

INTERLEUKIN 2 RECEPTOR-TARGETED CYTOTOXICITY
Interleukin 2 Receptor-mediated Action of a Diphtheria Toxin-related
Interleukin 2 Fusion Protein

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Over the past decade there has been considerable interest in the assembly of conjugate toxin molecules whose action is directed toward specific target cells. A wide variety of cell surface-directed ligands (e.g., mAbs, lectins, polypeptide hormones, growth factors) have been coupled through disulfide linkage to nontoxic fragments of plant and/or microbial toxins (reviewed in 1, 2). In particular, mAbs have been used to direct the cytotoxic action of ricin, both the A chain and intact toxin, and fragments of diphtheria toxin to specific antigen-bearing cells. This class of conjugate toxins are widely known as immunotoxins, and have been the subject of several recent reviews (3-6).

In contrast to chemical cross-linking to form conjugate toxins, our approach to the development of targeted toxins has involved genetic assembly of chimeric toxin genes that are expressed from recombinant strains of *Escherichia coli* K12 (6, 7). We have selected targeting ligands whose cell surface receptors are known to undergo obligatory receptor-mediated endocytosis in the design of chimeric toxins that are composed of portions of diphtheria toxin linked to peptide hormones and/or growth factors. We have used rDNA methodologies to construct fusion genes in which that portion of the diphtheria *tox* gene which encodes the receptor-binding domain has been genetically replaced with the cDNA encoding the ligand of choice. Murphy et al. (6) and Williams et al. (7) have described the genetic construction of fusion toxins in which α -melanocyte-stimulating hormone (α -MSH)¹ and IL-2 have served as the receptor-binding component of the hybrid. Since both peptides have been shown to bind to surface receptors and are internalized by receptor-mediated endocytosis (8-10), we reasoned that both α -MSH toxin and IL-2 toxin should be selectively toxic for α -MSH and high affinity IL-2-R-bearing cells, respectively. Indeed, the action

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¹ Abbreviations used in this paper: α -MSH, α -melanocyte-stimulating hormone; IC₅₀, 50% inhibition of protein synthesis.

TABLE I
Eukaryotic Cell Lines Used in This Study

Cell line	Species	Tissue type	IL-2-R	Source or reference
C91/PL	Human	Cord T cell HTLV-I transformed	+	11
C10/MJ	Human	Cord T cell HTLV-I transformed	+	11
MT-2	Human	Cord T cell HTLV-I transformed	+	41 Robert Schwartz (Tufts Medical School)
Hut 102/6TG	Human	T cell (ATL)	+	Kendall Smith (Dartmouth Medical School)
CTLL-2	Murine	T cell (cytotoxic)	+	42 ATCC (TIB 214)
CEM-EM3	Human	T cell (ALL)	-	ATCC (TIB 195)
CCRF HSB-2	Human	T cell (ALL)	-	ATCC (CCL 120.1)
K562	Human	Granulocyte (CML)	-	ATCC (CCL 243)

ALL, acute lymphoblastic leukemia; ATCC, American Type Culture Collection; ATL, adult T cell leukemia; CML, chronic myelogenous leukemia.

of these two chimeric toxins has been shown to be targeted to those cell lines that express the appropriate receptor. In each instance, cell lines that do not express the targeted receptor have been found to be resistant to the action of α -MSH toxin- or IL-2 toxin-mediated inhibition of protein synthesis.

In the present report, we demonstrate that the cytotoxic action of IL-2 toxin is mediated through the high affinity IL-2-R on both human and murine T cell lines in vitro. Since the action of IL-2 toxin is blocked by chloroquine, we conclude that after binding to IL-2-R and internalization, this chimeric toxin must pass through an acidic compartment to deliver its ADP-ribosyl transferase to the T cell cytosol. Finally, we demonstrate that IL-2 toxin-mediated inhibition of protein synthesis in high affinity IL-2-R-positive leukemic cell lines results from the classic diphtheria toxin fragment A-catalyzed ADP ribosylation of elongation factor 2.

Materials and Methods

IL-2 Toxin. IL-2 toxin was purified from periplasmic extracts of *E. coli*(pABI508) as previously described (7).

