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Immunology

Sequential effects of interleukin 2–diphtheria toxin fusion protein on T-cell activation

(T lymphocytes/interleukin 2 receptors/gene superinduction)

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ABSTRACT **The interleukin 2–diphtheria toxin-related fusion protein (IL-2–toxin) rapidly inhibits protein synthesis in IL-2 receptor (IL-2R)-bearing phytohemagglutinin-activated T cells but transiently stimulates DNA synthesis. At 7 hr after interaction with IL-2R$^+$ phytohemagglutinin-activated T cells, IL-2–toxin-treated cells bear augmented steady-state levels of c-myc, interferon γ, and IL-2R mRNA; these effects are indistinguishable from those produced by recombinant IL-2. Amplification of IL-2 sequences by the polymerase chain reaction reveals an increased level of IL-2 mRNA in cell cultures treated with recombinant IL-2, IL-2–toxin, and cycloheximide. These results suggest that IL-2–toxin can affect *de novo* IL-2 gene transcription/mRNA stabilization through independent mechanisms exerted by both the IL-2R binding domain and ADP-ribosyltransferase activity of the fusion protein. After 20 hr of culture, IL-2R mRNA was markedly decreased in both IL-2–toxin- and cycloheximide-treated phytohemagglutinin-activated T cells. Although interaction of IL-2–toxin with IL-2R$^+$ T cells initially mimics the stimulatory effects of IL-2 upon c-myc, interferon γ, IL-2R, and IL-2 gene expression, the consequences of inhibition of protein synthesis mediated by the ADP-ribosyltransferase activity of the toxin dominate after 7 hr and are indistinguishable from those effects mediated by cycloheximide.**

A fusion gene encoding the interleukin 2–diphtheria toxin fusion protein (IL-2–toxin) was constructed from a truncated diphtheria toxin gene by replacing DNA sequences coding for the toxin receptor binding domain with sequences coding for amino acids 2–133 of human IL-2 (1). The mature form of IL-2–toxin has a deduced molecular mass of 68 kDa, and the fusion protein retains at least some functional attributes of both its diphtheria toxin and IL-2 components. The fusion protein (*i*) binds with high affinity to the multimeric IL-2 receptor (IL-2R), (*ii*) is internalized by IL-2R-mediated endocytosis, and (*iii*) mediates the ADP-ribosylation of elongation factor 2 in the target-cell cytosol (2).

Binding of IL-2 to its high-affinity receptor rapidly activates intracellular processes that lead to gene activation and *de novo* gene transcription, DNA synthesis, and cell cycle progression (3–6). Even though IL-2–toxin rapidly inhibits protein synthesis in IL-2R-bearing phytohemagglutinin (PHA)-activated T cells, we now note that this chimeric toxin also transiently stimulates DNA synthesis (e.g., thymidine incorporation).

In the present study we demonstrate that binding of the IL-2–toxin to the high-affinity IL-2R *initially* produces effects in the PHA-activated target cell that are indistinguishable from those mediated by IL-2 itself. Subsequently, the fusion protein inhibits cellular protein synthesis. Additional indirect effects of IL-2–toxin upon T-cell gene activation, produced by the inhibition of protein synthesis, are characterized herein.

MATERIALS AND METHODS

Cell Cultures. Human peripheral blood mononuclear cells were isolated by means of Ficoll/Hypaque (Pharmacia) density centrifugation and were cultured in RPMI 1640 medium (M.A. Bioproducts) supplemented with 5 mM Hepes, penicillin (100 units/ml), streptomycin (100 μg/ml), 10% (vol/vol) heat-inactivated normal human AB serum (BioBee, Boston), and PHA (5 μg/ml) (Wellcome) for 72 hr. Cells were then washed three times, rested for 24 hr, and treated as indicated.

Materials. Recombinant IL-2–toxin was obtained and purified from extracts of *Escherichia coli* (pABI508) as previously described (1). A197/IL-2–toxin lacks ADP-ribosyltransferase activity due to a single amino acid change at position 52 (glycine to glutamic acid) and was used as a control (D. Williams and J.R.M., unpublished data). Human recombinant IL-2 (courtesy of Biogen) and cycloheximide (CHX) (Sigma) were used at the concentrations indicated.

