Deletion of Neoplastic B Cells by Immunotoxins Containing Ricin A Chains*

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Immunotoxins represent a new family of potentially useful reagents for scientists and clinicians. The term, immunotoxin, is used to designate a hybrid molecule: one portion is a toxic moiety and the other is either an antibody or an antigen. The purpose of using immunotoxins is to delete subsets of target cells that possess an antigen (or antibody) that will react specifically with the immunotoxin but not to delete bystander cells that lack the surface marker in question.[1] In this article, we will discuss recent experimental studies with immunotoxins and their implications with regard to problems in oncology.

During the last 10 years, there have been many attempts to apply this concept to the elimination of neoplastic and other target cells. One such toxin, ricin, like most toxic proteins produced by bacteria and plants, has a toxic polypeptide (A chain) attached to a cell binding polypeptide (B chain).[2] The B chain is a lectin that binds to galactose-containing glycoproteins or glycolipids on the cell surface. The presumed series of events that take place during intoxication of a cell by ricin are:[3] 1) binding of the toxin to the cell surface; 2) endocytosis; 3) translocation of the A chain across the endocytic membrane; and 4) inactivation of protein synthesis. The precise molecular and subcellular events underlying endocytosis and translocation are not known. There is evidence that the B chain can also facilitate the translocation of the A chain through the membrane of the endocytic vesicle, possibly by forming a pore.[4-7] In the cytoplasm, the A chain of ricin inhibits protein synthesis by enzymatically inactivating the EF-2 binding portion of the 60S ribosomal subunit. It is thought that one molecule of A chain in the cytoplasm of a susceptible cell can kill it.[3]

The A and B chains of ricin can be separated, purified and covalently linked to antibodies derivatized with the thiol-containing cross-linker, SPDP. In the case of A chain containing immunotoxins, the antibody portion substitutes for the lectin portion (B chain) thus allowing the specific targeting of the toxic A chain to the relevant target cells.

We have used a murine B cell leukaemia (BCL_1) as our experimental model. This mouse disease bears a close resemblance to the prolymphocytic form of chronic lymphocytic leukaemia in the human, *i.e.*, splenomegaly and severe leukaemia.[8,9] Injection of one BCL_1 cell into a normal BALB/c mouse results in leukaemia in approximately one-half of the recipients 12 weeks later.[10] Tumour bearing mice usually survive for 3-4 months after receiving 10^5 – 10^6 tumour cells. The BCL_1 tumour cells bear large amounts of cell surface IgMλ and traces of IgDλ, both of which have the same idiotype.

IN VITRO USE OF IMMUNOTOXINS

In initial experiments, immunotoxins containing anti-idiotypic antibody directed against the tumour-derived Ig were incubated with populations of BCL_1 tumour cells and control cells. The specific immunotoxin decreased protein synthesis in the populations containing tumour cells by 70-80%; the percentage of tumour cells in these populations was also 70-80%. Control immuno-

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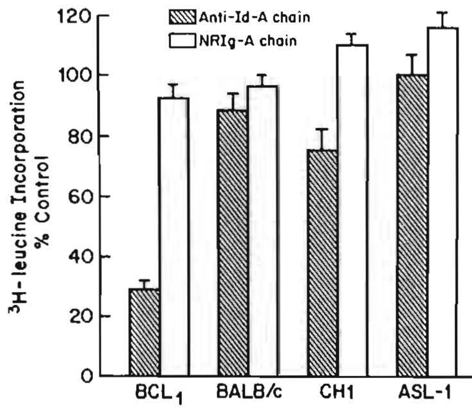

Fig. 1[10,11] Effect of anti-Id-A chain on protein synthesis in BCL_1 cells, normal BALB/c splenocytes, ASL-1 tumour cells, and CHl tumour cells. Anti-Id-A chain was used at 0.2 μg/ml. The CH1 cells express IgMλ on their surface but lack the BCL_1 idiotype. ▧, anti-Id-A chain; □, NRIg-A chain.

