of the IT after affinity purification averaged 20-40% (9). As assessed by radioimmunoassay (RIA), each IT contained 1-3 A or B chains

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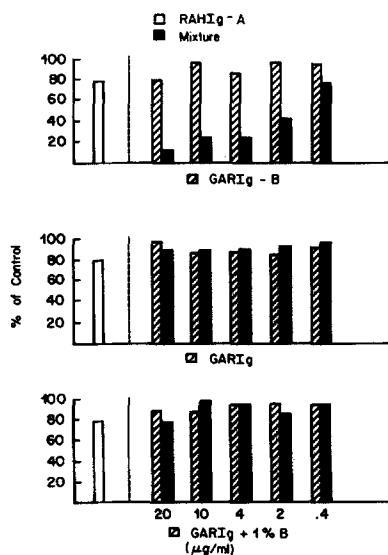


FIGURE 1. GARIg-B potentiates the killing of Daudi cells treated with RAHIg-A. (*Top*) Three treatment protocols were used: (a) Cells ( $1 \times 10^5$ /well) were treated for 15 min at  $4^\circ\text{C}$  in BSS (0.1 ml/well) containing RAHIg-A (final concentration,  $1.1 \times 10^{-8}$  M). Cells were washed and then treated for 15 min. at  $4^\circ\text{C}$  with BSS ( $\square$ ); (b) cells were treated for 15 min at  $4^\circ\text{C}$  with BSS, washed, and treated with BSS containing different concentrations of GARIg-B ( $1 \mu\text{g}/\text{ml} = 4 \times 10^{-9}$  M immunotoxin) ( $\square$ ); (c) cells were treated with BSS containing  $1.1 \times 10^{-8}$  M RAHIg-A, washed, and treated for 15 min at  $4^\circ\text{C}$  in BSS containing different concentrations of GARIg-B ( $\blacksquare$ ). Control cultures were treated with BSS in both steps. Cells were washed, cultured for an additional 22 h, and labeled for 6 h with [ $^3\text{H}$ ]leucine. Cells were harvested, counted, and the leucine incorporation was compared with that of the control cells. (*Middle*) Cells were treated as described in the top panel except that the GARIg was not attached to B chain. (*Bottom*) Cells were treated as described in the top panel except that GARIg + 1% free B chain was used instead of the GARIg-B. This is one representative experiment of six.

per molecule of antibody and was contaminated with <1% (and usually undetectable levels) of free A or B chains. Alternatively,  $\text{F}(\text{ab}')_2$  fragments were reduced, desalted on Sephadex G-25, and reacted with a 100-fold molar excess of 5,5'-dithiobis(2-nitrobenzoic acid) (Ellman's reagent). Contamination of  $\text{F}(\text{ab}')$  by  $\text{F}(\text{ab}')_2$  ranged from undetectable levels to  $\sim 0.1\%$ . The  $\text{F}(\text{ab}')\text{-Ellman's}$  was reacted with A chain and the  $\text{F}(\text{ab}')\text{-A}$  IT was purified as described previously (9).  $\text{F}(\text{ab}')\text{-A}$  chain contained one A chain per molecule of  $\text{F}(\text{ab}')$ .

*Evaluation of IT.* IT were assessed for the presence of active antibody and either A or B chain by a solid phase RIA (3, 9) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7% slab gels stained with silver (14).

*Treatment of Daudi Cells.*  $10^5$  Daudi cells in balanced salt solution (BSS) were distributed into wells ( $10^5$ /well) of a 96-well microtiter plate. Nontoxic dilutions of the RAHIg-A were added to triplicate wells for 15 min at  $4^\circ\text{C}$ . Cells were centrifuged and washed three times in BSS. Cells were then treated with the GARIg-B for 15 min at  $4^\circ\text{C}$ , washed three times in BSS, and cultured in 200  $\mu\text{l}$  of RPMI lacking leucine and containing 10% fetal calf serum (FCS) for 22 h at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator. Cells were labeled with [ $^3\text{H}$ ]leucine (New England Nuclear, Boston, MA) and harvested as described previously (9). At least nine wells of untreated cells were included as controls for each treatment group. The percent reduction in [ $^3\text{H}$ ]leucine incorporation as compared with the controls was used as the assessment of killing.

