required only 15 to 20 minutes to perform, representing a major advancement in the diagnosis of infections that previously required 3 to 6 days of culture to accomplish. In view of these advantages the continued development of techniques for the production and utilization of monoclonal antibodies should lead to great improvements in the quality of microbiological diagnosis.

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Immunotoxins: A New Approach to Cancer Therapy

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Approximately 75 years ago, Paul Ehrlich discussed the potential use of antibodies as carriers of pharmacologic agents (*1*). During the last decade, there has been considerable progress in the application of this concept to the elimination of cells that are reactive with antibodies coupled to toxic agents. In this article, we discuss work by ourselves and others concerning the elimination of normal and neoplastic target cells by conjugates containing antibody and toxin. We also present evidence that conjugates of toxin and antigen can induce specific immunologic unresponsiveness.

The term "immunotoxin" is used here to refer to a cell-binding antibody or antigen covalently bound to a plant or bacterial toxin. The toxin may be the whole molecule or a polypeptide portion carrying the toxic activity. Although much of our understanding of the mechanisms by which these toxins kill cells rests on studies of diphtheria toxin (*2*), the prevalence of diphtheria antitoxin in human populations renders this toxin unsuitable for clinical use. Therefore, most recent investigators have used ricin, a plant toxin. Like most toxic proteins produced by bacteria and plants, ricin has a toxic polypeptide (A chain) attached to a cell-binding polypeptide (B chain) (*3, 4*). The B chain is a lectin that binds to galactose-containing glycoproteins or glycolipids on the cell surface. By mechanisms that are not well understood, ricin A chain gains access to the cell cytoplasm. It is presumed, but has not been proved, that the route of entry is by receptor-mediated endocytosis (*5*) and that the A chain, which has a hydrophobic portion (*6*), penetrates the membrane of an endocytic vesicle or phagolysosome to enter the cytoplasm (*3*). By analogy with other toxins (*7*), it is possible that the B chain has a second function, namely, facilitating the translocation of the A chain through the membrane of the endocytic vesicle (*7–10*) by

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forming a pore in the membrane. In the cytoplasm, the A chain of ricin inhibits protein synthesis by enzymatically inactivating the EF2-binding portion of the 60*S* ribosomal subunit (*3*). A postulated model of this process is depicted in Fig. 1. Studies in vitro by Neville and Youle (*8*) and Thorpe and Ross (*9*) have established that different binding moieties (hormones, growth factor, or antibodies) can be substituted for the B chain to yield hybrid molecules in which the binding specificity is changed but the toxicity effected by the A chain is retained. Such immunotoxins are not as toxic as intact ricin (possibly because of the absence of the putative second function of the B chain) (*7–10*) but are nevertheless highly toxic compared to antibody or A chain alone. Since the A chain is nontoxic until it enters the cytoplasm, conjugates of antibody and A chain should be relatively nontoxic to nonphagocytic cells lacking the specific surface molecules to which the antibody is directed. In addition, the release of A chains from killed cells should not pose a significant problem for "bystander cells."

Some of the successful studies conducted in vitro with immunotoxins are summarized in Table 1 (*11–28*). Several generalizations can be gleaned from these investigations. (i) If antibodies are coupled to whole toxins that are galactose-binding lectins, such as ricin or abrin, then the presence of a high concentration of lactose (or galactose) will prevent lectin binding by the immunotoxin and thereby leave only its antibody specificity (*11–14, 23, 24, 27*). This approach is restricted to the deletion of target cells in vitro. Immunotoxins containing chemically derived A chains (ricin-A and abrin-A) (*15–22, 25, 26*) or naturally occurring A chains (gelonin) (*28*) do not require the use of galactose or lactose since the lectin binding chain is absent. (ii) Immunotoxins containing the antigen binding fragment F(ab)′ appear less toxic than those containing the same antibody in its $F(ab)'_2$ form (*20*). This finding indicates the importance of cross-linking and endocytosis in cell killing. (iii) Immunotoxins prepared with some monoclonal antibodies have poor toxicity (*7–9, 29*). This could be due to the inability of some antibodies to induce endocytosis because of their low binding affinity or because the target antigen is not readily taken up by endocytosis. Where internalization is slow or incomplete, the A chains in the endocytic vesicle may be below the concentration needed to traverse the membrane of the vesicle prior to degradation by enzymes in the phagolysosome.