DNA and Protein Synthesis. Cellular thymidine incorporation was measured by scintillation spectroscopy after a pulse with [^{3}H]thymidine (1 Ci/ml, 1 Ci = 37 GBq; NEN) during the last 6 hr of the 24-hr culture period. Total protein synthesis was quantitated by [^{14}C]leucine (NEN) uptake; after a 24-hr incubation, the medium was replaced with leucine-free medium (DMEM Selectamine; GIBCO) containing [^{14}C]leucine (2.5 μCi/ml) for 90 min. Results represent the mean ± SD of quadruplicate 200-μl wells.

Northern Blot Analysis. Total cellular RNA was prepared by the guanidine isothiocyanate method (7) and was quantitated by absorbance at 260 nm. RNA samples of 20 μg were size fractionated on a 1% formaldehyde/agarose gel. To ensure equal loading and transfer of RNA to nylon membranes (Hybond-N; Amersham), RNA samples were monitored by ethidium bromide staining. Hybridization was performed at 42°C in 50% (vol/vol) formamide/50 mM Na_2PO_4, pH 6.5/2× Denhardt's solution (1× Denhardt's = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/5× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate, pH 7.0)/0.1% SDS/denatured salmon sperm DNA at 250 μg/ml/10% (wt/vol) dextran sulfate. After washing under

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Abbreviations: IL-2, interleukin 2; IL-2R, IL-2 receptor; IL-2–toxin, IL-2–diphtheria toxin fusion protein; IFN-γ, interferon γ; CHX, cycloheximide; PCR, polymerase chain reaction; PHA, phytohemagglutinin.

stringent conditions (30 min at 65°C in 0.2× SSC/0.1% SDS), autoradiography was performed with an intensifying screen at −70°C. Full-length cDNA probes for IL-2 (courtesy of Biogen), CD3 (courtesy of C. Terhorst, Dana–Farber Cancer Institute, Boston), and interferon γ (IFN-γ) (courtesy of S. Clark, Genetics Institute, Cambridge, MA) as well as cDNA probes spanning the third exon of c-*myc* (courtesy of P. Leder, Harvard Medical School, Boston) and the fourth exon of IL-2R (Oncor, Gaithersburg, MD) were randomly primed with [^{32}P]dCTP (3000 Ci/mmol), and cDNA probes with 10^6 cpm were added to each milliliter of hybridization solution.

Polymerase Chain Reaction (PCR). PCR was employed to detect low-abundance mRNA encoding IL-2 in long-term peripheral blood mononuclear cell cultures. A 32-mer ("antisense") oligonucleotide (5′-GGCAGAAGCTTGGCCTGATATGTTTTAAGTGGG-3′), 22 nucleotides of which are complementary to the 3′ noncoding region of the IL-2 cDNA, and a 26-mer ("sense") oligonucleotide (5′-CAGTGTCTAGAAGAAGAACTCAAACC-3′), homologous to the IL-2 coding region at the *Xba* I restriction site, were synthesized on an Applied Biosystems 381A DNA synthesizer. First strand cDNA copies of the IL-2 mRNA were synthesized in a 10-μl reaction volume containing 10 μg of total RNA, 0.5 μM antisense oligonucleotide, and 200 units of Moloney murine leukemia virus reverse transcriptase (Bethesda Research Laboratories). The entire cDNA synthesis reaction volume was then combined in a 50-μl final reaction volume for PCR amplification containing each oligonucleotide primer at 0.25 μM and 1.5 units of *Thermus aquaticus* (*Taq*) DNA polymerase (Perkin–Elmer/Cetus) under conditions suggested by the manufacturer. Forty cycles of PCR amplification were performed with a DNA thermal cycler (Perkin–Elmer/Cetus). Each cycle consisted of 45 sec for denaturation at 94°C, 30 sec for annealing at 55°C, and 30 sec for enzymatic primer extension at 72°C. Specificity of the amplified IL-2 cDNA fragment was determined by Southern blot analysis, using the full-length IL-2 cDNA, and quantitated by densitometry.