Cell Culture. The eukaryotic cell lines used in this study are listed in Table I. C91/PL cells (11), derived from HTLV-I-transfected cord blood T lymphocytes, were cultured in RPMI 1640 medium supplemented with 15% FCS and 2 mM glutamine. Cells were subcultured at 3-4-d intervals. The IL-2-dependent murine CTLL-2 cell line (TIB-214; American Type Culture Collection, Rockville, Maryland) was maintained under similar conditions except that the medium was supplemented with 10% heat-inactivated FCS, 25 mM Hepes, 2 mM glutamine, and 40% rat T cell growth factor (12). All other cell lines used in this study were maintained in RPMI medium supplemented with 15% FCS and 2 mM glutamine.

Cytotoxicity Assays. Cells were seeded in 96-well V-bottomed plates (Nunc, Roskilde, Denmark) at a concentration of 10^4 /well (CTLL-2) or 10^5 /well in 100 μ l complete medium. Diphtheria toxin (Connaught Laboratories Ltd., Toronto, Canada) or IL-2 toxin at varying concentrations (10^{-12} - 10^{-6} M) in complete medium was added. Cells cultured with medium alone were included as the control. After a 24-h incubation at 37°C under

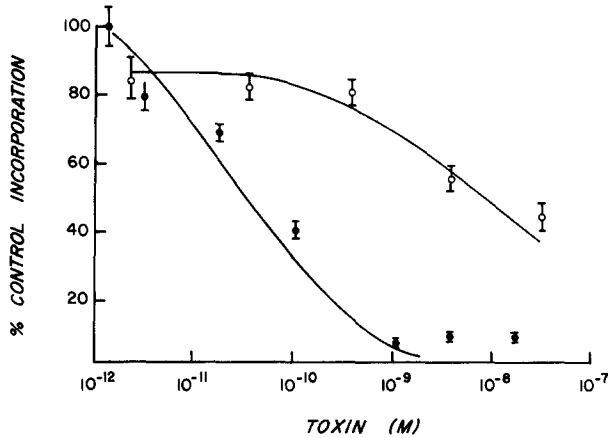


FIGURE 1. Inhibition of protein synthesis in IL-2-R-positive human C91/PL T cells by IL-2 toxin (●) and diphtheria toxin (○). Values are expressed as a percentage of [¹⁴C]leucine incorporation by untreated controls.

5% CO₂, the plates were centrifuged for 5 min at 200 g, the medium was removed and replaced with 100 μl leucine-free medium (DMEM Selectamine; Gibco, Grand Island, NY) containing 2.5 μCi/ml [¹⁴C]leucine (New England Nuclear, Boston, MA). Cells were then incubated at 37°C for 90 min and then collected on glass fiber filters using a cell harvester (Skatron Inc., Sterling, VA). Filters were washed, dried, and counted according to standard methods. All experiments were performed in quadruplicate.

Determination of Elongation Factor 2 Levels Available for ADP Ribosylation. The level of elongation factor 2 that was available for ADP ribosylation was determined essentially as described by Moynihan and Pappenheimer (13). Cells were seeded in 24-well plates at a concentration of 5×10^5 /well in 1 ml of medium. Diphtheria toxin or IL-2 toxin was added to the wells in 100 μl medium. After a 24-h incubation, the cell suspension was transferred to microcentrifuge tubes and centrifuged for 4 min. The cell pellets were lysed and assayed for the level of elongation factor 2 available for ADP ribosylation by the addition of purified diphtheria toxin fragment A to 2 μg/ml and [³²P]NAD to 1–2 Ci/ml. Assays were performed in triplicate.

Results

IL-2 Toxin Inhibition of Protein Synthesis in IL-2-R-positive Cells. Williams et al. (7) demonstrated that immunoaffinity-purified IL-2 toxin inhibits protein synthesis in a variety of IL-2-R-positive cell lines; whereas IL-2-R-negative cell lines were found to be uniformly resistant. We have now characterized the sensitivity of several HTLV-I-infected human T cell lines to IL-2 toxin by dose-response analysis. As shown in Fig. 1, exposure of human C91/PL T cells to IL-2 toxin at a concentration of 5×10^{-11} M for 24 h results in a 50% inhibition of protein synthesis (IC₅₀), compared to untreated control cultures. It is of interest to note that the IC₅₀ for native diphtheria toxin in the C91/PL T cell line is 4×10^{-8} M. Moreover, similar results were obtained in dose-response analysis of both IL-2 toxin and diphtheria toxin using the high affinity IL-2-R-positive C10/MJ, MT-2, and HUT102/6TG T cell lines in vitro (data not shown).

