Table 1. Essential features of the chicken B cell compartment.

1	Extensive cellular proliferation during bursal development
2	All specificities present at a very early stage
3	Involution of the bursa in the adult
4	Self-renewing capacity of differentiated cells
5	Persistence of expanded clones at the periphery
6	Lower adaptability of the system (compared with the mouse)

and followed by bursectomy, recovery is extremely slow, whereas in the unoperated animal it starts after 5 to 8 weeks (*30*). These observations imply that peripheral stem cells may not be able to generate somatic variants in a fashion comparable to their bursal equivalent, thus imposing on the system a very low adaptive capacity. When the main characteristics of the chicken B cell compartment are aligned, they clearly evoke the properties of the mouse thymic population (Table 1). However, it has been shown that new migrants from the adult mouse thymus still have considerable expansion capacity, implying that this system remains in a dynamic state during the life of the animal (*29*).

How the chicken B cell system can provide the overall immune surveillance and adaptability necessary throughout the life of the animal remains paradoxical. It would be essential to have more data on the diversity of this B cell repertoire particularly at the heavy chain level.

Immune systems seem to have arisen in primitive species from a cellular recognition device mediating self/non-self discrimination (*31*). One may envisage primitive B cell systems evolving from this preexisting cellular compartment. The chicken B cell population may in some ways provide a picture of this ancient event. It will obviously be important to understand the properties of B cell lineages below the avian species to obtain a clearer picture of this evolution.

REFERENCES AND NOTES

1. F. W. Alt, T. K. Blackwell, R. A. DePinho, M. G. Reth, G. D. Yancopoulos, *Immunol. Rev.* **89**, 5 (1986).
2. K. R. Hinds and G. W. Litman, *Nature (London)* **320**, 546 (1986); M. F. Flagnik, E. Hsu, J. K. Kaufman, L. Du Pasquier, *Immunol. Today* **8**, 58 (1987).
3. B. Glick, T. S. Chang, R. G. Jaap, *Poult. Sci.* **35**, 224 (1956); A. P. Mueller, H. R. Wolfe, R. K. Meyer, *J. Immunol.* **83**, 507 (1959); M. D. Cooper, W. A. Cain, P. J. Van Alten, R. A. Good, *Int. Arch. Allergy Appl. Immunol.* **35**, 242 (1969); N. L. Warner, J. W. Uhr, G. J. Thorbecke, Z. Ovary, *J. Immunol.* **103**, 1317 (1969).
4. M. A. S. Moore and J. J. T. Owen, *Nature (London)* **208**, 956 (1965); M. A. S. Moore and J. J. T. Owen, *Dev. Biol.* **14**, 40 (1966); N. M. Le Douarin, E. Houssaint, F. V. Jotereau, M. Belo, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2701 (1975).
5. E. Houssaint, M. Belo, N. M. Le Douarin, *Dev. Biol.* **53**, 250 (1976).
6. P. Toivanen and A. Toivanen, *Eur. J. Immunol.* **3**, 585 (1973); J. R. L. Pink, *Immunol. Rev.* **91**, 115 (1986).
7. J. A. Grant, B. Sanders, L. Hood, *Biochemistry* **10**, 3123 (1971); C.-A. Reynaud, A. Dahan, J.-C. Weill, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4099 (1983).
8. C.-A. Reynaud, V. Anquez, A. Dahan, J.-C. Weill, *Cell* **40**, 283 (1985).
9. C.-A. Reynaud, V. Anquez, H. Grimal, J.-C. Weill, *ibid.* **48**, 379 (1987).
10. J.-C. Weill, C.-A. Reynaud, O. Lassila, J. R. L. Pink, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 3336 (1986).
11. J. R. L. Pink, O. Vainio, A. M. Rijnbeek, *Eur. J. Immunol.* **15**, 83 (1985).
12. M. J. H. Ratcliffe, O. Lassila, J. R. L. Pink, O. Vainio, *Eur. J. Immunol.* **16**, 129 (1986).
13. M. J. H. Ratcliffe and J. Ivanyi, *Eur. J. Immunol.* **11**, 306 (1981).
14. J. R. L. Pink, M. J. H. Ratcliffe, O. Vainio, *Eur. J. Immunol.* **15**, 617 (1985).
15. M. G. Reth, S. Jackson, F. W. Alt, *EMBO J.* **5**, 2131 (1986); this is also true for the T cell receptor [K. Okazaki, D. D. Davies, H. Sakano, *Cell* **49**, 477 (1987)].
16. P. Lydyard, C. E. Grossi, M. D. Cooper, *J. Exp. Med.* **144**, 79 (1976).
17. S. Kim, M. Davis, E. Sinn, P. Patten, L. Hood, *Cell* **27**, 573 (1981).
18. C. B. Thompson and P. E. Neiman, *ibid.* **48**, 369 (1987).
19. M. Pech, J. Höchtl, H. Schnell, H. G. Zachau, *Nature (London)* **291**, 668 (1981).
20. A. Dahan, unpublished data.
21. D. Bucchini *et al.*, *Nature (London)* **326**, 409 (1987).
22. J. R. L. Pink, O. Lassila, O. Vainio, in *Avian Immunology*, A. Toivanen and P. Toivanen, Eds. (CRC Press, Boca Raton, FL, 1987), p. 65.
23. R. Woods and J. Linna, *Acta Pathol. Microbiol. Scand.* **64**, 470 (1965); O. Lassila, in preparation.
24. P. T. Jones, P. H. Dear, J. Foote, M. S. Neuberger, G. Winter, *Nature (London)* **321**, 522 (1986).
25. A. G. Amit, R. A. Mariuzza, S. E. V. Phillips, R. J. Poljak, *ibid.* **313**, 156 (1985).
26. A. Sette *et al.*, *ibid.* **328**, 395 (1987); H. M. Geysen, *Immunol. Today* **6**, 364 (1985).
27. M. Z. Atassi, W. Zablocki, *J. Biol. Chem.* **252**, 8784 (1977); M. Z. Atassi, *Immunochemistry* **15**, 909 (1978).
28. A. A. Fretas, B. Rocha, A. A. Coutinho, *Immunol. Rev.* **91**, 5 (1986).
29. B. B. Rocha, *J. Immunol.* **139**, 365 (1987).
30. J. Ivanyi and A. Salerno, *Immunology* **22**, 247 (1972); T. Hraba, I. Karakoz, J. Madar, *Folia Biol. (Prague)* **23**, (1977).
31. M. Cohn, *Cell* **33**, 657 (1983); R. K. Grosberg and J. F. Quinn, *Nature (London)* **322**, 456 (1986).