RESULTS

Effect of IL-2–Toxin on DNA and Protein Synthesis. Binding of IL-2 to its high-affinity receptor stimulates phosphorylation of several membrane proteins, internalization of the IL-2/IL-2R complex, and T-cell growth (5, 6, 8). IL-2 stimulation of [^{3}H]thymidine and [^{14}C]leucine incorporation of IL-2R$^+$ PHA-activated T cells is markedly inhibited by addition of 10^{-10}–10^{-9} M IL-2–toxin at 24 hr of culture (Fig. 1*A*). However, addition of IL-2–toxin alone to cultures of IL-2R$^+$ T cells resulted in a 5-fold increase of [^{3}H]thymidine uptake, despite a concomitant, dose-dependent decrease of [^{14}C]leucine incorporation (Fig. 1*B*). The stimulation of thymidine uptake suggested that IL-2–toxin initially acts as a IL-2R site agonist and may trigger, at least transiently, intracellular processes in an IL-2-like fashion.

Effect of IL-2–Toxin on Early T-Cell Activation Gene Expression. Northern blot analysis of total RNA extracted from IL-2R$^+$ PHA-activated T cells was performed to study the early effects of IL-2–toxin (10^{-9} M) on T-cell activation. After 7 hr of incubation, IL-2–toxin-treated cells exhibit increases in cytoplasmic mRNA coding for IL-2R, c-myc, and IFN-γ (Fig. 2*A*). These early effects were indistinguishable from those mediated by IL-2 or a mutant of IL-2–toxin, A197/IL-2–toxin, which lacks ADP-ribosyltransferase activity. Decreased levels of IL-2R, c-myc, and IFN-γ gene expression were observed in cells treated with CHX, an inhibitor of protein synthesis, and harvested at 7 hr of culture (Fig. 2*A*).

Amplification of cellular IL-2 mRNA by PCR revealed significantly increased steady-state levels of IL-2 mRNA in IL-2–toxin-treated, A197/IL-2–toxin-treated, or CHX-treated cells, when compared to untreated cultures (Fig. 3). Furthermore, IL-2–toxin-treated T cells contained signifi-

