observed electromechanical phenomena and that the transduction process may play an important role. The hypothesis that stereocilia stiffness is modified by current injection is not directly addressed by the data.

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Anti-T-Cell Reagents for Human Bone Marrow Transplantation: Ricin Linked to Three Monoclonal Antibodies

Abstract. *Three new reagents that react against human T cells were synthesized by covalently linking the toxin ricin to monoclonal antibodies recognizing differentiation antigens on the surface of T lymphocytes. Each of these immunotoxins selectively inhibited T-cell proliferation when the cells were incubated in the presence of lactose. Multipotent human stem cells were inhibited only at much higher concentrations. Mixtures of all three immunotoxins were more effective than any one alone. These reagents have the potential for preventing graft-versus-host disease in man.*

Bone marrow transplantation is used as an aggressive therapy for severe and life-threatening hematological disorders such as immunodeficiency diseases (*1*) and leukemia (*2*). Even when donors and recipients are matched with respect to their histocompatibility antigens (HLA), graft-versus-host disease (GVHD) may develop in which immunocompetent T cells in the donor graft react against the recipient's minor HLA antigens. In mice, GVHD can be prevented by eliminating T cells in the donor graft (*3–5*). In man, monoclonal antibodies to T cells have been used to treat donor cells before infusion, but the outcome has been disappointing (*6*). Antibody plus complement or lectin and sheep red blood cell fractionation (*7*) have also been used to deplete bone marrow of T cells. A new approach which is simple, rapid, and highly reproducible is to use conjugates of monoclonal antibody and toxins, which are capable of selectively killing T cells (*8*). These immunotoxins, when incubated with donor marrow in vitro, can protect mice in experimental models of lethal GVHD (*9, 10*). We have synthesized and tested three anti-T-cell immunotoxins that have potential for use in human bone marrow transplantation.

We selected the monoclonal antibodies TA-1, T101, and UCHT1 for our studies. TA-1 is an immunoglobulin G (IgG)2a antibody that binds to greater than 90 percent of peripheral blood T cells and monocytes, and approximately 70 percent of thymocytes when examined by indirect immunofluorescence (*11*). The cell surface molecule recognized by TA-1 is a two-chain, noncovalently linked glycoprotein complex of 170,000 and 95,000 daltons (*12*). TA-1 also recognizes monocytes and large granular lymphocytes with natural killer (NK) cell activity (*13*). Because these cells have been implicated in the pathology of GVHD (*14*), TA-1 was a particularly attractive antibody to link to ricin. T101, an IgG2a antibody, binds to all peripheral blood T cells (*15*). T101 recognizes a 65,000-dalton determinant on immature and mature normal T cells and T-cell lines. UCHT1, an IgG1 antibody, identifies a determinant on all human peripheral T lymphocytes and on a minority of thymocytes (*16*). UCHT1 precipitates the same glycoprotein as OKT3, that is, a complex of approximately 19,000 daltons (*17*). These three monoclonal antibodies were covalently linked to intact ricin by way of a thioether linkage by slight modification of a previously published procedure (*8*).

The plant seed toxin ricin contains two disulfide-linked subunits that have distinct roles in killing cells. The A chain enzymatically inactivates 60*S* ribosomes, inhibiting protein synthesis (*18*). The B chain binds to galactose-containing cell surface receptors (*18*) and increases the rate of A chain transport to ribosomes by an unknown mechanism (*19, 20*). Intact ricin (*8*) and ricin A chain (*20, 21*) linked to antibodies have been shown to specifically kill antigen-bearing cells. Immunotoxins made with ricin often exhibit more cell killing than those made with A chain alone (*10, 20, 22*). Immunotoxins made with intact ricin require the presence of 100 m*M* lactose to block the ricin binding site and thus cannot be used in vivo. Since procedures for bone marrow transplantation permit the treatment of donor cells in vitro, we elected to use the more potent, intact ricin immunotoxins.