## Results

*Ability of GARIg-B to Potentiate the Killing of RAHIg-A.* As seen in Fig. 1, *top*,

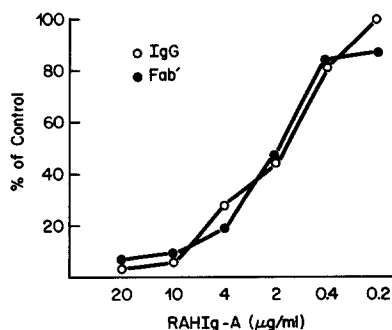


FIGURE 2. Both divalent and univalent fragments of RAHIg-A kill Daudi cells. Cells ( $1 \times 10^5$ /well) were treated for 15 min at  $4^\circ\text{C}$  in BSS (0.1 ml/well) with the indicated concentrations of either divalent (RAHIg) ( $1 \mu\text{g/ml} = 4.2 \times 10^{-9}$  M) or univalent [F(ab')] RAHIg-A ( $1 \mu\text{g/ml} = 7.2 \times 10^{-9}$  M). The cells were cultured and labeled as described in Fig. 1. This is one representative experiment of three.

neither  $1.1 \times 10^{-8}$  M RAHIg-A or GARIg-B at  $8 \times 10^{-8}$  to  $2 \times 10^{-9}$  M inhibited protein synthesis. However, when the cells were treated with RAHIg-A, washed, and then treated with different concentrations of GARIg-B, there was significant potentiation of cytotoxicity. When GARIg alone was used (Fig. 1 *middle*) or when GARIg were mixed with 1% free B chains (the maximum estimated contamination of the secondary IT) (Fig. 1 *bottom*), no potentiation of the killing by RAHIg-A was observed. These experiments demonstrate that the potentiation of killing is dependent upon the covalent attachment of the B chain to the secondary antibody.

An irrelevant secondary IT (GA-OVA-B) was ineffective at potentiating killing by the RAHIg-A (data not shown). In addition, an irrelevant primary IT (RA-OVA-A) or antibody (RAHIg) followed by the GARIg-B did not inhibit protein synthesis (data not shown).

*Ability of A Chain-Containing F(ab') Fragments of RAHIg to Kill Daudi Cells.* As seen in Fig. 2, F(ab') fragments of RAHIg-A were highly toxic to Daudi cells, confirming earlier reports (15, 16). The titration curves using F(ab')-RAHIg-A chains and intact RAHIg-A were virtually identical. This was unexpected since the F(ab') fragments, in contrast to intact antibody, should not effectively cross-link cell surface Ig (sIg). The possibility was considered that the high content of A chain in this preparation [F(ab')/A ratio, 3:1] caused cross-linking of the surface Ig and rapid endocytosis of the Ig-IT complex. However, F(ab')-A-IT prepared using Ellman' reagent [F(ab')/A, 1:1] killed the Daudi cells in a comparable manner (data not shown) and immunofluorescence studies did not show any patching of sIg on F(ab')-RAHIg-A-treated cells (data not shown). The effectiveness of F(ab')-A, compared with intact RAHIg-A-IT, may indicate that these different ligands enter different endocytic vesicles with different potentials for A chain translocation.

*Ability of GARIg-B to Potentiate the Killing by F(ab') Fragments of RAHIg-A.* As shown in Fig. 3, *top*, nontoxic concentrations of the F(ab')-RAHIg-A caused only a slight reduction in protein synthesis, and none of the concentrations of the GARIg-B were inhibitory. However, when cells were treated with a nontoxic concentration of F(ab')-RAHIg-A, washed, and treated with different amounts

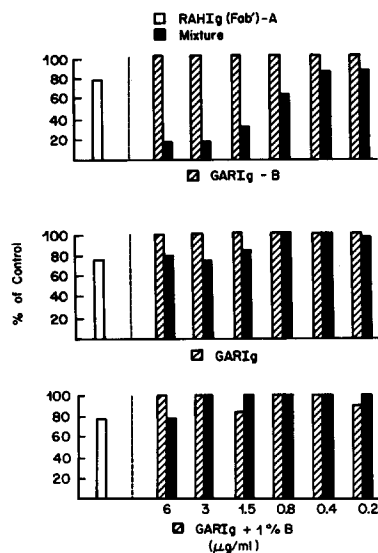


FIGURE 3.

FIGURE 3. GARIg-B potentiates the killing of Daudi cells treated with F(ab')-RAHIg-A. See Fig. 1 for experimental details. In this experiment, the F(ab')-RAHIg-A (final concentration,  $7.2 \times 10^{-9}$  M) was used in place of the intact RAHIg-A (Fig. 1). This is one representative experiment of four.

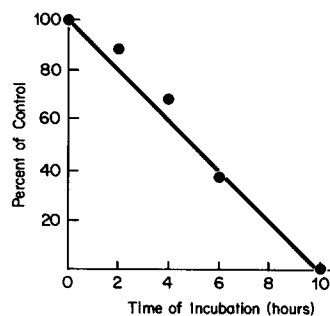


FIGURE 4.