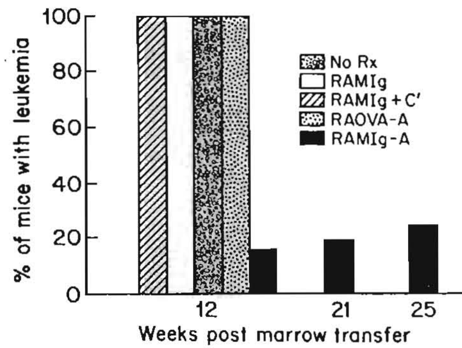

Fig. 2[15] Adoptive transfer into lethally irradiated recipients of BCL_1-containing bone marrow cells treated with rabbit antibody (Ab) to mouse Ig conjugated with A chain. Bone marrow cells containing 10 to 15 percent tumour cells were injected into groups of 20 mice at 10^6 marrow cells per mouse. Every two weeks after adoptive transfer the mice were examined for leukaemia. At 25 weeks, all surviving mice were killed and 10^6 spleen cells were adoptively transferred into normal recipients. The spleen cells from one of the mice caused a tumour in these recipients 10 weeks later. Thus, this mouse is scored as leukaemic at 25 weeks.

toxins containing irrelevant antibodies had no effect on BCL_1 cells nor did specific immunotoxins have an effect on normal splenocytes, on T cell tumours, or on another B cell tumour bearing a different idiotype (Fig. 1). Anti-idiotype antibody by itself did not affect protein synthesis in BCL_1 cells. These results indicate that immunotoxin-mediated killing of neoplastic B cells in a mixed population is specific.[11]

Similar studies were performed using a tumour infiltrated bone marrow[12] (containing 15% BCL_1 cells) because of the clinical implications of removing tumour cells from marrow. In addition, it was possible to evaluate the nonspecific killing of stem cells by adoptively transferring the treated cells into lethally irradiated recipients. In these studies, anti-Ig immunotoxin was used since, the only requirement for the specificty of the immunotoxin was that it kill all the tumour cells but *not* the stem cells. Thus, it was possible to use a polyvalent antibody against Ig rather than an anti-idiotypic antibody. The results of these experiments (Fig. 2) indicate that 1) the haematopoietic system of all the animals was reconstituted because all lethally irradiated mice survived. 2) 15 of 20 mice treated with tumour reactive immunotoxin did not develop tumour over a period of 25 weeks of observation. Of the 5 animals that relapsed, all had idiotype positive cells that were susceptible to the *in vitro* lethal effect of anti-Ig containing immunotoxins. Hence, no evidence was obtained for the emergence of an immunotoxin-resistant variant. Rather, the results of immunotoxin treatment in these studies was consistent with the survival of 1 cell per 1×10^6 cells injected. Results similar to ours have been obtained by Thorpe *et al*[13] using antibody-*ricin* conjugates in the presence of lactose to delete tumour cells from rat bone marrow. Furthermore, we have recently extended this approach to the removal of neoplastic B cells from human bone marrow and demonstrated that the tumour cells are killed but that the CFU_{GM}, BFU_E and CFU_E are not.[14]

IN VIVO USE OF IMMUNOTOXINS[15]

For these experiments, mice bearing massive tumour burdens (20% of body weight or approximately 10^{10} tumour cells) were employed. The strategy was to reduce the tumour burden by at least 95% using nonspecific cytoreduction and to eliminate the remaining tumour cells with immunotoxins directed against either the idiotype or the δ chain of sIgD on the BCL_1 cells. The rationale for using anti-δ is that sIgD is present on a large proportion of B cell tumours and, therefore, would present a more practical reagent for clinical therapy. Furthermore, after cytoreductive therapy, there are virtually no sIgD-positive normal B cells or serum IgD to bind the immunotoxin. Normal B cells can also be regenerated from $sIgD^-$ cells. In these experiments, nonspecific cytoreduction was accomplished with a combination of splenectomy and fractionated total lymphoid irradiation (TLI). Animals receiving no further treatment other than TLI and splenectomy were dead within 8 weeks (Fig. 3). The injection of these cytoreduced mice with control immunotoxins or antibody alone did not prolong their survival. In contrast, animals receiving anti-δ immunotoxins appeared disease-free as judged by the absence of detectable idiotype-positive cells 12 to 18 weeks later in 3 of 4 experiments. In one experiment, treated mice relapsed at 8-10 weeks after immunotoxin therapy. It should also be noted that 14 weeks after such immunotoxin treatment, mice in remission had normal or above normal levels of sIgD-bearing B lymphocytes. Hence, stem cells, pre-B cells or $sIgD^-$ lymphocytes had fully restored the virgin B cell compartment.