The sensitivity of the three immunotoxins to T cells was determined by incubating them at various concentrations with peripheral blood mononuclear cells (PBMC) for 2 hours in the presence of lactose. The cells were then washed and cultured for 3 days in the presence of the T-cell mitogen phytohemagglutinin (PHA). All three immunotoxins—TA-1–ricin, T101-ricin, and UCHT1-ricin—inhibited the PHA response more than 90 percent at 300 ng/ml (Fig. 1).

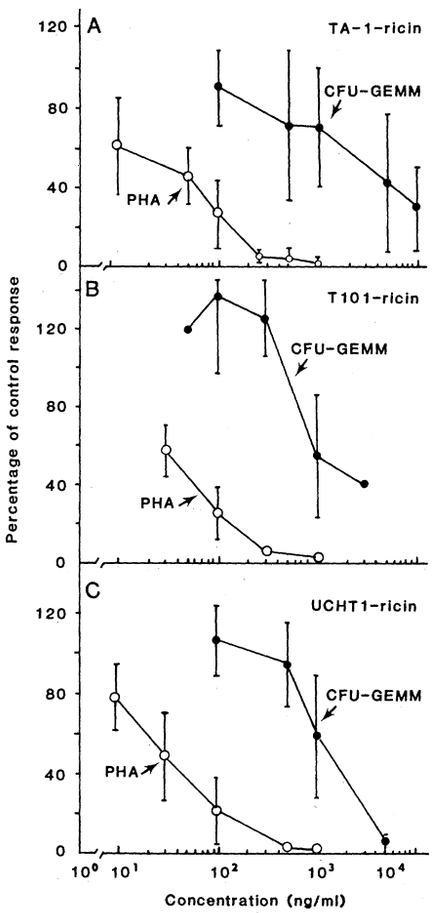
Stem cells must be preserved in the donor marrow for engraftment to occur in the recipient. Therefore, we examined the selectivity of the immunotoxins for different cell types by determining their toxicity to pluripotential stem cells using the CFU-GEMM assay (colony-forming units, granulocyte, erythroid, monocytic, megakaryocytic) (*23, 24*). Figure 1 shows that the concentration of immunotoxin that inhibits T cells more than 90 percent, 300 ng/ml, does not significantly inhibit the growth of CFU-GEMM. If the doses of immunotoxin that inhibit the PHA response and CFU-GEMM colony formation by 50 percent are compared, it is evident that T101-ricin and UCHT1-

ricin are 35-fold and TA-1–ricin is 100 times more toxic to T cells than stem cells. We independently measured erythroid precursers (BFU-E) in a plasma clot assay (*25*) and found them as resistant to the toxic effects of the immunotoxins as the CFU-GEMM (data not shown). We also assayed stem-cell and T-cell activity in mixtures of bone marrow and peripheral mononuclear cells treated with TA-1–ricin. The results confirmed the separate assays (Fig. 1) as T cells were inhibited at concentrations of immunotoxin that did not inhibit the CFU-GEMM (data not shown).

In an additional test of specificity, we found that free monoclonal antibody could compete for the toxicity of immunotoxin to T cells. Table 1 shows that excess TA-1 antibody will block TA-1–ricin inhibition of PHA responsiveness when cells are pretreated with either 100 or 300 ng of TA-1–ricin per milliliter. Control antibodies (IgG2a myeloma or T101) did not block TA-1–ricin toxicity. Moreover, similar findings were obtained with other immunotoxins in that T101 antibody, but not TA-1, blocked T101-ricin toxicity and UCHT1 antibody, but not control myeloma, blocked UCHT1-ricin toxicity (Table 1, experiments 2 and 3). Also, a control anti-Thy 1.2 immunotoxin that did not bind human T lymphocytes did not inhibit PHA responses at appropriate concentrations (not shown). Taken together, these findings show that the specificity of the anti-T-cell immunotoxins was determined by the antigen-binding region of the antibody.

To increase the selective elimination of T-cell populations, we tested the effect of mixtures of the three immunotoxins (Fig. 2). For each independent immunotoxin the results were expressed by plotting the log response against dose. In such plots, processes that are first order with respect to concentration are linear. We found that the dose response curves for the individual immunotoxins leveled off at higher concentrations. Such findings might be explained by resistant T-cell populations that lack appropriate determinants or by the antibody binding sites becoming saturated above 300 ng of conjugate per milliliter (*26*).