Eukaryotic cell lines of murine origin are markedly resistant to diphtheria toxin even though their elongation factor 2 is sensitive to ADP ribosylation in vitro (14). Since the CTLL-2 murine T cell line expresses high affinity, growth-promoting IL-2 receptors, it was of interest to examine the sensitivity of these cells to IL-2 toxin. As depicted in Fig. 2, the IC₅₀ for IL-2 toxin is $\sim 2 \times 10^{-10}$

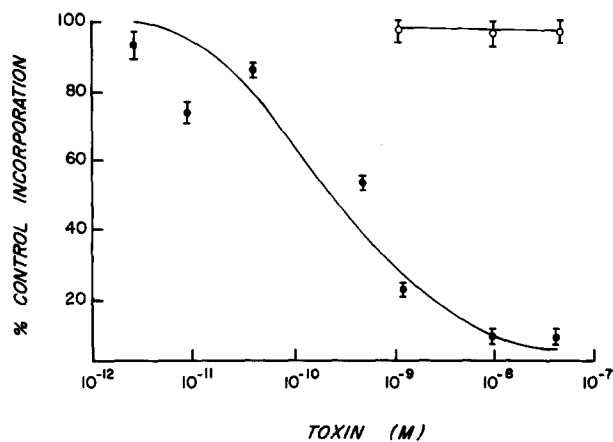


FIGURE 2. Inhibition of protein synthesis in IL-2-R-positive murine CTLL-2 T cells by IL-2 toxin (●) and diphtheria toxin (○). Values are expressed as a percentage of [¹⁴C]leucine incorporation by untreated controls.

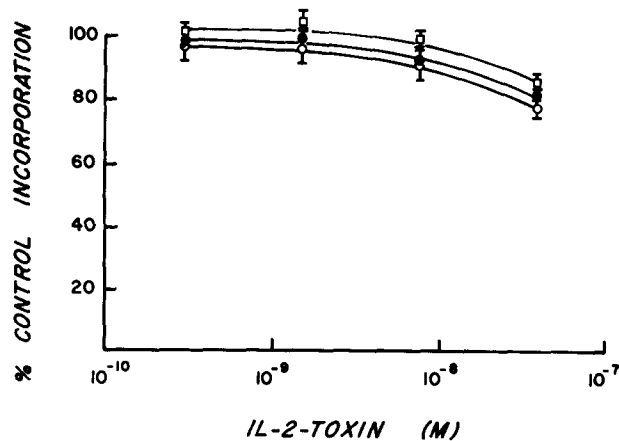


FIGURE 3. Inhibition of protein synthesis in IL-2-R-negative CEM-EM3 (□) and CCRF HSB-2 (●) human T cells, and the K562 (○) human granulocyte cell line. Values are expressed as a percentage of [¹⁴C]leucine incorporation by untreated controls.

M. In striking contrast, concentrations of diphtheria toxin as high as 10^{-6} M fail to inhibit protein synthesis in CTLL-2 cells in vitro.

Williams et al. (7) have shown that several eukaryotic cell lines that are devoid of the high affinity IL-2-R are resistant to the action of IL-2 toxin. We have extended this observation to a total of 22 different eukaryotic cell lines of human, monkey, murine, rat, hamster, and feline origin. Fig. 3 shows representative IL-2 toxin dose-response curves for three different IL-2-R-negative cell lines. The inhibition of protein synthesis observed at higher concentrations of IL-2 toxin are likely to result from nonreceptor site-specific fluid phase uptake into endocytic vesicles. Indeed, similar dose-response curves are seen in the case of the ADPR-transferase-positive, receptor-binding domain-negative CRM45 mutant of diphtheria toxin (15).