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Redesigning Nature's Poisons to Create Anti-Tumor Reagents

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Immunotoxins are conjugates of cell-reactive antibodies and toxins or their subunits. In this report, the chemistry, biology, pharmacokinetics, and anti-tumor effects of first generation immunotoxins; the preparation of improved second generation immunotoxins that display greater anti-tumor efficacy; and the role of genetic engineering in creating third-generation immunotoxins are discussed.

THE MOST REMARKABLE FEATURE OF THE MAMMALIAN IMmune system is the virtually unlimited repertoire of antibody molecules of different specificities that can be generated by a single individual. The advent of monoclonal antibody technology (*1*) has made it possible to "tap" this repertoire by immortalizing single B cells. The resultant hybridoma cells produce large amounts of homogeneous antibody of a single desired specificity, such as

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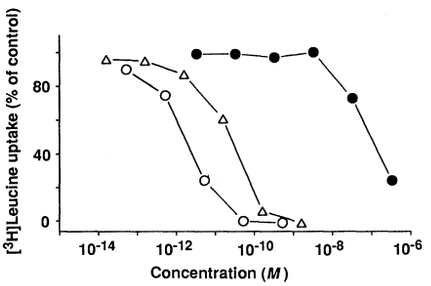

Fig. 1. Toxicity of an IT-A (anti-Thy-1.1), ricin, or A chain on a murine Thy-1.1–bearing thymoma (AKR-A) cultured for 48 hours with the reagents indicated. [^{3}H]leucine incorporation was determined during the last 24 hours of culture. Controls consisted of untreated cells cultured for the same period of time. The IC_{50} is the concentration that inhibits protein synthesis by 50% [adapted from Blakey *et al.* (*17*)]. ○, IT-A ($IC_{50} = 9 \times 10^{-13}$); △, ricin ($IC_{50} = 4 \times 10^{-11}$; ●, A chain ($IC_{50} = 9 \times 10^{-8}$).

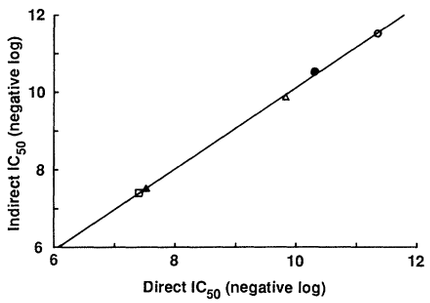

Fig. 2. A comparison of the direct and indirect killing assays. A human neoplastic B lymphocyte line (Daudi) bearing the CD22 antigen was cultured either (i) in the indirect assay with various antibodies followed by Fab-goat anti–mouse Ig–A chain; or (ii) in the direct assay using IT-As prepared with the same antibodies. Three different monoclonal anti-CD22 antibodies (H-1, H-2, and H-3) and two controls (anti–Thy-1.1 or MOPC-21) were used (*33*). ○, anti-CD22 (H-1); ●, anti-CD22 (H-2); △, anti-CD22 (H-3); ▲, anti–Thy-1.1; □, MOPC-21.

those directed against determinants on microorganisms, neoplastic cells, virally infected cells, or subsets of normal cells. Such antibodies can be used as carriers of pharmacologic agents, such as toxins, and thereby provide an elegant strategy for creating cell-specific cytotoxic agents (*2*).

Toxins, like antibodies, are products of nature usually produced by bacteria and plants (*3*). The concept of utilizing antibody-toxin conjugates, or "immunotoxins" (ITs) for targeting is simple, but the development of effective clinical reagents and regimens is complex and encompasses the fields of biochemistry, cell biology, immunology, pharmacology, oncology, and molecular biology.

Structure and Function of Toxins

The toxins produced by plants show remarkable conservation in structure and function despite large phylogenetic differences (*3*). All the plant toxins that inhibit protein synthesis are disulfide-bonded heterodimers. The binding chain (B) is usually a galactose-specific lectin of approximately 30 kD; the toxic chain (A) is an enzyme of the same size that catalytically inactivates the 60*S* ribosomal subunit of eukaryotic cells by modifying one or two nucleoside residues of 28*S* ribosomal RNA (*4*). In the case of ricin, produced by the beans of the plant *Ricinus communis*, both chains have oligosaccharide moieties containing a high content of mannose and the A chain contains fucose (*5*). A subdomain of the B chain of ricin shows homology with a bacterial galactose-binding protein (*6*), indicating that the primordial gene is over 2 billion years old.

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The sequence of intracellular events underlying toxin-mediated killing of eukaryotic cells is partially understood. After ricin binds to the cell, the complex of toxin and the cell surface glycoprotein or glycolipid to which it is bound is endocytosed, the disulfide bond between the A and B chains is reduced, and the A chain translocates across an endocytic membrane to gain access to the cytosol (*3*). A single molecule of A chain in the cytosol may be sufficient to kill a cell (*7*). The precise events involved in membrane translocation of A chains are not understood, but it is likely that a stretch of 25 hydrophobic amino acids in the middle of the molecule is responsible for penetration into the lipid bilayer. There is evidence to indicate that B chains can markedly facilitate this translocation (*8*) and, by analogy with diphtheria toxin (*9*), one could postulate that the B chains of ricin aggregate and form a pore in the membrane through which the A chain traverses.

Other toxins (and their A chains) that have been studied include bacterial toxins (for example, diphtheria toxin) and ribosome-inactivating proteins (RIPs) produced by plants (for example, gelonin). RIPs are single A chains that lack B chain equivalents and, in vitro, are not toxic unless they are targeted to cells by ligands (*10*).

Construction of Immunotoxins

This review will focus on ITs prepared with ricin (IT-Rs) or its A chain (IT-As) because many studies, including our own, have used these components and because the results obtained are, in many respects, prototypic of those obtained with other toxins, their A subunits, or RIPs. Ricin A chain is usually coupled to a cell-reactive antibody by the cross-linker, *N*-succinimidyl-3-(2-pyridydithio)propionate (SPDP) (*11*), which contains a disulfide bond. If the disulfide bond is replaced by a more stable one, such as a thioether, cytotoxicity is markedly reduced (*12*). By analogy with ricin, it is presumed that the disulfide bond between A chain and antibody must be cleaved before the A chain translocates into the cytoplasm.