If the individual immunotoxins within a mixture act independently, a theoretical response can be plotted by multiplying the response of three separate com-



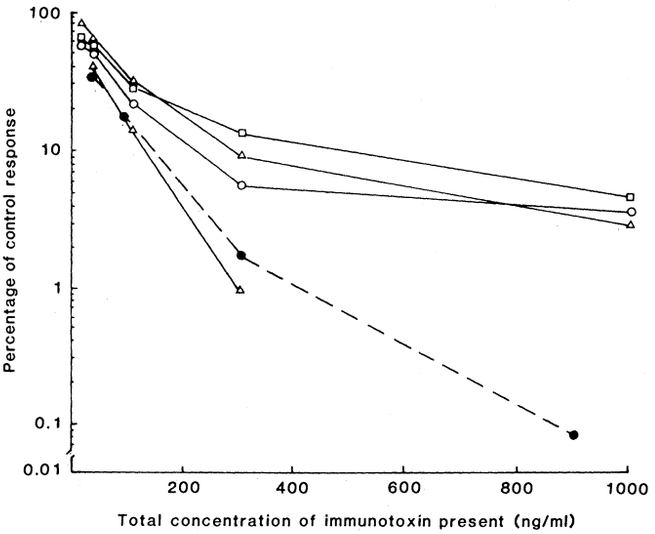


Fig. 1 (left). Effects of three different immunotoxins on human peripheral blood T cells and bone marrow stem cells. Ficoll-Hypaque isolated PBMC (10^7 per milliliter) from normal donors were treated with either TA-1–ricin, T101-ricin, or UCHT1-ricin, each in 75 percent RPMI 1640 medium, 0.2 percent $NaHCO_3$, 2 percent human serum albumin (HSA), and 100 m*M* lactose at 37°C for 2 hours in 5 percent CO_2 and 95 percent air. Isoosmotic medium containing lactose had no effect on PHA or stem cell assays. Treated suspensions were centrifuged (400*g*) for 10 minutes and washed once with 75 percent RPMI 1640, 1 percent HSA, 100 m*M* lactose and again with RPMI 1640, 1 percent HSA, and 15 m*M* lactose. For PHA assays, cells were cultured in triplicate (10^5 per well) in 0.2 ml of medium (RPMI 1640 supplemented with 20 percent pooled human serum, 2 m*M* glutamine, penicillin, and streptomycin) in 96-well flat-bottom culture plates and stimulated with 2.0 μg of PHA per well. Cultures were exposed to tritiated thymidine 18 hours before being harvested on day 3. Incorporation of thymidine was quantitated by standard liquid scintillation counting methods. Unstimulated control cultures in all conjugate experiments averaged 609 ± 599 count/min; the addition of PHA caused a 50- to 100-fold increase in thymidine incorporation. The data for each point (○) represented the mean values from pooled experiments in which each point is derived by calculating the percentage control response (that is, the response of immunotoxin-treated cultures divided by the response of lactose control cultures multiplied by 100). To measure the effect of the immunotoxins on human stem cells, we treated bone marrow mononuclear cells isolated on Ficoll-Hypaque in a manner identical to the PBMC and analyzed them using CFU-GEMM clonogenic assays. Treated cells were suspended in quadruplicate in Iscove's modified Dulbecco's medium containing 30 percent fetal calf serum, 5 percent PHA-conditioned medium, 5×10^{-5} *M* 2-mercaptoethanol, 1 I.U. of human urinary erythropoietin, antibiotics, and 0.9 percent methylcellulose in 35-mm plastic petri dishes (*24*). Cultures were incubated for 15 days at 37°C, in 5 percent CO_2 and 95 percent air at 100 percent humidity. Mixed colonies were counted microscopically. Untreated control cultures for all the CFU-GEMM experiments shown above averaged 12.0 ± 4.0 CFU-GEMM per 10^5 plated cells per dish. The data for CFU-GEMM experiments show the mean percentage control response (●) and were calculated in the same manner as the PHA data (*28*). Fig. 2 (right). The effect of immunotoxin mixtures on T cells. Cell suspensions were treated with immunotoxins and tested in the PHA assay as described in Fig. 1. Cells were treated with TA-1–ricin (□), T101-ricin (△), UCHT1-ricin (○), or a mixture of all three in which each immunotoxin is one-third of the total concentration present (▲). Each point is the mean percentage control response derived from four independent experiments (donors). Mean ± 1 standard deviation units are as follows: for TA-1–ricin at 30, 100, and 300 ng/ml, 55 ± 15, 29 ± 7, and 14 ± 10, respectively. For T101-ricin at 30, 100, and 300 ng/ml, 63 ± 18, 30 ± 12, and 10 ± 4, respectively. For UCHT1-ricin at 30, 100, and 300 ng/ml, 51 ± 14, 22 ± 4, and 6 ± 2, respectively. For mixtures at 30, 100, and 300 ng/ml, 42 ± 4, 15 ± 3, 1 ± 1 respectively. Untreated control cultures in all experiments averaged 603 ± 260 count/min; the addition of PHA caused a 68- to 135-fold increase in stimulation. The percentage control response is plotted logarithmically on the ordinate whereas concentration is plotted linearly on the abscissa as total concentration of immunotoxin. For mixtures, the theoretical product of individual immunotoxin treatments is plotted (●).