IL-2-R-mediated Toxicity. To demonstrate that the action of IL-2 toxin is mediated by IL-2-R, the effect of a number of competitive and noncompetitive inhibitors of IL-2-R site binding were examined. As shown in Table II, incubation of C91/PL cells in the presence of 4×10^{-9} M IL-2 toxin for 24 h inhibits [¹⁴C]leucine incorporation to 14% of control levels. Addition of excess free rIL-2 to the incubation mixture completely blocks the inhibitory effect of IL-2 toxin on

TABLE II
*Incorporation of [¹⁴C]Leucine by Human C91/PL IL-2-R⁺ T Cells
 after a 24-h Exposure to IL-2 Toxin*

IL-2 toxin concentration	Addition (10 ⁻⁷ M)	Control incorporation
<i>M</i>		
—	—	100
4 × 10 ⁻⁹	—	14
4 × 10 ⁻⁹	IL-2	111
4 × 10 ⁻⁹	33B.3*	72
4 × 10 ⁻⁹	Transferrin	17
4 × 10 ⁻⁹	4F2†	15

* mAb to IL-2-R.

† mAb to the early activation antigen 4F2.

leucine incorporation by C91/P1 cells. In addition, coincubation with the 33B3.1 anti-IL-2-R mAb (16), which binds to the p55 subunit of the IL-2-R, was found to partially block the action of IL-2 toxin. In direct contrast, coincubation with agents that interact with cell surface structures other than IL-2-R (e.g., transferrin, mAb 4F2 directed towards an early activation antigen on the T cell surface, and antithrombospondin) have no effect on IL-2 toxin-mediated inhibition of protein synthesis in C91/PL cells in vitro. We have found that rIL-2 competitively blocks the action of IL-2 toxin on murine CTLL-2 T cells, while transferrin has no effect (data not shown). mAbs to murine IL-2-R (e.g., M7/20 [reference 17]) themselves inhibit the growth of CTLL-2 cells in vitro; therefore, this receptor-binding control was not performed.

IL-2 Toxin Internalization. After binding to specific receptors on the cell surface, diphtheria toxin is concentrated in coated pits and internalized by receptor-mediated endocytosis (18, 19). Many ligands that enter cells by receptor-mediated endocytosis can be detected in vesicles that rapidly become acidified. Indeed, acidification of the vesicle is essential for the membrane translocation of fragment A of diphtheria toxin to the cytosol (20–22). A wide variety of lysosomotropic agents that prevent acidification of the endosome blocks diphtherial intoxication (23). To further characterize the mechanism by which IL-2 toxin inhibits protein synthesis in high affinity IL-2-R-bearing T cells, we have found that the addition of chloroquine completely blocks the action of IL-2 toxin on C91/PL T cells in vitro (Table III) and murine CTLL-2 cells (data not shown). Hence, after binding to IL-2-R and internalization, IL-2 toxin must pass through an acidic vesicle in order to deliver its ADP ribosyl transferase to the cytosol.

IL-2 Toxin ADP-ribosylates Elongation Factor 2. Diphtheria toxin-mediated inhibition of protein synthesis in sensitive eukaryotic cells involves the fragment A-catalyzed ADP ribosylation of elongation factor 2 (15). Since it is possible, albeit unlikely, that IL-2 toxin-mediated inhibition of [¹⁴C]leucine incorporation in high affinity IL-2-R-bearing T cells may have been due to steric hindrance of the receptor, we have directly measured the elongation factor 2 that was available for ADP ribosylation in C91/PL and CTLL-2 cells that were exposed to either diphtheria toxin or IL-2 toxin. As shown in Table IV, murine CTLL-2 T cells that are resistant to diphtheria toxin, but sensitive to IL-2 toxin, have

TABLE III
Incorporation of [¹⁴C]Leucine by C91/PL Cells after Exposure to IL-2 Toxin in the Presence and Absence of Chloroquine

Addition	[¹⁴ C]Leucine incorporation	
	cpm ± SEM	% Control
None	36,136 ± 724	100
Chloroquine	30,553 ± 453	84
IL-2 toxin	13,692 ± 333	38
IL-2 toxin + chloroquine	27,543 ± 855	90

Chloroquine was added to a final concentration of 6×10^{-6} M; IL-2 toxin was added to a final concentration of 1×10^{-9} M.