There have been two recent technical advances in the preparation of IT-As. (i) Pure A chains have been prepared biochemically (*13*) or by recombinant DNA technology (*14*). Such A chains, although retaining their full ribosome-inactivating function, are nontoxic to cells in vitro at concentrations that are typically 10^5- to 10^6-fold higher than ricin itself [A chain cannot bind to cells effectively without B chain (*13*)]. "Purified" A chains used in earlier studies were frequently contaminated by trace amounts of B chains resulting in inaccurate estimations of both cytotoxicity to target cells and toxicity to animals. (ii) "Free" antibody can now be readily removed from preparations of IT-As by affinity chromatography on either Sepharose conjugated with monoclonal antibodies to the A chain (anti–A chain Sepharose) (*15*) or Blue Sepharose (*16*). Contamination of ITs with free antibody in past studies may have reduced their potency in vivo due to the fact that free antibody has a much longer blood half-life than the IT (*17*) and, therefore, with increasing time after administration, it becomes a major competitor for binding sites on target cells.

IT-Rs are generally formed by introducing thiol groups into the antibody and an alkylating function into the toxin. The mixture of the two produces an IT with a thioether linkage (*18*). The A and B chains of the toxin remain disulfide-bonded and this bond can be cleaved intracellularly, thereby preserving cytotoxicity.

IT-Rs are highly cytotoxic, but are only specific for the target cells when they are used in the presence of galactose or lactose to block the lectin activity of the B chain (*18*). In contrast, IT-As have exquisite specificity, but show unpredictable cytotoxicity because of the absence of B chain–mediated potentiation of A chain translocation (*2, 8*). Thus, some show little toxicity; however, others can be

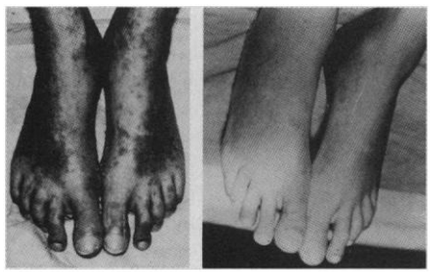

Fig. 3. The skin of a patient with acute graft versus host disease before (left) and after (right) 14 daily infusions of a T cell–reactive anti–CD5-A chain (XomaZyme®-H65). This figure was kindly provided by R. Gingrich (University of Iowa) and V. Byers (Xoma Corp., Berkeley, California).

as toxic as ricin itself (Fig. 1). Some investigators have stressed the relative ineffectiveness of IT-As compared to IT-Rs (*19*) and concluded that IT-As are poor candidates for in vivo use. This viewpoint does not take into account the fact that IT-As are 10^4-fold less lethal to mice than IT-Rs, and that some IT-As are highly potent (Fig. 1). Hence, a large dose of a potent IT-A should show considerable efficacy in killing target cells in vivo. (Efficacy is the maximal therapeutic effect of a drug.) In contrast to IT-As, IT-Rs must be modified to reduce their toxicity to animals before they can be expected to display similar efficacy in vivo.

Efficacy of IT-Rs

Because of their potency, IT-Rs have found their greatest use in vitro where the nonspecific lectin activity of the B chain can be blocked by the addition of free sugars as mentioned above. IT-Rs have proven highly effective at killing T cells in the bone marrow of rodents and humans prior to transplantation for the treatment of malignancy and immunodeficiency diseases (*20*). In Minneapolis, 26 patients have been transplanted with T cell–depleted bone marrow (*21*) and the usually fatal complication of donor T cell–induced graft versus host disease was markedly inhibited. Paradoxically, the anti–T cell ITs may have been too effective at T cell depletion, since recent studies suggest that some of the resulting graft failures (*21*) may be due to killing subsets of donor T cells that suppress radioresistant recipient T cells that cause the rejection. At present, IT-Rs appear to be prohibitively toxic for systemic administration, although in one report, they were efficacious in treating an experimental intraperitoneal tumor when injected into the same site (*22*). In addition, IT-Rs in which the lectin sites are sterically hindered by the coupled antibody have been generated (*23*). These "blocked" IT-Rs are highly and specifically toxic to target cells in vitro and show moderate reduction of nonspecific toxicity in vivo.

In Vitro Potency of First-Generation IT-As

We define first-generation IT-As as intact antibodies coupled via SPDP to native A chain. As mentioned above, they are prime candidates for clinical use. It is important, therefore, to consider the factors that account for the variability in cytotoxicity of IT-As. Perhaps the most critical factors are the cell surface antigen to which the targeting antibody is directed and its resultant pathway of internalization after binding to the IT-A (*24*). There is evidence to suggest that IT-As specific for target antigens that are routed via endosomes to the Golgi complex are effective (*25*). In contrast, IT-As specific for antigens that are routed to the lysosomes are usually less effective, presumably because the A chain is rapidly degraded. Another important factor is the epitope on the target antigen that is recognized by the antibody (*26*). One could speculate that such an epitope must be sufficiently near the endocytic membrane to allow the hydrophobic segment of the A chain to penetrate the membrane (*27*) and thereby initiate the cascade of events resulting in translocation. Generally, the antibodies of highest affinity (*28*) that are directed against critical epitopes on a suitable target molecule appear to be the most effective carriers of A chain. Nevertheless, different cells carrying the same antigenic determinant may vary in their susceptibility to IT-As (*28, 29*). In some cases, this is dependent on the density of the specific antigen on the target cell (*30*). However, cell types expressing a similar density of a given target antigen can vary markedly in their susceptibility to ITs for reasons that are not understood (*28, 29*). For example, with the murine B cell tumor (BCL_1), IT-As directed against sIgD are highly effective, even though the cells express barely detectable levels of sIgD. Finally, cells in G_0 may be less susceptible to killing by IT-As than dividing cells (*31*). Thus, when protein turnover is slow and the half-life of proteins necessary for cell survival is long, ITs may be less toxic to the target cells. However, such cells may endocytose the IT so that when activation occurs and an increased rate of protein synthesis is necessary to maintain viability, the cells die.

Since IT-As have variable potency as cytotoxic agents, it was essential to develop a simple screening method to circumvent the laborious task of large-scale production and purification of monoclonal antibodies, coupling to A chain, purification of the IT, and in vitro testing on target cells. Such a method was first described by Weltman *et al.* (*32*), and developed further into a highly sensitive assay by Till, *et al.* (*33*). This method involves treating the target cells with dilutions of the tissue culture supernatant or with ascites containing the monoclonal antibody. Treated cells are then cultured with anti–mouse Ig–A chain. Till *et al.* (*33*) used the Fab fragment of anti–mouse Ig–A chain to treat the cultured cells in order to avoid cross-linking the bound test antibody by the secondary antibody and, hence, possibly rerouting the primary antibody to a different intracellular site. By testing a panel of eight cell lines (or fresh cells) and 17 antibodies, it was found that the correlation in potency between this "indirect" killing assay and a direct killing assay (utilizing the primary antibody as an IT-A) was virtually 100%. Thus, it is possible to rapidly and accurately predict which antibodies will make effective ITs (Fig. 2).