These considerations suggest that improved efficacy of immunotoxins may depend on increasing both endocytosis of the conjugate and the ability of the A chain to traverse the membrane of the endocytic vesicle before inactivation by enzymes. Thus, toxicity can, in some cases, be improved by (i) more effectively cross-linking the immunotoxin with a second layer of antibody to facilitate endocytosis (*30*), (ii) using antibodies of higher affinity (*7*), (iii) incubating the immunotoxins for longer periods with the target cells (*7–8*), (iv) adding agents that raise the *p*H of the phagolysosome (for example, NH_4Cl) (*7*) and perhaps inactivating enzymes that degrade A chain (*17*), or (v) adding free B chains to the immunotoxin (*8–10*).

Summary. Conjugates of tumor-reactive antibody and toxins (immunotoxins) have been used to eradicate tumor cells in vitro and in vivo. Such immunotoxins are highly effective in killing murine leukemic cells in infiltrated bone marrow and should be useful in the bone marrow rescue approach for the treatment of cancer. Similar immunotoxins injected parentally can help to induce prolonged remissions in leukemic mice, and antigen-containing immunotoxins can induce immunologic unresponsiveness in vitro in normal murine splenocytes. Thus, long-range goals for the parental use of immunotoxins include the killing of cancer cells in vivo and the modulation of the immune response for therapeutic purposes.

Preparation of Immunotoxins

The strategy for preparing ricin A chain is to: (i) reduce the interchain disulfide bond (*4–6*), (ii) purify A chains by chromatography on DEAE-cellulose (*4–6*), and (iii) remove contaminating B chains (or ricin) by either lectin affinity chromatography (with the use of a galactose-containing solid matrix) or by antibody affinity chromatography (with Sepharose-coupled antibody to B chain in the presence of lactose) (*7, 30*). The purified A chains should show no evidence of contaminating B chains when analyzed by electrophoresis on sodium dodecyl sulfate gels. However, when tested for toxicity in mice, trace contamination with B chain or ricin can occasionally be detected. Prior to being coupled with antibody, the capacity of purified A chain to inhibit protein synthesis can be assessed in a cell-free translation system (*9*). Coupling of A chain to antibody can be achieved by any one of several chemical methods (*31*). The most common strategy is to use the heterobifunctional cross-linking agent *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP), which reacts with free amino groups on the antibody through the succinimide ester portion of the cross-linking agent (*7–9*). The PDP-derivatized antibody is then mixed at neutral *p*H with A chains to allow disulfide exchange. The resulting conjugates are separated from both free A chain and uncoupled antibody by a combination of gel filtration (*7–9*) and affinity chromatography with Sepharose bound to the antigen or Sepharose bound to an antibody to the A chain (*30*). The immunotoxins can be assayed for antibody activity and active A chain by radioimmunoassay (*32*) and by killing of the relevant target cells in vitro (*4, 7–10, 32*).

Testing the Efficacy of Immunotoxins

The murine BCL_1 model for studies in vitro. We chose the plant toxin ricin as the toxic agent for our studies because of its potency (one molecule in the cytoplasm of a cell will kill it) (*3*) and because ricin inhibits protein synthesis and can therefore kill nondividing cells (*4*). This latter attribute is important for successful therapy of human malignancies in which eradication of small metastatic foci containing nondividing cells may be critical. We have used a murine leukemia involving B cells bearing surface immunoglobulin (Ig) molecules to study in vitro and in vivo the effects of immunotoxins containing antibodies to Ig. Our choice of this model was based on the fact that B cell tumors are monoclonal (*33–38*) and that each clone synthesizes a distinct V_H V_L Ig (there are 10^6 or more clones of normal B cells in each mouse). The clone-specific antigenic determinants of a particular V_H V_L combination are called, collectively, the idiotype, and antibodies can be raised against them (anti-idiotypic) (*33–38*). Therefore, the surface Ig of each B cell tumor has a unique idiotype that can be viewed as a tumor-specific antigen. Hence, anti-idiotypic antibody represents an ideal targeting vehicle for guiding toxic agents to neoplastic B cells. The BCL_1 tumor arose spontaneously in an elderly BALB/c mouse (*39*), and healthy mice injected with as few as one to ten cells

from this tumor develop severe splenomegaly and leukemia (*32, 39*). In many respects the BCL_1 disease resembles the prolymphocytic variant of chronic lymphocytic leukemia in humans (*39–41*). Mice bearing the tumor normally survive for 3 to 4 months after receiving 10^5 to 10^6 tumor cells. The BCL_1 tumor cells bear Fc receptors, IgMλ, IgDλ, Ia antigens, and H-2 antigens (*39*). The cells lack complement receptors, suggesting they are analogs of immature B cells (*39*). This analogy is further supported by the functional properties of the BCL_1 cells (*42*).