TABLE IV
Murine CTLL-2 and Human C91/PL Elongation Factor 2 Available for ADP-ribosylation after 24-h Exposure to Either Diphtheria Toxin or IL-2 Toxin

Cell line	Toxin and concentration	Control level of EF-2 available for ADP ribosylation
		%
CTLL-2	Diphtheria (10^{-7} M)	98
	IL-2 toxin (10^{-8} M)	8
C91/PL	Diphtheria (10^{-6} M)	<5
	IL-2 toxin (10^{-8} M)	<5

reduced levels of elongation factor 2 available for ADP ribosylation after a 24-h incubation with IL-2 toxin. As expected, incubation of CTLL-2 cells with 10^{-7} M diphtheria toxin for 24 h fails to inhibit protein synthesis and does not result in reducing the level of elongation factor 2 available for ADP ribosylation. In the case of human C91/PL T cells, which are sensitive to both diphtheria toxin and IL-2 toxin, reduced levels of elongation factor 2 available for ADP ribosylation were found after incubation with either toxin. Moreover, since the growth of C91/PL cells in vitro is IL-2 independent, we conclude that the inhibition of target cell protein synthesis is mediated through the action of IL-2 toxin rather than nonspecific steric hindrance of IL-2-R.

Discussion

We have used protein engineering and DNA techniques for the genetic assembly of a fusion gene in which the diphtheria toxin receptor-binding domain has been replaced with IL-2 sequences (7). This genetic construction is analogous to a previous report in which the diphtheria toxin receptor-binding domain has been replaced with the α -MSH (6). The chimeric IL-2 toxin has been found to be selectively toxic for only those eukaryotic cell lines that bear high affinity surface receptors for IL-2. In the present report, we have demonstrated that IL-2 toxin is specifically targeted toward IL-2-R on a variety of HTLV-I-infected transformed human T cell lines, as well as the IL-2-dependent murine CTLL-2

T cell line. In both instances, IL-2 toxin-mediated inhibition of protein synthesis in target T cells can be blocked by agents which bind to the IL-2-R; e.g., a molar excess of rIL-2 or anti-IL-2-R mAb. In contrast, agents that bind to other cell surface receptors, or mAbs that bind to other early lymphocyte activation antigens, do not block IL-2 toxin-mediated inhibition of cellular protein synthesis. In addition, we have shown that chloroquine completely blocks the cytotoxic action of both diphtheria toxin and IL-2 toxin. These results strongly suggest that IL-2 toxin, like diphtheria toxin (20-22), must be internalized and pass through an acidic vesicle in order to deliver its ADP ribosyl transferase to the T cell cytosol.

Once IL-2 is bound to its high affinity receptor it has been shown to be rapidly internalized (24); in addition, recent evidence suggests that the high affinity IL-2-R is not recycled (25). Two cell surface proteins that bind IL-2 have been characterized: a 55,000- and a 70,000-dalton glycoprotein. The Tac antigen, the 55,000-dalton low affinity IL-2-binding protein (p55), has been defined by many mAbs including 33B3.1 (16), and in the case of the murine IL-2-R, M7/20 (17); however, interaction of IL-2 or anti-IL-2-R mAbs with the p55 subunit does not readily promote internalization of the ligand (9, 10). Moreover, interaction of IL-2 with the p55 low affinity receptor does not stimulate DNA synthesis (26, 27). The 70,000-dalton subunit of IL-2-R has been identified and shown to bind IL-2 with moderate affinity (28-32). A recent report suggests that IL-2 bound to the p70 subunit is internalized into the cell (33). Given these observations, it is of interest to note that IL-2 toxin is selectively cytotoxic for only those T cell lines that bear high affinity (p55 + p70) IL-2-R (C. Waters, unpublished observations).