Pharmacokinetics and Toxicity

Numerous studies in which ITs have been injected into normal animals have demonstrated that the majority of the IT is cleared from the blood with an initial rapid phase [α phase; half-life ($T_{1/2}$) = several minutes to hours] (*17*). The short $T_{1/2}$ represents rapid uptake of the IT-A by Kupffer cells in the liver and equilibration with the extravascular compartment. Subsequently, a slower phase is observed (β phase; $T_{1/2}$ = several hours to several days). This is due to removal of residual IT-A by the reticuloendothelial system (RES), continued slow equilibration with the extravascular compartment, and progressive splitting of the linker between the A chain and antibody (*17*).

The above conclusions have been drawn largely from pharmacokinetic studies in normal animals and can be misleading when applied to animals (or humans) bearing a large tumor burden. In such cases, tumor-reactive ITs are even more rapidly removed because the tumor acts as an antigenic sink.

A quantitative expression of the efficacy of a particular IT is its therapeutic index; namely, the ratio of the dose that results in toxicity to the host to the dose that displays therapeutic efficacy [for example, the dose that causes death in 50% of mice (LD_{50}) divided by the dose that causes tumor remission in 50% of mice]. At present, the pathophysiological mechanisms that cause death from

ricin intoxication are not known. There is a prevailing concept that hepatic damage might cause death in patients treated with IT-As because ricin causes hepatotoxicity in rodents (*34*). For reasons to be discussed below, an alternative interpretation is that a life-supporting tissue other than the liver is the critical organ (or organs) that determines survival. This organ could be the vascular endothelium or the heart.

In Vivo Anti-Tumor Effects of First-Generation IT-As

Jansen and his co-workers reported the first successful use of an IT-A (ricin) in vivo (*35*). However, since both the tumor and the IT were injected within hours of each other into the same body cavity, the experiments did not test the potential of systemic IT therapy for disseminated neoplastic disease. Subsequently, Krolick *et al.* (*36*) treated mice carrying an advanced B cell tumor, BCL_1 (20% of body weight), with total lymphoid irradiation and splenectomy prior to the administration of an IT-A directed against either the BCL_1 idiotype or the δ chain of sIgD. Both the cytoreductive therapy and the specific IT-A therapy were essential in rendering the animals disease-free for periods of observation as long as 8 months. Nevertheless, tissues from such animals (obtained after they were killed) adoptively transferred tumor to normal mice, indicating that the treated animals, although clinically "cured," harbored dormant tumor cells (*37*). These studies indicate that with some tumors, it may not be necessary to eliminate *every* tumor cell to achieve a prolonged remission or cure and that the immune response of the host, acting in concert with IT therapy, may be critical for inducing and maintaining remission.

Treatment of solid tumors with IT-As in animal models has met with some success (*38*). Successful studies have utilized nude mice bearing human tumors. The peculiar growth characteristics of such tumors and the frequently used procedure of intratumor administration of ITs, raise concerns about the relevance of these studies to the treatment of metastatic tumors, the major problem of human cancer.

In Vivo Clinical Trials

A clinical trial in which ricin or abrin was used in patients with cancer was reported (*39*), but only one patient showed a convincing therapeutic effect with these untargeted poisons. Nevertheless, patients showed relatively little symptomatology; that is, a flu-like syndrome that lasted 1 to 2 days at maximally tolerated doses. Furthermore, these doses of ricin did not result in serious hepatic damage. Maximally tolerated doses of ricin were predictable from prior studies in mice which indicate the usefulness of animal models in predicting toxicity in humans.

Several clinical trials with first-generation IT-As have been performed. The major one involved a phase II clinical trial of 46 patients with advanced metastatic melanoma (*40*). There was one complete remission and three partial responses. Side effects were modest and reversible; these included allergic reactions and hypoalbuminemia resulting in edema. In another study, patients with T or B cell leukemia treated with an IT-A showed a very transient decrease in leukemia despite the demonstration that IT-A had saturated the circulating leukemic cells (*41*). In contrast, IT-A therapy was efficacious in far-advanced, steroid-resistant graft-versus-host disease, a complication of allogeneic histocompatibility antigen (HLA)–matched bone marrow transplantation that is fatal in many patients. Thus, 12 of 15 patients showed significant reduction in their disease in at least one organ (Fig. 3) at 7 days (*42*); at 28 to 40 days, 3 of 10 patients were classified as complete responders. In our view, the factors leading to greater success of this clinical trial, as compared to the melanoma trial, were predictable: (i) the target cells were readily accessible to the circulation; (ii) the T cells were probably highly susceptible to the IT because they were activated by host antigens and were rapidly dividing, thereby requiring protein synthesis; and (iii) alteration of the immune response is probably more readily accomplished than eradication of malignancy.

Problems of in Vivo Therapy and Development of Second-Generation ITs

IT trials in animals and humans have highlighted a number of issues. The accessibility of the target cell to the circulation has been mentioned above. One possible solution is the use of smaller IT constructs such as those prepared with Fab′ fragments of antibody (Fab′-A). It has also been suggested that lower affinity antibodies may be more effective in vivo in percolating through a tumor mass to reach cells in its center because the ITs are less likely to be irreversibly bound by the more accessible cells in the periphery of the tumor (*43*). Fab′-IT-As have reduced avidity because of their univalency.

A second problem is instability of the linker used to conjugate the ligand to the A chain (*17, 44*). New linkers have been synthesized that generate "hindered" disulfide groups that are not readily cleaved by thiols or other reducing agents in the blood or tissues, but are effectively cleaved in the target tissue (*45*). It is also possible, with appropriate chemistry, to couple the A chain directly (without a spacer) to the cysteine of an Fab′ fragment (*46*) that had been used to form the inter–H chain disulfide bond in the intact IgG. The resultant disulfide bond should be protected by the protein components, and hence more stable.