ponents. Such a curve is plotted in Fig. 2 and is the product of the individual immunotoxin curves. When mixtures of equal amounts of the three immunotoxins were used to treat cell suspensions our actual findings closely paralleled the theoretical results. With 300 ng of mixture per milliliter, the PHA response was only 1 percent of control. When the same concentration of mixture was tested against bone marrow cells in human stem cell assays, CFU-GEMM was reduced to 78 percent of the control response in a single experiment whereas BFU-E was reduced to 75 and 99 percent in two experiments (data not shown). Thus, 300 ng of mixture per milliliter might be considered for use in human bone marrow transplantation.

For use in humans we need to know the potential toxicity of ricin-treated bone marrow before it is infused into a recipient. We labeled the ricin moiety of the immunotoxins with ^{125}I and determined the amount of ricin remaining in the marrow after the 2 hours of incubation and two washes. When all three immunotoxins were incubated separately at 1000 ng/ml, 80 ng/ml or less remained after the second wash. On the basis of this result we calculated that treatment of 10^8 bone marow cells per kilogram of body weight of the recipient with 10^{-6} g of immunotoxin per 10^7 cells would result in a final administration (after incubation and washing) of 8×10^{-7} g of immunotoxin per kilogram of body weight. Humans have been treated with ricin therapeutically at 3.8×10^{-6} g/kg without lethal effects (*27*). Our calculated dosage of 8×10^{-7} g/kg is only one-fifth of the doses previously tested in humans. Furthermore, our immunotoxins contain only 28 percent ricin and are 100 times less toxic to mice than ricin. The treatment appears safe from ricin toxicity.

Table 1. Inhibition of T-cell proliferation by immunotoxins blocked by the addition of unconjugated antibody. Peripheral blood mononuclear cells (10^7) were treated with the unconjugated Staph A–purified antibodies (TA-1, T101, UCHT1, or a control IgG2a myeloma protein (10^5 ng/ml) at 4°C for 30 minutes (in an attempt to block conjugate binding). Treated cells were centrifuged and the pellet was resuspended in medium containing immunotoxins plus 100 m*M* lactose under conditions described in Fig. 1. The cells were then cultured in a PHA assay as described. Cultures were harvested in triplicate and counted by standard methods.