The only reaction that diphtheria toxin fragment A is known to catalyze in the eukaryotic cell cytosol is the ADP ribosylation of elongation factor 2 (15). Moynihan and Pappenheimer (13) have shown that fragment A of toxin is rapidly delivered across the cell membrane, and the rate of ADP ribosylation of elongation factor 2 increases with increasing concentrations of toxin. We have shown that the IL-2 toxin-mediated inhibition of protein synthesis in both murine CTLL-2 and human C91/PL T cells primarily results from ADP ribosylation of elongation factor 2. This observation demonstrates that the inhibition of protein synthesis in high affinity IL-2-R-bearing T cells results from the toxin fragment A-catalyzed reaction, and not from simply blocking the IL-2-R, from the action of IL-2 itself.

It is of particular interest to note that IL-2 toxin is active against the murine CTLL-2 T cell line. Cell lines derived from diphtheria toxin-resistant species (e.g., mouse and rat) are resistant to diphtheria toxin (15). Since elongation factor 2 derived from these cells is sensitive to ADP ribosylation by fragment A *in vitro*, it is not known whether the resistance of whole cells is due to a nonfunctional diphtheria toxin receptor, a defect in the internalization of diphtheria toxin, or alternatively to a defect in a putative processing event that may be involved in the intoxication process. Sensitivity of murine CTLL-2 cells to IL-2 toxin clearly demonstrates that the binding of this diphtheria toxin-related fusion protein to a receptor that is known to be capable of ligand binding and subsequent internalization results in the entry of the ADP ribosyl transferase of

IL-2 toxin into the cytosol. These results suggest that the diphtheria toxin receptor on murine cells is either not present or is not functional. The small decrease in sensitivity of CTLL-2 cells (IC_{50} 2×10^{-10} M) to IL-2 toxin relative to human C91/PL cells (IC_{50} 5×10^{-11} M) may be related to the decreased affinity of the human IL-2 component of the fusion protein for the murine IL-2 receptor, or to low levels of requisite IL-2 in the medium.

There are several factors that make the high affinity IL-2-R an attractive target for cytotoxic therapeutic agents. The cellular distribution of the high affinity heterodimeric p55/p70 IL-2-R appears to be largely limited to activated proliferating T lymphocytes, some recently activated B cells, and perhaps activated macrophages (26, 27, 34, 35). Hematopoietic stem cells do not express the high affinity IL-2-R, and only rare "resting" or memory T cells appear to do so (36).

Since the expression of IL-2 and high affinity IL-2-Rs marks a critical and pivotal event in the development of the immune response, it has been of considerable interest to examine the therapeutic effectiveness of mAbs directed against this receptor. In the case of both mAbs that recognize the IL-2-R on the surface of murine T cells (M7/20) and rat T cells (ART 18), a single 10-d course of therapy was found to greatly extend allograft survival (37, 38). Moreover, after treatment, immunologic tolerance was often noted. In addition, Kelley et al. (39) have shown that treatment with anti-IL-2-R mAbs are immunosuppressive in a delayed type hypersensitivity model in the mouse.

In the case of HTLV-I-associated adult T cell leukemia, the 42,000-mol-wt transactivator, *tat*, gene product appears to induce the expression of large numbers of high affinity IL-2-Rs on the leukemic cell surface (40). We have shown that IL-2 toxin is extremely selective and potent (IC_{50} ranging between 10 and 50 pM) against a number of human T cell lines in vitro. As such, IL-2 toxin is currently being developed as a prototype biologic agent for the treatment of adult T cell leukemia and other IL-2-R-bearing malignancies.

Summary

The IL-2 toxin-mediated inhibition of protein synthesis in high affinity IL-2-R-positive murine and human T cell lines has been examined. Both excess free IL-2 and mAb to the Tac epitope of the p55 subunit of IL-2-R are shown to block the action of IL-2 toxin; whereas, agents that interact with other receptors or antigens on the T cell surface have no effect. We show that IL-2 toxin, like diphtheria toxin, must pass through an acidic vesicle in order to intoxicate target T cells. Finally, we demonstrate that the IL-2 toxin-mediated inhibition of protein synthesis in both human and murine T cells that bear the high affinity IL-2-R is due to the classic diphtheria toxin fragment A-catalyzed ADP ribosylation of elongation factor 2.

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