Another key problem noted above is that the liver rapidly removes IT-As from the circulation primarily because the Kupffer cells express receptors for the mannose-rich carbohydrates of the A chain. Removal of IT-As by these cells prevents them from reaching the target cells. The solution to this problem has been addressed in several ways. Thorpe, Foxwell, and co-workers have chemically destroyed or enzymatically removed the mannose (and fucose) residues in the A chain (*5, 47*) and have shown that ITs prepared with deglycosylated A chain (dgA) show greatly reduced liver uptake resulting in an improved anti-tumor effect (*17*). Thus, IT-dgAs are excellent candidates for second-generation ITs. Several investigators have infused mannose-containing molecules with an IT-A to compete for mannose binding of the IT-A by the RES (*17*). These maneuvers were effective in vivo. Finally, recombinant A chain lacking carbohydrate moieties has been expressed in *Escherichia coli* (*14, 22*).

Another problem is the development of an antibody response against either the A chain or the monoclonal antibody of the IT-A (*48*). Such antibody neutralizes IT-A either directly or by increasing markedly its rate of removal from the blood. Antibody responses against IT-As may be a major obstacle for their use in cancer therapy, in which multiple courses of treatment with conventional chemotherapeutic agents have been essential to achieve cures. It is important, therefore, to explore maneuvers such as the use of immunosuppressive drugs to prevent this immune response. Another potential solution would be to use different types of ITs directed against the same antigen for multiple courses of treatment. For example, the A chain of ricin could be used initially and a non-cross-reactive A chain of another plant toxin could be used subsequently. In addition, another monoclonal antibody from a different species

directed against the same antigen would prevent interference of the action of a second IT-A by anti-idiotypic or anti-isotypic antibodies directed against the first IT-A.

Pharmacokinetics and in Vivo Efficacy of Second-Generation IT-As

Recent pharmacokinetic studies have focused on two ITs that utilize the dgA chain and that have more stable linkers, namely, Fab'-dgA (*49*) and IgG-SMPT-dgA [SMPT, 4-succinimidyloxycarbonyl-α-methyl-α(e-pyridyldithio)toluene] (*50*). The major points to emerge are that both ITs have shown markedly greater stability in the blood compared to IgG-SPDP-ITs, as predicted; neither is targeted rapidly to the liver, in contrast to the same ITs prepared with native A chains. In BCL_1-bearing mice, Fab'-dgA was more effective than IgG-SPDP-dgA or Fab'-A at localizing to tumor cells in the spleen (*51*). This is probably because of its smaller size or lower avidity (or both) and the lack of liver targeting. Finally, 90% of the ITs are removed from the blood in BCL_1-bearing mice by 1 hour. However, free antibody that has dissociated from the IgG-SPDP-ITs is not detected until about 6 hours after injection. Hence, instability of linkage between antibody and A chain may be irrelevant to the treatment of neoplasms that are readily accessible to the systemic circulation. Linkage stability might be important, however, for treatment of solid tumors with a small blood supply or those tumors whose malignant stem cells are not readily accessible to the blood. Moreover, for such tumors, it may also be important to have a relatively long $T_{1/2}$ in the circulation and maximal target cell toxicity. This would favor the use of IgG-SMPT-dgA instead of Fab'-dgA.

Anti-δ Fab'-dgA has been administered to BCL_1-bearing animals in a model that allows quantification of killing in the tumor-bearing spleen. There was an impressive anti-tumor effect observed over the first 24 hours where as many as 10^9 cells were killed (Fig. 4) (*49*). However, about 1 to 10% of viable tumor cells remained in the spleen. This may be due to some cells lacking sufficient concentrations of sIgD, other intracellular resistance mechanisms or anatomical barriers.

Fab'-anti-δ-dgA is approximately two- to threefold more toxic to mice than Fab'-anti-δ-A as determined by LD_{50}'s. The greater toxicity of IT-dgAs that do not home readily to the liver supports our hypothesis that damage to nonhepatic tissues is the cause of death from IT-A and probably ricin itself. However, the in vivo ID_{50} (the dose that kills 50% of tumor cells) of Fab'-anti-δ-dgA is fivefold lower (Fig. 5). Hence, Fab'-dgA gives a two- to threefold increase in the therapeutic index.

Particular Problems in Cancer Therapy

There are additional obstacles in using ITs as anti-tumor reagents:

1) Is the IT specific for the neoplastic renewal cell? As exemplified by hematopoietic malignancies, the malignant stem cells (that is, the malignant cells that can renew themselves to perpetuate the disease) can differentiate further to give rise to cells that do not divide, but constitute the bulk of cells seen in the disease. A prime example is chronic lymphocytic leukemia (CLL) in which the neoplastic lymphocytes are not dividing, indicating that the renewal cell is a more immature cell (*52*). The primary objective of IT therapy is to direct the IT against antigens on the renewal cell to eradicate the malignancy. However, this cell has not yet been identified and immunophenotyped in CLL or in the majority of malignant diseases. Thus, it is important to utilize antibodies as ITs directed against more immature cells in the lineage of the tumor cell.

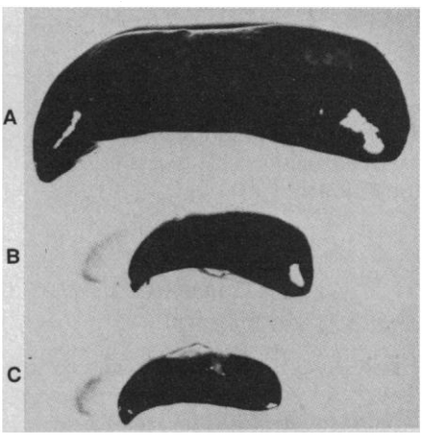

Fig. 4. Spleens removed from BCL_1-bearing mice 24 hours after intravenous injection with buffer (**A**) or 100 μg Fab'–anti-δ-A (**B**). A normal mouse spleen is shown in (**C**) (*49*).

2) Cross-reactivity between tumor-associated antigens and normal cells. The problem of damaging normal life-sustaining tissues represents a major concern of IT-A therapy. The notion of tumor-specific antigens that was generally accepted for decades has gradually been replaced by the concept that the majority of such antigens are normal differentiation antigens present on minor subsets of normal cells from the tissue of origin, for example, idiotypes on T and B cell tumors. Exceptions may be abnormal oligosaccharides or retrovirus-encoded antigens. With regard to ITs, cross-reactivity with some normal tissues might be acceptable if the tissues are not life-sustaining. Therefore, it is important to determine cross-reactivities with normal tissues before using ITs in vivo.