Immunotoxin (ng/ml)	Blocking antibody	Radioactivity (count/min per well)*	Percent of control response†
	Experiment 1: TA-1–ricin		
0	None	51016	100
100	None	29030	57
300	None	3881	8
0	TA-1	45808	100
100	TA-1	44012	96
300	TA-1	25153	55
0	CMP‡	41064	100
100	CMP	11150	27
300	CMP	3599	9
0	T101	50832	100
100	T101	16006	31
300	T101	6470	13
	Experiment 2: T101-ricin		
0	None	80718	100
100	None	16460	20
300	None	9666	12
0	T101	85354	100
100	T101	75375	88
300	T101	86201	101
0	TA-1	54217	100
100	TA-1	6958	13
300	TA-1	3891	7
	Experiment 3: UCHT1-ricin		
0	None	154212	100
30	None	74876	49
100	None	16810	11
300	None	1419	1
0	UCHT1	148347	100
30	UCHT1	125981	85
100	UCHT1	84057	57
300	UCHT1	30376	20
0	CMP	105419	100
30	CMP	48825	46
100	CMP	6653	6
300	CMP	575	1

*Data are reported as counts per minute per well minus blank values. For TA-1–ricin the blank values did not exceed 2892 count/min. Standard deviation did not exceed ±20 percent of the mean values in these studies. †Percent of control response is determined by comparing the PHA response of immunotoxin- and lactose-treated cultures to lactose-treated cultures without immunotoxin. Cultures showing 100 percent of the control response, in addition to lactose, also contain free antibody depending on the nature of the blocking antibodies used in the experiment. ‡Control IgG2a myeloma protein.

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28. The standard deviations in Fig. 1 are due to variations between experiments. (A) Eighteen different experiments with TA-1–ricin over 11 months were pooled to yield the averages (one experiment was omitted because of exceptionally low numbers of CFU-GEMM colonies). (B) Two different experiments with T101-ricin were pooled. (C) Three experiments performed with UCHT1-ricin. One experiment that was different is not shown in (C) since both the PHA and CFU-GEMM experiments were very sensitive to immunotoxin, with both responses showing a tenfold decrease. Possible explanations are large donor variations or an undetected error. For each experiment in (B) and (C) we used the same donor for peripheral blood and bone marrow cells. Only two experiments in (A) used the same donor cells. The experimental error between experiments (shown) is of the same magnitude as the error within replicates of one experiment (not shown). In control experiments, ricin with or without lactose displayed similar toxicity toward T cells and stem cells measured in these assays, showing that the selectivity of the immunotoxin was not due to cell type differences in ricin sensitivity.
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Predatory Capture of Toads by Fly Larvae

Abstract. *A natural occurrence of predation upon toads (*Scaphiopus multiplicatus*) by fly larvae* (Tabanus punctifer*) is described. The larvae lie buried in mud, seize the toads with hooked mandibles, pull them partly into the mud, and kill them by feeding on their body fluids. The larvae may ordinarily subsist mostly on invertebrates and take Amphibia only opportunistically.*

On the evening of 27 August 1982, by a small pond near Portal, Chochise County, Arizona, we observed thousands of spadefoot toads (*Scaphiopus multiplicatus*) which, having metamorphosed in close synchrony from the aquatic tadpole stage, were emerging from the water and congregating on the muddy shores. Spaced only centimeters apart in places, they were all of minimal adult size (body length, 1.5 to 2 cm). Conspicuous among them were toads that were dead or dying, apparently having been seized by a predator in the mud and drawn partly into the substrate, until only their head, or head and trunk, projected above ground. We counted dozens of such semisubmerged toads (Fig. 1E) along a stretch of several meters of shoreline. Most were near the edge of the water, where the mud was soft and wet. There were also remains of toads killed on some previous night and dried by daytime exposure.

When we attempted to pull fresh carcasses from the mud with forceps, we always felt a counterpull, which persisted until almost the moment of extrication of the toads. The predator remained concealed below the surface and seemed capable of quick evasive burrowing. By sifting through mud from around captive toads we found it to be a large grublike insect larva, subsequently identified as that of the horsefly *Tabanus punctifer*. Roughly equal in size to the toad itself (Fig. 1F), it occurred with fresh carcasses only, and always singly.