In initial experiments, immunotoxins containing anti-idiotypic antibody were incubated with populations of BCL_1 tumor cells, cells from a different murine B cell tumor (CH1), or normal B cells (*16*). Protein synthesis was not inhibited in normal splenocytes or in a B cell tumor (CH1) bearing the same surface immunoglobulin isotype (IgMλ) as BCL_1 but a different idiotype. In contrast, anti-idiotype-containing immunotoxins decreased protein synthesis by 70 to 80 percent in spleen cell populations from BCL_1-bearing mice (70 to 80 percent of the cells in these spleens are of tumor origin). Control immunotoxins (containing irrelevant antibodies) had no effect on BCL_1 cells. These results indicate that the exquisite specificity of antibody is reflected in the specificity of killing the cells in vitro by antibody-containing immunotoxins.

Elimination of BCL_1 cells from bone marrow. To test further the precision of immunotoxin-mediated killing, we conducted similar experiments using an adoptive transfer system to assess the number of viable tumor cells remaining in the treated population. We had shown previously that by 12 weeks after the intravenous injection of ten BCL_1 cells, a tumor was detectable in virtually all recipient mice (*39–43*). About half of the mice injected with one BCL_1 cell had detectable leukemia at this time (*32, 43*). (This percentage might have been higher if technical maneuvers had ensured that a single cell was actually injected into each mouse.) Thus, experiments in vitro with spleens from BCL_1-bearing animals were performed as above. Immunotoxin-treated cells were transferred to normal recipient animals (10^4 cells per mouse) and the mice were observed for 12 weeks. Since none of the recipient mice had leukemia at 12 weeks, the results indicate that the immunotoxin could eliminate all tumor cells from a population of cells treated in vitro or, alternatively, that a host antitumor response was transferred with the tumor cell populations and was holding a small number of surviving tumor cells in check.

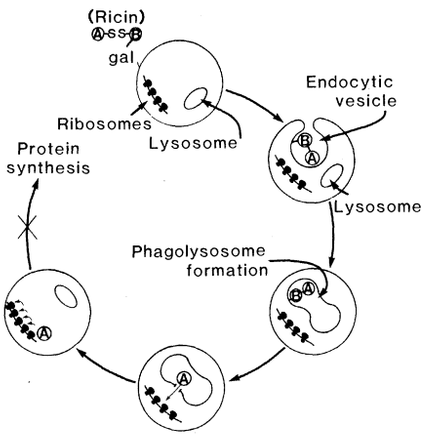

Fig. 1. A model for the cytotoxic action of ricin [based on data from (*3–10*)].

We performed similar studies on tumor-infiltrated bone marrow because of the clinical implications of removing tumor cells from marrow. Thus, a form of therapy for certain types of leukemias and other forms of cancer is the autologous bone marrow rescue approach (*44*). In this form of treatment, conventional chemotherapy is used to induce a remission in a patient bearing a tumor. During remission, a portion of the patient's bone marrow is removed and frozen. If the patient relapses, he or she receives supralethal therapy, that is, high doses of irradiation or chemotherapy in order to kill all tumor cells in the body. The result of this therapy, however, is the obliteration of the patient's own bone marrow. The patient is then "rescued" from the lethal effects of the therapy by reinfusion of his or her own bone marrow. Although the supralethal therapy is frequently sufficient to kill all tumor cells remaining in the individual, the reinfused bone marrow may contain small numbers of viable tumor cells that will then cause recurrence of the cancer. Our objective was to use a conjugate of tumor-reactive antibody–ricin A chain to destroy such tumor cells in a preparation of tumor-infiltrated bone marrow (*45*). Thus, bone marrow containing 15 percent BCL_1 cells was treated in vitro with anti-Ig immunotoxin and the treated cells were adoptively transferred to lethally irradiated animals. It is important to stress that the antibody used in these studies (antibody to Ig) was tumor reactive but not tumor specific. Thus, the only requirement for success in this approach was that the immunotoxin kill all the tumor cells but not the stem cells. The target antigens must be expressed on all the tumor cells but not the stem cells.