FIGURE 4. The effect of delaying the time of addition of GARIg-B on the potentiation of the killing of Daudi cells by F(ab') RAHIg-A. Daudi cells were treated with a nontoxic concentration of F(ab')-RAHIg-A ( $7.2 \times 10^{-9}$  M) as described in Fig. 1. The washed cells were incubated at  $37^\circ\text{C}$  in complete medium for the indicated time intervals before being washed and treated with GARIg-B (final concentration,  $2.5 \times 10^{-8}$  M), as described in Fig. 1. Cells were washed, cultured, and harvested as described in Fig. 1. Results are presented as the percentage of potentiation obtained when GARIg-B addition was not delayed. This is one representative experiment of three.

of GARIg-B, there was significant potentiation of cytotoxicity. This potentiation was not observed with GARIg (Fig. 3, *middle*) or GARIg mixed with 1% B chains (Fig. 3, *bottom*).

*Effect of the Time Interval Between the Addition of the Primary and Secondary IT on In Vitro Killing of the Daudi Cells.* An important objective was to demonstrate that F(ab') fragments of RAHIg-A could remain on the cell surface so that GARIg-B could kill such cells when administered after an interval of several hours. Therefore, cells were treated for 15 min at  $4^\circ\text{C}$  with nontoxic concentrations of F(ab')-RAHIg-A, washed, and cultured at  $37^\circ\text{C}$  for various time intervals. Cells were then washed and treated for 15 min at  $4^\circ\text{C}$  with the GARIg-B, washed, and cultured at  $37^\circ\text{C}$  for 22 h. As seen in Fig. 4, the effectiveness of potentiation with increasing time intervals between treatments with the F(ab')-RAHIg-A and the GARIg-B decreased in a linear fashion. 50% of the control potentiation was observed at an interval of 5 h but no potentiation was observed after an interval of 10 h.

### Discussion

Three approaches have been used in vitro to exploit the function of the B chain in potentiating killing by ricin A-IT: (a) the use of ricin-containing IT in which the lectin-binding site of the ricin is sterically blocked by its binding to

antibody (17); (b) the addition of free B chains to A-IT (7, 18); and (c) the addition of B-IT to A-IT in which both IT are directed against the same cell surface molecule (9). Such B-IT have greatly reduced lectin-binding affinities (9).

The present results have established the feasibility of using B-IT reactive with the univalent antibody of the A-IT to potentiate its specific toxicity. Furthermore, significant potentiation can be achieved after an interval of ~5 h.

Full toxicity is achieved by the union of two different molecular species, only one of which, F(ab')-A chain, should be toxic to the reticuloendothelial system (RES). Furthermore, F(ab') fragments of the A-IT should not bind to Fc receptors on nontarget tissue. Hence, it should be possible to mark target cells *in vivo* with this IT. Since F(ab') fragments are not reabsorbed by the kidney, the serum half-life is extremely short and 5 h later much of the excess F(ab')-A chain should have been excreted (19). At this time, B-IT directed to determinants on the F(ab') fragments could be administered. B-IT have little or no inherent toxicity. However, their uptake by target cells coated with the F(ab')-A chain IT should result in potentiation of specific toxicity.

There are several potential obstacles which must be overcome before using this strategy *in vivo*. (a) It may be necessary to develop B-IT that have an even lower binding affinity for galactose. This might be accomplished by altering the lectin-binding site on the B chain. (b) IT are taken up by the mannose receptors on macrophages and cells of the RES (20) because of the mannose-rich oligosaccharide of the ricin chains (2). This potential problem could be overcome by removal of the carbohydrate moieties from the A and B chains. (c) It is unclear whether intact B-IT can gain access to cells in lymphoid organs rapidly enough to bind to A-IT before they are removed from the cell surface. Hence, further information concerning the *in vivo* distribution, kinetics of clearance, and toxicity of these IT is needed to properly design effective therapeutic regimens for *in vivo* use.

### Summary

*In vitro* killing of the human Daudi cell line by either univalent [F(ab')] or divalent (IgG) forms of rabbit anti-human Ig (RAHIg) coupled to ricin A chain can be specifically potentiated by a "piggyback" treatment with ricin B chain coupled to goat anti-rabbit Ig (GARIg). When cells are treated with univalent immunotoxin (IT) [F(ab') RAHIg-A] and then cultured, IT can be detected on the cell surface for at least 5 h, since GARIg-B can still enhance killing at this time. These results provide a strategy for *in vivo* use of A chain- and B chain-containing IT.

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