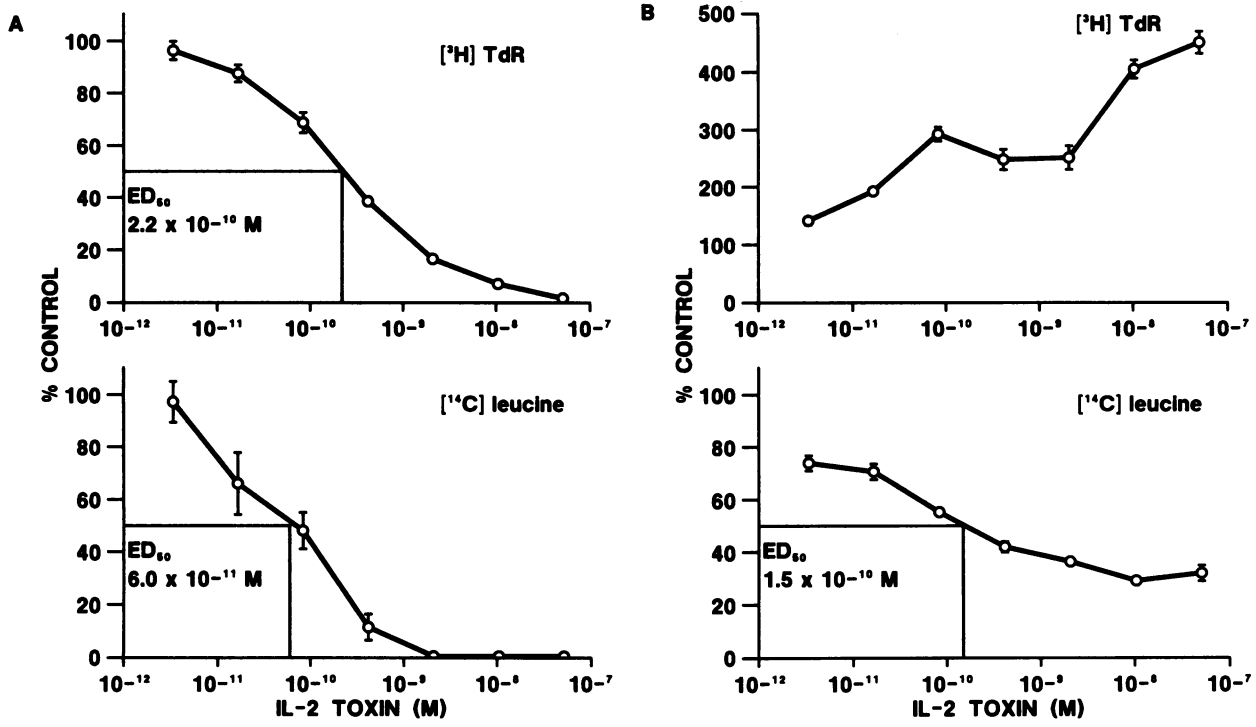

FIG. 1. Effect of IL-2–toxin on DNA and protein synthesis. (*A*) IL-2R$^+$ T cells were incubated with IL-2 (50 units/ml) and increasing concentrations of IL-2–toxin. [^{3}H]Thymidine ([^{3}H]TdR) and [^{14}C]leucine uptake are significantly inhibited above 10^{-9} M IL-2–toxin at 24 hr of culture. (*B*) Addition of IL-2–toxin to IL-2R$^+$ T cells alone resulted in a dose-dependent increase of [^{3}H]thymidine incorporation despite a concomitant decrease in [^{14}C]leucine uptake at 24 hr of culture.