As shown in Fig. 2, the results indicate the following. (i) The hematopoietic system of all the animals was reconstituted, because all lethally irradiated mice survived after the administration of bone marrow cells. This finding together with earlier dosage experiments shows that no more than 10 percent of the bone marrow stem cells were damaged by the immunotoxins. (ii) Eighty-five percent of the animals (17 out of 20) treated with tumor-reactive immunotoxin did not develop tumors within 12 weeks although animals in all the control groups became leukemic (*45*). At 21 weeks after injection with treated cells, another mouse relapsed. At 25 weeks, spleen cells from the remaining mice were examined by analysis on the fluorescence activated cell sorter (FACS) for idiotype-positive cells. Although none were detected, spleen cells from one mouse caused leukemia in a second adoptive recipient. Thus, of the 20 mice receiving treated bone marrow, 5 out of 20 or 25 percent received marrow with at least one viable tumor cell. The one animal with late relapse and the other animal harboring "dormant" BCL_1 cells may have developed immunity to the BCL_1 tumor either from the administration of putative immune cells in the bone marrow itself or as a result of the challenge with the "killed" BCL_1 cells in the bone marrow inoculum. Of the three animals that relapsed within 12 weeks, all had idiotype positive cells that were susceptible to the lethal effect of Ig antibody-containing immunotoxins in vitro.

The simplest interpretation is that all malignant BCL_1 cells bear surface immunoglobulin (sIg). The use of higher concentrations of immunotoxin or different conditions for treatment might have resulted in complete elimination of tumor cells from the bone marrow. In addition, since many antigens on tumor cells (that are not stem cell malignancies) are not represented on stem cells, it might be possible to make a "cocktail" of monoclonal antibodies reactive with different antigens on the tumor cells to maximize the probability of complete killing. A similar approach in which cells are exposed to antibody-ricin conjugates in the presence of lactose has been used by others to delete tumor cells (*13*) from rodent bone marrow.

A different strategy for the treatment of cancer patients by means of bone marrow rescue is to transplant allogeneic marrow from which T cells have been eliminated (*46–51*). Deletion of T cells may avoid the life-threatening syndrome called graft versus host disease. Current methods for eliminating T cells from

bone marrow have met with some success, but they are technically limited. Such methods require the use of large amounts of complement; complement batches are difficult to standardize, and killing is frequently incomplete.

Vallera *et al.* (*52*) have recently used an anti-T cell–ricin immunotoxin to treat mouse bone marrow. The allogeneic recipients of such bone marrow did not develop graft versus host disease, indicating that the T cells had been efficiently eliminated and that the stem cells remained viable. Should this strategy work in humans it would obviate the necessity of using tumor-infiltrated autologous marrow and would also be applicable to diseases in which the bone marrow fails to generate cells (for example, aplastic anemia).

Therapy of BCL_1 in vivo

In these experiments (*43*) we used mice with massive tumor burdens (20 percent of body weight; approximately 10^{10} tumor cells). The rationale was to use experimental animals that would resemble most closely the clinical situations that would be faced initially with therapy in humans. Our strategy was to reduce the tumor burden by at least 95 percent by using nonspecific cytoreductive methods, and to eliminate remaining tumor cells with immunotoxins directed against either the idiotype or the δ chain of the sIgD on the BCL_1 cells. (The anti-idiotype would be the more specific of the two reagents.) The rationale for using

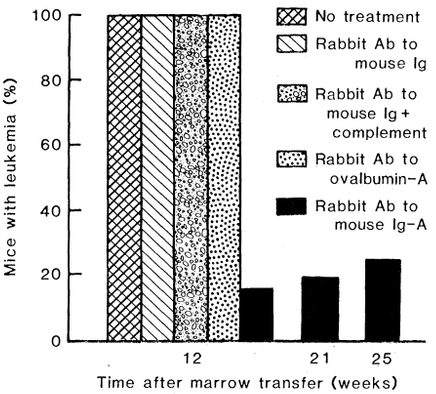

Fig. 2. Adoptive transfer into lethally irradiated recipients of BCL_1-containing bone marrow cells treated with rabbit antibody (*Ab*) to mouse Ig conjugated with A chain. Bone marrow cells containing 10 to 15 percent tumor cells were injected into groups of 20 mice at 10^6 marrow cells per mouse. Every 2 weeks after adoptive transfer the mice were examined for leukemia. 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 tumor in these recipients 10 weeks later. Thus, this mouse is scored as "leukemic" at 25 weeks.