3) Heterogeneity of tumor cells. There is abundant evidence that, apart from the issue of renewal, tumor cells are heterogeneous with respect to such features as cell surface markers, state of differentiation, and genetic abnormalities; a major problem in cancer therapy is the escape of genetic and phenotypic variants (*53*). It is undoubtedly important to prepare a cocktail of highly effective ITs containing monoclonal antibodies directed against several different target antigens represented on the cell lineage of the tumor. Mutant cells that are resistant to A chain–mediated ribosome inactivation have not yet been described because A chains may bind to the elongation factor-2–binding site on the 60*S* ribosomal subunit and mutations in this site may be lethal.

4) Tumors may shed their surface antigens into the circulation. This has two undesirable consequences: (i) immune complexes will be formed which, in the case of ITs, could potentially damage the kidney; and (ii) such soluble tumor-associated antigens will bind to the IT and thereby decrease its localization to the target tissue. This problem might be partially overcome by plasmaphoresis prior to administration of the IT.

The Role of the Immune Response

There is considerable experimental and clinical evidence that the immune response can play a critical role in preventing tumorigenesis or influencing the progression of a tumor. As mentioned above, early observations in IT-A–treated BCL_1-bearing mice suggested synergy between IT therapy and a putative host immune response in the treated mice (*37*). Further studies with mice congenic at the IgH locus indicate that tumor immunity to BCL_1 can be achieved and that such immunity is dependent in part on an anti-idiotypic response (*54*). In addition, immunization of syngeneic mice with monoclonal BCL_1-IgM has induced an anti-idiotypic response that creates transient tumor dormancy after later injection of viable BCL_1 cells (*55*). The possibility raised by these studies is that an anti-

tumor immune response may potentiate the effects of IT-mediated killing of tumor cells and, even more importantly, that in one experimental tumor model not all tumor cells have to be killed by the IT to maintain a disease-free state (*36, 37*). Along these lines, Levy *et al.* (*48*) administered monoclonal anti-idiotypic antibody into patients with B cell tumors and found a strong correlation between the levels of T cells infiltrating the tumor prior to treatment and the capacity of the administered antibody to induce a remission, suggesting that the antibody therapy is most effective when aided by an anti-tumor response.

Potentiation of the Toxicity of IT-As

In the future, if potent ITs are not available for the tumor cells in question, it may be possible to increase the potency of existing agents.

One approach is to use so-called lysosomotropic agents, such as chloroquine, which perturb membranes and raise endosomal *p*H. They have been used in vitro to potentiate the toxicity of ITs-containing ricin or IT-As (*56*). However, potentiation is not observed with all IT-As. Where enhancement is observed, it could (in some cases) be due to contaminating B chain, since these agents enhance the potentiating effect of B chains on IT-As (*13, 57*). The effect of these agents on the therapeutic index is not known. Another approach is to exploit the ability of B chains to potentiate the translocation of A chains. In vitro, ITs-containing B chain (IT-Bs), specific for the same cell surface antigen (*58*) or to the antibody of the IT-A (*59*) ("piggyback" approach), have been added to cells previously treated with cell-reactive IT-A. Toxicity of the IT-As was markedly enhanced, presumably because a portion of both ITs were routed to the same endocytic vesicle and the B chain facilitated A chain translocation. "Free" B chains have been used in a similar manner both in vitro (*8*), and with some success, in vivo (*60*). Nevertheless, the lectin activity of "free" B chains and IT-Bs could result in nonspecific binding to other cells in vivo such that optimal delivery to IT-A–coated cells would not occur. It would be desirable, therefore, to eliminate this lectin activity. Biochemical studies indicate that the two activities of the B chain can be separated, that is, lectin activity can be abolished and potentiating activity can be retained when such B chains are used as IT-Bs (*61*).

Development of Third-Generation IT-As by Recombinant DNA Technology

With regard to improvement of ITs, the finding of an occasional, very potent IT-A is important (Fig. 1). Such an IT-A can be as potent in an in vitro assay as ricin, demonstrating that its antibody component can substitute effectively for the B chain in facilitating A chain toxicity. Yet, it has 10^4-fold less systemic toxicity for mice than ricin. Therefore, our tampering with nature has resulted in a 10^4-fold increase in therapeutic index.

By cloning the genes for relevant toxins and antibodies and manipulating these genes, nature's molecules can be redesigned in a more rational manner. Ricin toxin, and its A and B chains, have all been separately cloned and the recombinant A and B chains have been expressed (*14, 62*). With regard to B chain, the contact residues in the galactose-binding sites have been identified (*63*) so that lectin activity can be abolished by site-directed mutagenesis. If potentiating activity remains as predicted from the biochemical studies (*61*), then such mutagenized B chains may be important new therapeutic agents as separate ITs or possibly as fusion proteins with A chain. Fusion proteins produced by ligating toxin genes and growth factor

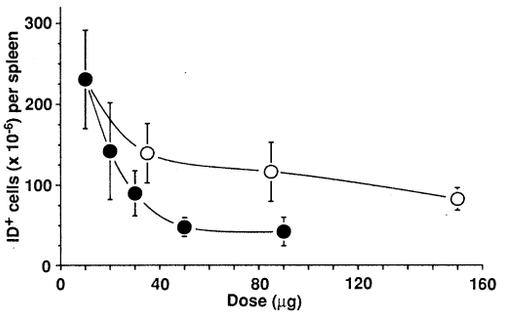

Fig. 5. The capacity of Fab'–anti-δ-IT injected intraperitoneally to kill tumor cells in the spleens of BCL_1-bearing mice. The figure depicts the average ± SD of three experiments (three mice per experiment) showing the number of BCL_1 tumor cells (ID^+) remaining in the spleens of mice 24 hours after treatment with Fab'–anti-δ-As prepared with native (○) or dgA (●) chain. The LD_{50}'s of the Fab'–anti-δ-dgA and the Fab'–anti-δ-A in BCL_1-bearing mice were 0.3 and 0.8 mg, respectively (*49*).

genes (*64*) show promise as cytotoxic agents. In particular, deletion of the nucleotide sequence of the toxin gene that encodes the cell-binding site allows the fusion protein to display the specificity of the ligand. With respect to the ricin A chain, it would be useful to prepare a smaller version of the A chain that can be ligated via a DNA-encoded linker to the DNA encoding the antigen-combining site of the antibody (Fv region) and expressed in *Escherichia coli*. This would result in a smaller molecule that might be less immunogenic and also might penetrate tissues and tumors more readily. Immunogenicity could be further decreased by utilizing human genes encoding the constant regions of the heavy and light chains of immunoglobulin and by substituting human amino acid residues in the conserved framework of the variable region, leaving murine residues only in the critical hypervariable regions (*65*). Apart from altering the IT molecule, it would be desirable to use recombinant DNA technology to prepare homogeneous ITs in the large amounts required for clinical use.