These results suggest that 1)

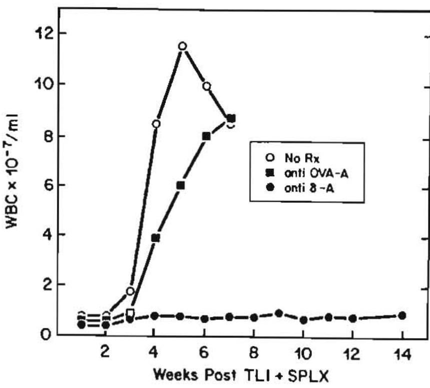

Fig. 3[12] Effect of TLI, splenectomy, and administration of immunotoxin on leukaemic relapse of BCL_1-bearing mice. After nine doses of TLI and splenectomy, mice were injected with two doses of 20 μg of anti-δ or control immunotoxin or were not injected. There were nine mice per group. Leukaemic relapse was monitored by determining the number of white cells in the blood of the treated mice. The control mice were all dead at 7 wk after TLI. The rabbit anti-mouse δ-A chain-treated group was monitored for a period of 14 wk post-TLI, at which point the experiment was terminated.

either remaining tumour cells had been eradicated in the animals that appeared tumour-free or that some viable tumour cells remained but were "held in check" by host resistance mechanisms. 2) Immunotoxin to a normal tissue component, in this case sIgD, can be used to render animals disease-free and the host can survive the effects of such cross-reactivity and can reconstitute the B cell compartment. To determine whether the animals were disease-free, tissues were then transferred from disease-free animals 25 weeks after treatments. All animals adoptively transferred tumour into normal mice indicating that the animals were not tumour-free and suggesting that host resistance had developed.

The partial success of these experiments was probably due to the fact that nonspecific cytoreduction was successful in reducing the number of remaining tumour cells to a level which could be effectively killed by a non-lethal dose of the immunotoxin. In addition, the immunotoxins in this instance did not kill all the remaining tumour cells yet prolonged remissions occurred. Presumably, the remaining viable tumour cells did not produce progressive disease because of a tumour-specific immune response.

USE OF B CHAIN-CONTAINING IMMUNOTOXINS TO POTENTIATE THE SPECIFIC TOXICITY OF A CHAIN-CONTAINING IMMUNOTOXINS

It is known that in many cases ricin conjugates are significantly more toxic than antibody-A chain conjugates.[4-7] In addition, free B chains can synergise *in vitro* with A chain-containing immunotoxins in specifically killing target cells.[5] It is postulated, therefore, that the greater toxicity of ricin-containing immunotoxins as compared to A chain-containing immunotoxins is due to the ability of the B chain to facilitate the entry of A chain into the cytoplasm.[5] It would be desirable to develop a strategy in which the putative transport role of the B chain could be preserved while eliminating or minimising its function as a lectin. We have recently described a new approach to accomplish this objective. The idea is to add to the incubation mixture B chain-containing immunotoxins. These are made using the same procedure for generating A chain-containing immunotoxins. Thus, using the two immunotoxins, the two subunits of the ricin toxin could thereby be delivered *independently* to the same target cell.

As seen in the upper panel of Figure 4,[16] when Daudi cells were treated with either a low dose of rabbit anti-human Ig-A chain (RαHIg-A) *or* different doses of rabbit anti-human Ig-B chain (RαHIg-B), no toxicity was observed. However, when the RαHIg-A was *mixed* with various concentrations of RαHIg-B there was significant cytotoxicity. It is of interest that this treatment of the Daudi cells with the mixture of immunotoxins was performed in medium lacking galactose. As shown in the bottom panel of Figure 4, when Daudi cells were treated with a low dose of RαHIg-A, a variety of doses of rabbit anti-ovalbumin-B (RαOVA-B) or mixtures of the two, no toxicity was observed except at the highest dose of the RαOVA-B. These results indicate that the target cell specificity of the antibody combining site of the immunotoxin is essential for synergy.

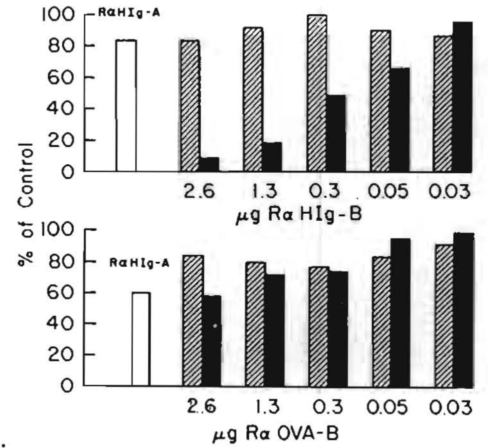

Fig. 4[16] The use of mixtures of A chain and B chain containing immunotoxins to kill Daudi cells *in vitro*. Daudi cells were cultured with a nontoxic concentration of either RαHIg-A □, or nontoxic doses of either RαHIg-B (upper panel) ▨, or RαOVA-B (lower panel) ▨. The solid bar ■, represent mixtures of the single dose of the RαHIg-A plus different concentrations of either RαHIg-B (upper panel), or RαOVA-B (lower panel). Cells were treated with immunotoxin for 15 minutes at 4°C, washed, and cultured for 16 hours at 37°C in medium containing immunotoxin. Cells were labeled for 4 hours with ^{3}H-leucine and harvested. The controls were not treated with immunotoxins but were incubated and labeled in the same manner.