antibody to δ is that sIgD is present on BCL_1 cells and on more than 50 percent of B cell tumors in humans. Antibody to δ, therefore, would represent a more practical reagent for clinical therapy. It was realized that treatment with anti-δ–A chain would eliminate virtually all virgin B cells, but it was reasoned that stem cells, pre-B cells, or immature IgD^- (IgM^+) cells all had the capacity to repopulate the virgin B cell compartment of the animals. Furthermore, since IgD is present in very low concentrations in the serum of both humans and mice, and since cytoreduction of BCL_1-bearing mice eliminates most mature B cells, the normal B cells and serum IgD would not represent major sources of competition for the anti-δ immunotoxins.

Nonspecific cytoreduction was accomplished with a combination of fractionated total lymphoid irradiation (TLI) (*53*) and splenectomy (*40, 54*). Animals receiving no treatment other than TLI and splenectomy were dead within 7 weeks. The injection of these cytoreduced mice with control immunotoxins did not prolong their survival. In contrast, animals receiving the anti-δ immunotoxin appeared tumor free as judged by the absence of detectable idiotype positive cells 12 to 18 weeks later (three of four such experiments). A successful experiment is depicted in Fig. 3. In one of the three successful experiments, 3×10^6 blood cells were transferred from animals in remission to normal animals and leukemia did not develop in the normal animals by 12 weeks, suggesting an absence of tumor cells in the blood of the donor animals. By 14 weeks, mice in remission had normal or above-normal levels of IgD-bearing normal lymphocytes (*43*). These results suggest that either the remaining tumor cells were eradicated in the animals that appeared tumor free, or the tumor cells remained in some organs but were held in check by a host resistance mechanism. However, such cells would have to be present in tissues other than blood,

Table 1. Immunotoxins used to kill target cells in vitro.

Toxin moiety	Antibody*			Target cell	Reference
	Heterologous	Monoclonal	Specificity		
Ricin (R)		+	Mouse T cells (Thy-1.2)	EL-4, WEHI-7	*11, 12*
		+	Rat T cells (W3/25)	T cells	*13*
		+	Mouse T cells (Thy-1.1)	T cells (AKR SL3)	*14*
R-A chain	+		DNP	TNP-HeLA	*15*
	+		Mouse μ chain	B cells	*16, 17*
	+		Mouse B cell leukemia (BCL_1) idiotype	BCL_1	*16*
	F(ab)′		Mouse B cell leukemia	L120	*18*
	F(ab)′, $F(ab)'_2$		Human Ig	Daudi	*19, 20*
		+	IgD allotypes	B cells	*16*
		+	Human colorectal cancer cells	SW1116, SW948	*21*
		+	Human leukemia (CALLA)	Nalm-1	*22*
Diphtheria toxin (DT)	+ and $F(ab)'_2$		Human lymphocytes	Daudi	*23, 24*
DT-A chain	+		Con A	3T3-ConA	*25*
	F(ab)′		Mouse B cell leukemia (L1210)	L1210	*26*
		+	Human colorectal cancer cells	SW1116	*21*
		+	Mouse T cells (Thy-1.2)	T cells	*17*
Abrin	+		Human lymphocytes	Daudi	*27*
Gelonin		+	Mouse T cells (Thy-1.1)	T cells (AKR-A, BW5147)	*28*

*Plus signs indicate that the antibody is intact.

because transfer of blood to normal recipients did not cause tumors.

In ongoing experiments with long-term survivors (25 to 30 weeks after immunotoxin treatment), tissues (liver, lung, kidney, bone marrow, and lymph nodes) adoptively transferred into normal mice did cause tumors 6 to 12 weeks later, suggesting that host resistance had developed. In the one experiment where the treated mice relapsed at 10 weeks, idiotype positive cells were detectable in the blood indicating that remission was prolonged but was incomplete. The partial success of these experiments was probably directly related to the fact that cytoreduction (TLI and splenectomy) was successful in reducing the number of remaining tumor cells to a level that could be effectively killed by a nonlethal dose of the immunotoxin. Moreover, it appears that the immunotoxins need not kill every tumor cell in vivo for prolonged remissions to occur, since a few remaining tumor cells may be permanently held in check by the immune system. Nevertheless, it was clear from preliminary experiments that inadequate cytoreduction (for example, TLI without splenectomy) left too many tumor cells to be effectively handled by such doses of immunotoxin. Thus, in considering the use of immunotoxins for treating human cancer, the tumor burden must be a major consideration—at least until the therapeutic index of immunotoxins can be significantly improved.