Time Frame for the Development of IT-As

After the advent of conventional chemotherapy in 1945, approximately two decades of experimentation were required before treatment regimens were developed that could cure some patients with particular cancers. Improvements in such therapy are still taking place. The development of IT therapy is an even more formidable task; the molecule is about 1000-fold larger than conventional chemotherapeutic agents and has three distinct components; there is less known about the chemistry, biology and pharmacokinetics of ITs; and there are relatively few scientists who are investigating the basic biology and biochemistry of ITs. It is likely that it will take 5 to 10 years to delineate the limitations and potentials of this approach. Further, ITs may synergize with other treatment modalities, both conventional and those in their developmental stages. For example, chemotherapeutic agents can enhance the specific cytotoxicity of ITs (*66*). Most importantly, we suggest that elucidation of the elements of an effective host anti-tumor immune response and the harnessing of that immune response, together with IT therapy, may offer the brightest hope for successfully treating the cancer patient.

REFERENCES AND NOTES

1. G. Köhler and C. Milstein, *Nature (London)* **256**, 495 (1975).
2. Reviewed in *Immunol. Rev.* **62** (1982).
3. S. Olsnes and A. Pihl, in *Molecular Action of Toxins and Viruses*, P. Cohen, and S. van Heyningen, Eds. (Elsevier, New York, 1982), p. 503.
4. Y. Endo, M. Kazuhiro, M. Motizuki, K. Tsurugi, *J. Biol. Chem.* **262**, 5908 (1987).
5. M. Funatsu *et al.*, *Proc. Jap. Acad.* **47**, 718 (1971); S. Nanno, M. Ishiguro, G.