Placed in mud in aquariums, the larvae buried themselves (Fig. 1, A to D) in seconds by forcing themselves into the soil rear-end first, until their front end, which bears the mouthparts (Fig. 1G), was flush or nearly flush with the surface (Fig. 1D). They remained thus for hours or repositioned themselves by moving about underground periodically.

Scaphiopus that we offered to the larvae singly were all eventually caught when they came to rest upon the mouthparts of semisubmerged larvae. The larvae caught them with their pointed hinged mandibles and dragged them partway into the mud within minutes. Of 20 toads that were captured by the 12 larvae

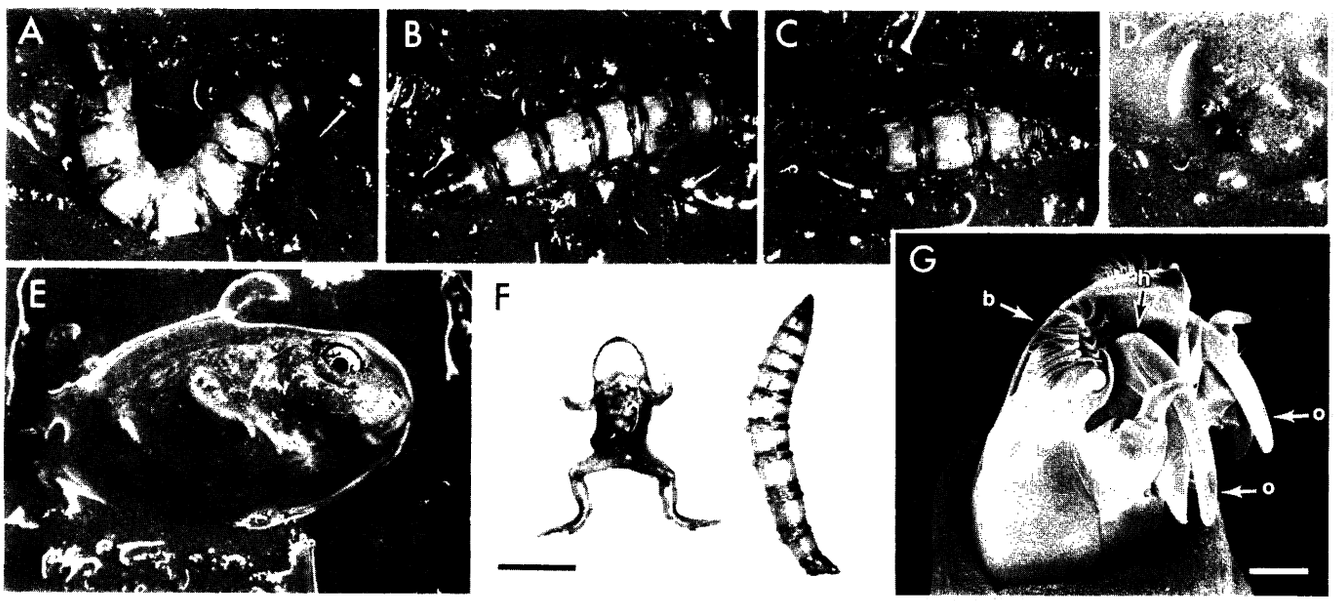

Fig. 1. (A to D) *Tabanus punctifer* larva, burying itself in mud. The larva withdraws until only the mouthparts (D) project from the surface. (E) Moribund toad (*Scaphiopus multiplicatus*) being fed upon by a *T. punctifer* larva that caught it and pulled it partway into the mud. (F) *Tobanus punctifer* larva, beside the toad upon which it was feeding; the abdominal bulge at the base of the right hind leg of the toad marks the site through which the larva was imbibing the body fluids of its prey. (G) Scanning electronmicrograph of head capsule of *T. punctifer* larva showing the hooked mandibles by which the prey are caught (*h*, hinge of mandible). The cephalic brushes (*b*) are presumed to help the larva anchor itself to its prey. The orifices (*o*) on the mandibles denote the openings of the presumed venom glands [morphological interpretation based on Teskey (*2*)]. Scale bars: (F) 1 cm and (G) 0.2 mm.