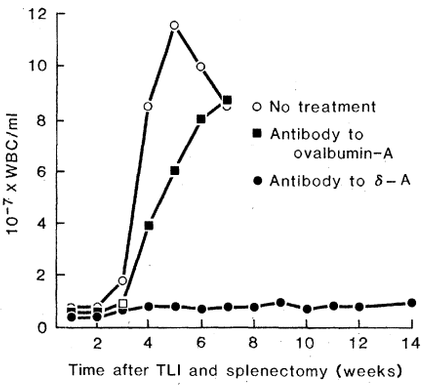

Fig. 3. Effect of total lymphoid irradiation (*TLI*), splenectomy, and administration of immunotoxin on leukemic relapse of BCL_1-bearing mice. After receiving nine doses of TLI and splenectomy, the mice were injected with two doses of 20 micrograms of antibody to δ or control immunotoxin (antibody to ovalbumin-A chain), or they were not injected. There were nine mice per group. Leukemic relapse was monitored by determining the number of white cells in the blood (*WBC*) of the treated mice. The control mice were all dead at 7 weeks after TLI (*43*).

Induction of Specific Immunologic Unresponsiveness

A potential problem in the repeated administration of immunotoxins in cancer patients is the generation of an antibody response to the injected immunotoxin (that is, antibody to the antibody or the A chain). The problem could arise in humans from the administration of rodent monoclonal antibodies or even human monoclonal antibodies as carriers of toxins; the latter could stimulate an anti-idiotypic response. Theoretically, an antibody to the immunotoxin should not develop because the B cells bearing antigen-binding determinants reactive with the antibody or A chain should be eliminated after binding the immunotoxin. Nevertheless, it was important to test this possibility directly and to develop a strategy for induction of specific immunological tolerance by means of immunotoxins.

In the first series of experiments along these lines, we attempted to eliminate a subset of B lymphocytes in vitro by a brief exposure to antigen-containing immunotoxin [that is, dinitrophenylated human serum albumin–A chain (DNP-HSA-A chain)]. We then tested the immune responsiveness of this treated cell population by transferring the cells to immunoincompetent irradiated recipients. The recipients were challenged with an immunogen [keyhole limpet hemocyanin (DNP-KLH)] containing the specific hapten to determine if DNP-specific cells had been eliminated from the injected cells and with sheep red blood cells (SRBC) as a control immunogen. Figure 4 shows the results of a representative experiment. The data indicate that the hapten antibody response of mice receiving cells treated with an irrelevant antigen-A chain (HSA-A chain) was unimpaired. However, the specific antigen-A chain (DNP-HSA-A chain) reduced the hapten antibody response by approximately 95 percent but did not effect the response to SRBC. Thus, specific immunologic unresponsiveness can be induced in vitro by such conjugates. The use of hapten as the antigenic determinant in these experiments suggests that B cells were rendered tolerant since it is known that B cells are specific for the hapten in a hapten-carrier conjugate (*55*). However, this tentative conclusion remains to be proved. It will also be important to determine the conditions for inducing unresponsiveness in vivo, the duration of such unresponsiveness, and, if it wanes, the effect of readministration of the specific immunotoxin. Similar studies recently reported by Volkman *et al.* (*56*) suggest that antigen-binding human B cells can be eliminated in vitro by using antigen bound to ricin in the presence of lactose.

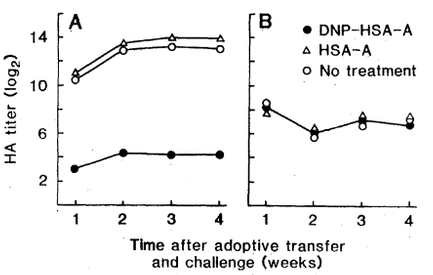

Fig. 4. Serum hemagglutination titer (*HA*) of irradiated mice injected with 10^7 cells and treated as indicated. Spleen cells were obtained from mice previously injected with DNP-keyhole limpet hemocyanin and sheep red blood cells (SRBC). The cells were treated for 15 minutes at 4°C with immunotoxins (50 μg per 10^6 cells), protein, or nothing, and 10^7 washed cells were injected into irradiated mice. These mice were injected with antigen 2 hours later and the hemagglutination titer of the serum was evaluated 1 to 4 weeks later. (A) Antibody to trinitrophenyl, (B) antibody to SRBC. Although not shown in the figure, incubation of cells with antigen alone had no effect on their subsequent responsiveness in vivo.