Funatsu, M. Funatsu, *Agric. Biol. Chem.* **39**, 1651 (1975); B. M. J. Foxwell, T. A. Donovan, P. E. Thorpe, G. Wilson, *Biochim. Biophys. Acta* **840**, 193 (1985).
6. J. D. Robertus and M. P. Ready, *J. Biol. Chem.* **259**, 13953 (1984).
7. L. Carrasco, C. Fernandez-Puentes, D. Vazquez, *Eur. J. Biochem.* **54**, 499 (1975).
8. R. J. Youle and D. M. Neville, Jr., *J. Biol. Chem.* **257**, 1598 (1982); D. P. McIntosh *et al.*, *Fed. Eur. Biochem. Soc.* **164**, 17 (1983).
9. B. L. Kagan, A. Finkelstein, M. Colombini, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4950 (1981); J. J. Donovan, M. I. Simon, R. K. Draper, M. Montal, *ibid.*, p. 172.
10. L. Barbieri and F. Stirpe, *Cancer Surveys* **1**, 489 (1982); F. Stirpe and L. Barbieri, *FEBS Lett.* **195**, 1 (1986).
11. J. Carlsson, D. Drevin, R. Axen, *Biochemistry* **173**, 723 (1978).
12. Y. Masuho, K. Kishida, M. Saito, N. Umemoto, T. Hara, *J. Biochem.* **91**, 1583 (1982); F. K. Jansen *et al.*, *Immunol. Rev.* **62**, 185 (1982).
13. R. J. Fulton *et al.*, *J. Biol. Chem.* **261**, 5314 (1986).
14. M. O'Hare *et al.*, *FEBS Lett.* **216**, 73 (1987).
15. E. S. Vitetta, unpublished observations.
16. P. P. Knowles and P. E. Thorpe, *Anal. Biochem.*, in press.
17. D. C. Blakey, G. J. Watson, P. P. Knowles, P. E. Thorpe, *Cancer Res.* **47**, 947 (1987); B. J. Bourrie, P. Casellas, H. E. Blythman, F. K. Jansen, *Eur. J. Biochem.* **155**, 1 (1986); V. S. Byers *et al.*, *Cancer Res.*, in press.
18. R. J. Youle and D. M. Neville, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5483 (1980); L. L. Houston, R. C. Nowinski, *Cancer Res.* **41**, 3913 (1981).
19. D. M. Neville, Jr., and R. J. Youle, *Immunol. Rev.* **62**, 75 (1982).
20. D. A. Vallera, R. J. Youle, D. M. Neville, Jr., J. H. Kersey, *J. Exp. Med.* **155**, 949 (1982); D. A. Vallera *et al.*, *Science* **222**, 512 (1983); A. H. Filipovich, R. J. Youle, D. M. Neville, Jr., D. A. Vallera, *Lancet* **I-1984**, 469 (1984).
21. F. M. Uckun *et al.*, in *Molecular and Cellular Biology*, B. Bonavida and R. J. Collier, Eds. (Liss, New York, 1986), vol. 45.
22. E. O. Gregg *et al.*, *J. Immunol.* **138**, 4502 (1987).
23. P. E. Thorpe *et al.*, *Eur. J. Biochem.* **140**, 63 (1984).
24. P. Casellas *et al.*, *Int. J. Cancer* **30**, 437 (1982).
25. O. W. Press *et al.*, *Cell. Immunol.* **102**, 10 (1986); V. Raso and M. Basala, in *Receptor-Mediated Targeting of Drugs*, G. Gregoriadis, G. Poste, J. Senior, A. Trouet, Eds. (NATO ASI Series A, Plenum, New York, 1984), pp. 73–86; J. M. Lambert, P. D. Senter, A. Yau-Young, W. A. Blattler, V. S. Goldmacher, *J. Biol. Chem.* **260**, 12035 (1985).
26. O. Press, P. Martin, E. S. Vitetta, P. E. Thorpe, unpublished observations.
27. N. Beugnier, P. Falmagne, J. Zanen, F. K. Jansen, *Arch. Int. Physiol. Biochem.* **90**, B93 (1982); B. Ischida, D. B. Cawley, K. Reue, B. J. Wisnieski, *J. Biol. Chem.* **258**, 5933 (1983).
28. S. Ramakrishnan and L. L. Houston, *Cancer Res.* **44**, 201 (1984).
29. M. J. Bjorn, D. Ring, A. Frankel, *ibid.* **45**, 1314 (1985); V. Raso, J. Ritz, M. Basala, S. F. Schlossman, *ibid.* **42**, 457 (1982).
30. T. W. Griffin, L. R. Haynes, L. V. Levin, in *Receptor-Mediated Targeting of Drugs*, G. Gregoriadis, G. Poste, J. Senior, A. Trouet, Eds. (Plenum, New York, 1984), pp. 187–200; M. J. Embleton *et al.*, *Cancer Res.* **46**, 5524 (1986).
31. R. J. Fulton, R. D. May, M. A. Till, J. W. Uhr, E. S. Vitetta, unpublished observations.
32. J. K. Weltman *et al.*, *Biotechniques* **4**, 224 (1986).
33. M. A. Till, R. D. May, P. E. Thorpe, J. W. Uhr, E. S. Vitetta, in preparation.
34. P. Ehrlich, in *The Collected Papers of Paul Ehrlich* (Pergamon, New York, 1957), p. 21; S. Flexner, *J. Exp. Med.*, **2**, 197 (1897).
35. F. J. Jansen *et al.*, *Immunol. Lett.* **2**, 97 (1980).
36. K. A. Krolick, J. W. Uhr, S. Slavin, E. S. Vitetta, *J. Exp. Med.* **155**, 1797 (1982).
37. E. S. Vitetta, K. A. Krolick, M. Miyama-Inaba, W. Cushley, J. W. Uhr, *Science* **219**, 644 (1983).
38. K. Kishida, Y. Masuho, M. Saito, T. Hara, H. Fuji, *Cancer Immunol. Immunother.* **16**, 93 (1983); D. J. FitzGerald, M. C. Willingham, I. Pastan, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 6627 (1986); K. M. Hwang, K. A. Foon, P. H. Cheung, J. W. Pearson, R. K. Oldham, *Cancer Res.* **44**, 4578 (1984); H. Hara and B. Seon, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3390 (1987).
39. O. Fodstad *et al.*, *Cancer Res.* **44**, 860 (1984).
40. L. E. Spitler, in *Immunotoxins*, A. E. Frankel, Ed. (Nijhoff, Boston, in press).
41. G. Laurent, *et al.*, *Blood* **67**, 1680 (1986); A. E. Frankel *et al.*, in *Second International Conference on Monoclonal Antibody Immunoconjugates for Cancer*, I. Royston and R. O. Dillman, Eds. (USCD Cancer Center, San Diego, CA, 1987), p. 19.
42. V. S. Byers *et al.*, in *Second International Conference on Monoclonal Antibody Immunoconjugates for Cancer*, I. Royston and R. O. Dillman, Eds. (USCD Cancer Center, San Diego, CA, 1987), p. 40; V. S. Byers *et al.*, in preparation.
43. J. N. Weinstein, in *Second International Conference on Monoclonal Antibody Immunoconjugates for Cancer*, I. Royston and R. O. Dillman, Eds. (USCD Cancer Center, San Diego, CA, 1987), p. 30.
44. N. R. Worrell *et al.*, *Anti-Cancer Drug Design* **1**, 179 (1986); N. L. Letvin *et al.*, *J. Clin. Invest.* 77, 977 (1986).
45. E. J. Wawrzynczak and P. E. Thorpe, in *Immunoconjugates: Antibody Conjugates in Radioimaging and Therapy of Cancer*, C. W. Vogel, Ed. (Oxford Univ. Press, New York, in press).
46. G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959); V. Raso, T. Griffin, *J. Immunol.* **125**, 2610 (1980); R. J. Fulton, J. W. Uhr, E. S. Vitetta, *ibid.* **136**, 3103 (1986).
47. P. E. Thorpe *et al.*, *Eur. J. Biochem.* **147**, 197 (1985).
48. R. Levy and R. A. Miller, *Fed. Proc.* **42**, 2650 (1983); S. Harkonen *et al.*, *Cancer Res.* **47**, 1377 (1987).
49. R. J. Fulton, J. W. Uhr, E. S. Vitetta, in preparation.
50. P. E. Thorpe *et al.*, *Cancer Res.*, in press.
51. R. J. Fulton, T. Tucker, E. S. Vitetta, J. W. Uhr, in preparation.
52. T. S. Zimmerman, H. A. Godwin, S. Perry, *Blood* **31**, 277 (1968).
53. E. A. Boyse, E. Stockert, L. J. Old, *Proc. Natl. Acad. Sci. U.S.A.* **58**, 954 (1967); M. J. Glennie *et al.*, *J. Exp. Med.*, in press; V. S. Goldmacher, in *Immunotoxins*, A. E. Frankel, Ed. (Nijhoff, Boston, in press).
54. R. P. Ciavarra, E. S. Vitetta, J. Forman, *J. Immunol.* **137**, 1371 (1986).
55. A. J. George, A. L. Tutt, F. K. Stevenson, *ibid.* **138**, 628 (1987).
56. K. Sandvig and S. Solnes, *J. Biol. Chem.* **257**, 7504 (1982); P. Casellas, B. J. P. Bourrie, P. Gros, F. K. Jansen, *ibid.* **259**, 9359 (1984); S. Ramakrishnan and L. L. Houston, *Science* **223**, 58 (1984).
57. R. J. Fulton, J. W. Uhr, E. S. Vitetta, *J. Immunol.* **136**, 3103 (1986).
58. E. S. Vitetta, W. Cushley, J. W. Uhr, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 6332 (1983).
59. E. S. Vitetta, R. J. Fulton, J. W. Uhr, *J. Exp. Med.* **160**, 341 (1984).
60. S. A. Eccles *et al.*, *Cancer Immunol. Immunother.* **24**, 37 (1987).
61. E. S. Vitetta, *J. Immunol.* **136**, 1880 (1986).
62. F. I. Lamb, L. M. Roberts, J. M. Lord, *Eur. J. Biochem.* **148**, 265 (1985); M. S. Chang, D. W. Russell, J. W. Uhr, E. S. Vitetta, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5640 (1987).
63. W. Monfort *et al.*, *J. Biol. Chem.* **262**, 5398 (1987).
64. J. R. Murphy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8258 (1986); V. K. Chaudhary, D. J. FitzGerald, S. Adhya, I. Pastan, *ibid.*, in press.
65. S. L. Morrison, M. J. Johnson, L. A. Herzenberg, V. T. Oi, *ibid.* **81**, 6851 (1984); P. T. Jones *et al.*, *Nature (London)* **321**, 522 (1986).
66. F. M. Uckun *et al.*, *J. Immunol.* **134**, 3504 (1985).
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