A second 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-an-

tibody to the B chain. The univalent A chain-containing immunotoxin should remain on the target cell for an extended period of time

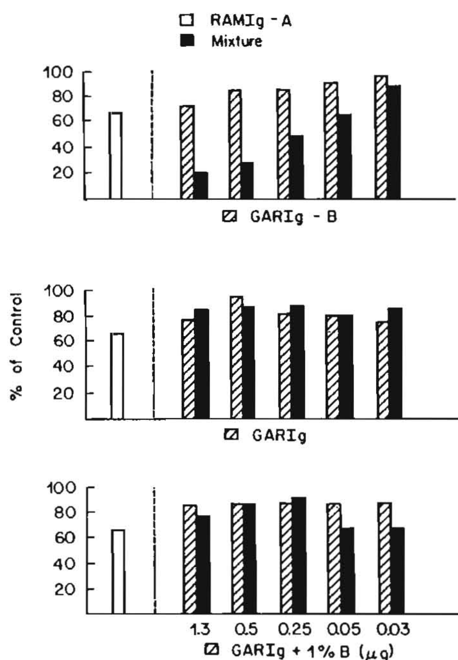

Fig. 5[17] GARIg-B potentiates the killing of Daudi cells treated with (Fab′) fragments of RαHIg-A (upper panel). Three treatment protocols were used: (a) cells ($1x10^5$ /well) were treated for 15 min at 4°C in balanced salt solution (BSS) containing (Fab′) fragments of RαHIg-A ($7.2x10^{-9}$M). Cells were washed and then treated for 15 min at 4°C □; (b) cells were treated for 15 min at 4°C with BSS, washed and treated with BSS containing different concentrations of GARIg-B (1 μg/ml = $4x10^{-9}$M immunotoxin) ▨; (c) cells were treated with BSS containing $7.2x10^{-9}$M (Fab′) RαHIg-A, washed and treated for 15 min at 4°C in BSS containing different concentrations of GARIg-B ■ Control cultures were treated with BSS in both steps. Cells were then washed, cultured for an additional 22 hrs and labeled for 6 hrs with ^{3}H-leucine. Cells were harvested, counted and the leucine incorporation was compared to that of the control cells. Middle panel: Cells were treated as described in the upper panel except that the GARIg was not attached to B chain. Lower panel: Cells were treated as described in the upper panel except the GARIg plus 1% free B chain were used instead of GARIg-B.

and the anti-antibody immunotoxin-B could be administered several hours later. Only cells coated with the A chain-containing immunotoxin that bind the B chain containing immunotoxin should be killed. As shown in the upper panel of Figure 5,[17] the secondary antibody (GαRIg) coupled to B chain (GαRIg-B) potentiates the ability of (Fab′) fragments of RαHIg-A to kill Daudi cells. The use of secondary antibody alone (middle panel) or the antibody mixed with the maximal amounts of free B chain estimated to be present in the affinity purified conjugate does not potentiate the cytotoxic activity of the (Fab′) fragments of RαHIg-A. Other results[17] have demonstrated that the (Fab′) fragments of RαHIg-A will remain on the cell surface for a period of up to five hours and that the GαRIg-B will potentiate the killing of the immunotoxin-A chain-coated cells during this time. Since (Fab′) fragments should not bind to Fc receptors on non-target tissue and should have a relatively short serum half-life, this approach may be feasible for *in vivo* use.

The subcellular events that are responsible for synergy between immunotoxin-A and immunotoxin-B are not known. One possibility is that a portion of the two immunotoxins that bind to the same target cell are endocytosed together and are present in the same endosome. Therein, interchain disulphide bonds may be split and free A and B chains released in the endocytic vesicle. The B chain would then facilitate translocation of the A chain into the cytoplasm with resultant cell death. These results represent a new strategy for utilising the potential toxic property of the A chain and the translocating ability of the B chain in a manner which retains the specific toxicity conferred by the antibody

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