These results on the induction of tolerance with soluble antigen-containing immunotoxins have implications at the clinical level. The induction of immunologic unresponsiveness to specific antigens would be useful not only in cancer patients that are to receive immunotoxins containing tumor-reactive antibody but also in treating autoimmune diseases. For example, in the case of an autoimmune disease in which the antigen has been identified, it may be possible to delete the B cells responsive to this autoantigen by injecting autoantigen-A chain.

Other Possibilities for Modulating the Immune Response

There are additional possibilities for using immunotoxins to manipulate the immune response for therapeutic purposes. Immunotoxins directed against T suppressor cells (*57*), which constitute a minor subpopulation of T lymphocytes, should be effective in stimulating immune responsiveness when desirable, for example, tumor immunity or immune deficiency due to hyperreactivity of the T suppressor system. This maneuver might also be desirable for certain per-

sistent viral infections, such as herpes simplex, in which the objective might be to transiently eliminate suppression in order to stimulate the host antiviral response and thereby bring the viral infection under control. Conversely, immunotoxins directed against various inducer and helper T cells might be desirable for controlling autoimmune diseases in which there is a plethora of autoantibodies, for example, lupus erythematosus. Alternatively, one could delete cytokine-producer cells which sustain the growth of tumor cells or autoreactive cells. The use of this strategy will require a more complete understanding of the target cell specificity of these cytokines and the nature of the cells producing them.

Problems and Future Considerations

Pharmacokinetics. Serum half-life, tissue distribution, and toxicity of immunotoxins have not been investigated in depth. Preliminary studies by ourselves and others (*15*) indicate that immunotoxins containing normal Ig's have a relatively low toxicity. The organ systems that sustain significant tissue damage appear to be the intestine and the reticuloendothelial system (*15*), that is, macrophages in the liver and spleen that presumably remove the immunotoxin from the circulation. Since these cells can be replaced by stem cells, it is likely that this type of damage will be reversible. Indeed, past experimental results suggest that it is virtually impossible to exhaust the reticuloendothelial system because of replacement of macrophages from cells in the bone marrow (*58*). Surprisingly, immunotoxins do not appear to cause major damage in the kidney.

Studies of the metabolic half-life and tissue distribution of immunotoxins are also of a preliminary nature. A relatively short serum half-life for immunotoxins (30 minutes) has been reported (*15*). This may be due to the hydrophobicity of the A chain. This short half-life might be undesirable in that persistent serum titers of immunotoxin may be critical in permitting its access to tumor cells in sites where there is a blood-tissue barrier that takes time to penetrate. It may be important, therefore, to block uptake of immunotoxins by prior injection of macromolecules that temporarily cause RES blockade, for example, aggregated Ig.

Cross-reactive target antigens on normal tissues. Another major problem in predicting the efficacy of immunotoxins in any type of therapy in vivo is the possible representation of target antigens on normal tissues. For example, it is generally acknowledged that "tumor-specific antigens" are rare, if they exist at all. Most tumor-specific antigens are probably differentiation antigens that are expressed on subsets of cells in normal tissues of the same organ and, possibly, other organs. If such tissues are vital to survival, then it will be crucial to determine whether the unwanted tissue damage is acceptable. At present, there is insufficient information to answer this question.

Solid tumors. Another major issue is whether immunotoxins can gain access to cancer cells that form solid tumors, particularly those with a dense connective tissue component. Two possible approaches are to make the immunotoxin molecule smaller, that is, use an Fv or F(ab)′ fragment that contains only the combining site. This fragment might then be attached to an active fragment of the A chain. An alternative approach would be to bind a vasodilator, such as histamine, to the immunotoxin molecule to facilitate its penetration of the blood-tissue barriers.

Tumor cell heterogeneity. The problem of tumor cell heterogeneity, including the emergence of mutants lacking particular surface antigens or resistant to the toxic effects of ricin A chain, represents a major challenge to immunotoxin therapy. Here, the critical issue will be the therapeutic index of immunotoxins. If they have a high index, use of a combinatorial approach, namely, a mixture of immunotoxins directed against a variety of different cell-surface, tumor-associated antigens, might overcome this problem. If it is not possible to eradicate all tumor cells even by this combinatorial approach, a second approach may be to increase the host's immune response to the tumor by using immunotoxins (and other modalities) to provide the host with sufficient immunity to deal with the small number of tumor cells that may remain after administration of tumor antibody-containing immunotoxins.

Antigens in the circulation. Another obstacle to the treatment of cancer or induction of tolerance in autoimmune disease is the presence of tumor-associated antigens or autoantibody, respectively, in the circulation. These might have to be partially removed by plasmaphoresis or the injection of unconjugated antibody (or antigen in the case of autoimmune disease) before injecting the appropriate immunotoxin. However, it is not yet known whether low concentrations of such tumor-associated antigens necessarily represent a major problem. Thus, even under conditions in which immunotoxins exist primarily as serum antigen–antibody complexes, transient binding of a very small number of immunotoxin molecules to surface receptors can occur and may suffice for cell killing.

Conclusions

Immunotoxins represent a new approach to pharmacology. Rather than relying on the innate tissue "specificity" of pharmacologic agents, immunotoxins harness the exquisite specificity of antibodies (or antigens) to direct the pharmacologic agent to cells bearing a particular surface receptor. It is clear that considerable additional information of the chemistry, biology, and physiology of immunotoxins is essential to provide a firm foundation for designing regimens of immunotoxin therapy. With regard to treatment of cancer, the use of immunotoxins in vitro to eradicate either tumor cells from bone marrow or T cells from allogeneic marrow probably represents the initial approaches most likely to be helpful. In our judgment, the most rewarding future possibilities lie in the direction of modulation of the immune response. The findings that specific immunologic unresponsiveness can be induced in vitro with antigen-containing immunotoxins and that immunotoxins containing antibody to δ can eliminate sIgD positive lymphocytes in vivo underscores the potential of this approach to manipulate the immune response for therapeutic purposes.

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Protein Sequence Analysis: Automated Microsequencing

Michael W. Hunkapiller and Leroy E. Hood

Summary. The automated microsequencing of proteins can now be carried out at the 5- to 10-picomoles (submicrogram) level on polypeptides obtained directly from one- and two-dimensional gel electrophoresis. The techniques are applicable to polypeptides ranging in size from small peptides (less than 10 residues) to large proteins (more than 1000 residues).

Since its introduction 25 years ago, the Edman degradation has been the most widely used method for the direct determination of the primary structure of proteins and peptides (*1*). However, the introduction of rapid, simple methods of DNA sequencing, by which protein sequences are obtained indirectly, has raised questions about the utility of protein sequencing. Several years ago in one leading scientific journal, an editorial appeared under the title *The Decline and Fall of Protein Chemistry* (*2*). However, the advent of modern micromethods requiring as little as a few picomoles of proteins and peptides for sequence analysis has firmly established the importance of protein sequencing as a tool for biochemistry and molecular biology.

Chemistry

The Edman chemistry is shown in Fig. 1. One cycle, which results in removal of one amino acid from the amino-terminal end of a peptide and generation of a new peptide that is one amino acid shorter, consists of two separate chemical steps. In the first (coupling), phenyl isothiocyanate is coupled under basic conditions to the amino end of the peptide to form a phenylthiocarbamyl peptide. In the second (cleavage), treatment with a strong, anhydrous acid removes the derivatized amino acid as its anilinothiazolinone. The latter is usually converted in a third reaction (conversion) into the more stable phenylthiohydantoin (Pth) for subsequent analysis.

Repetition of this sequence, in theory, allows one to proceed from the amino-terminal to carboxyl-terminal end of a protein to define its primary structure. In practice, side reactions, incomplete reactions, and loss of sample usually limit successful degradations in a single run to 30 to 70 cycles. The sequence of the remainder of the protein is determined after fragmentation by chemical or enzymic methods (or both) to generate a set of overlapping peptides that are individually analyzed by the Edman chemistry.

Instrumentation

Manual. The Edman degradation was originally developed as a manual method. Despite the subsequent popularity and success of automated methods, there remain many applications where the manual approach is suitable, particularly when there is need for rather limited sequence information and there is no access to automated instruments. Improvements in technique (*3*) and chemistry (*4*) make it particularly attractive for initial screening of a large number of small- to medium-sized peptides to select those most suitable for further, extensive sequence analysis by automated methods. Experienced protein chemists can

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