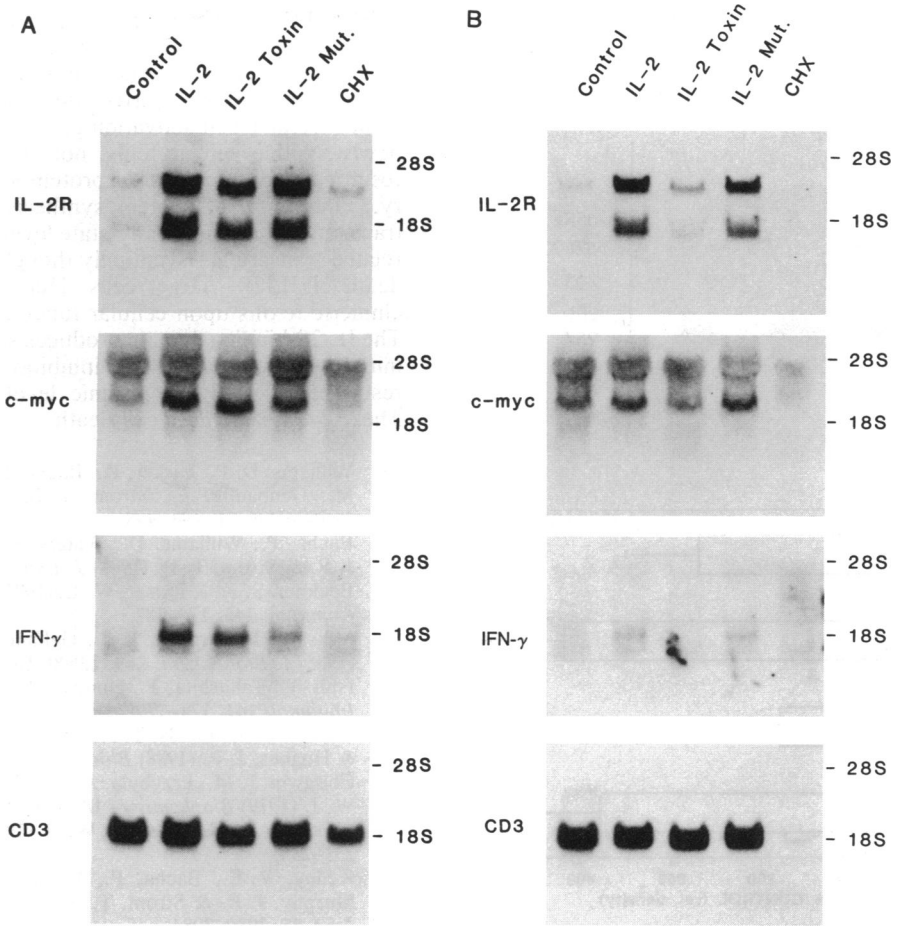

FIG. 2. (*A*) Northern blot analysis of total RNA from IL-2R$^+$ T cells treated for 7 hr as indicated. IL-2–toxin- or A197/IL-2–toxin (IL-2 MUT)-treated cells exhibit an increase in cytoplasmic mRNA coding for IL-2R, c-myc, and IFN-γ that is indistinguishable from that obtained from IL-2-treated cells, whereas transcripts in CHX-treated cultures are significantly reduced. (*B*) At 20 hr of culture, IL-2–toxin-treated cells exhibited reduced mRNA levels for c-myc, IL-2R, and IFN-γ that are comparable to the mRNA levels resulting from CHX treatment, while these transcripts remain detectable in IL-2- or A197/IL-2–toxin-treated cultures. The constitutively expressed CD3 mRNA is reduced in CHX-treated cultures.

cantly higher levels of IL-2 mRNA than either IL-2- or A197/IL-2–toxin-treated cultures ($P < 0.05$). These results suggest that the independent effects of the IL-2R binding domain and the ADP-ribosyltransferase region of IL-2–toxin act synergistically to effect *de novo* IL-2 transcription and/or mRNA stabilization.

Effect of IL-2–Toxin on Late T-Cell Gene Expression. Whereas IL-2R and c-myc transcripts remained elevated in IL-2- and A197/IL-2–toxin-treated cell cultures, IL-2–toxin-treated cells revealed decreased IL-2R and c-myc steady-state mRNA levels following 20 hr of incubation (Fig. 2*B*). This decrease was comparable to that obtained in cells that were treated with CHX for 7 hr, indicating an arrest of protein synthesis in IL-2–toxin-treated cells at 20 hr of culture.

DISCUSSION

Fusion proteins, such as IL-2–toxin, that are selectively targeted toward specific cell surface receptors are an attractive therapeutic alternative for treating patients with IL-2R$^+$ tumors, organ transplant recipients, or patients afflicted with an autoimmune state (1, 9–11). Hence, it is of importance to characterize the biological events that are produced following the ligand–receptor interaction.

Binding of recombinant IL-2 to its high-affinity receptor rapidly induces a series of intracellular events, including protein phosphorylation, protein kinase C membrane translocation (8), activation of the Na^+/H^+ antiport and cytosolic alkalinization (4), inhibition of adenylate cyclase activity (3), calcium influx (12), and internalization of the ligand–receptor complex (refs. 5 and 6; reviewed in ref. 13). These processes support IL-2-stimulated *de novo* gene transcriptional events, as documented for the p55 subunit of the IL-2R, c-myc, and heat shock protein genes (14, 15).

The results presented in this study show that the early effects of IL-2–toxin upon PHA-activated T cells precisely mimick the effects mediated by IL-2. Despite inhibition of protein synthesis (Fig. 1*A*), IL-2–toxin-treated PHA-activated T lymphoblasts do incorporate thymidine during the first 24 hr of culture (Fig. 1*B*).

At 7 hr of culture, IL-2–toxin stimulates accumulation of cytoplasmic IL-2R, c-myc, and IFN-γ mRNA in PHA-activated T lymphoblasts (Fig. 2*A*), also noted with IL-2 treatment (14, 15). In contrast, steady-state levels of these transcripts were significantly reduced in cell cultures treated for 7 hr with CHX (Fig. 2*A*). However, the detection of low-abundance mRNA by PCR revealed an increase in cytoplasmic IL-2 mRNA levels in IL-2–toxin-, IL-2-, and CHX-treated T lymphoblasts at 7 hr of culture (Fig. 3). IL-2 gene superinduction in mitogen-stimulated T cells by CHX has been reported to result entirely from increased mRNA stability (15–18). Indeed, there is considerable evidence that 3′ A+U-rich sequences are targeted for selective mRNA degradation in a variety of cytokine and cytokine receptor transcripts (19, 20). However, it is possible that IL-2 augments accumulation of cytoplasmic IL-2 mRNA through a

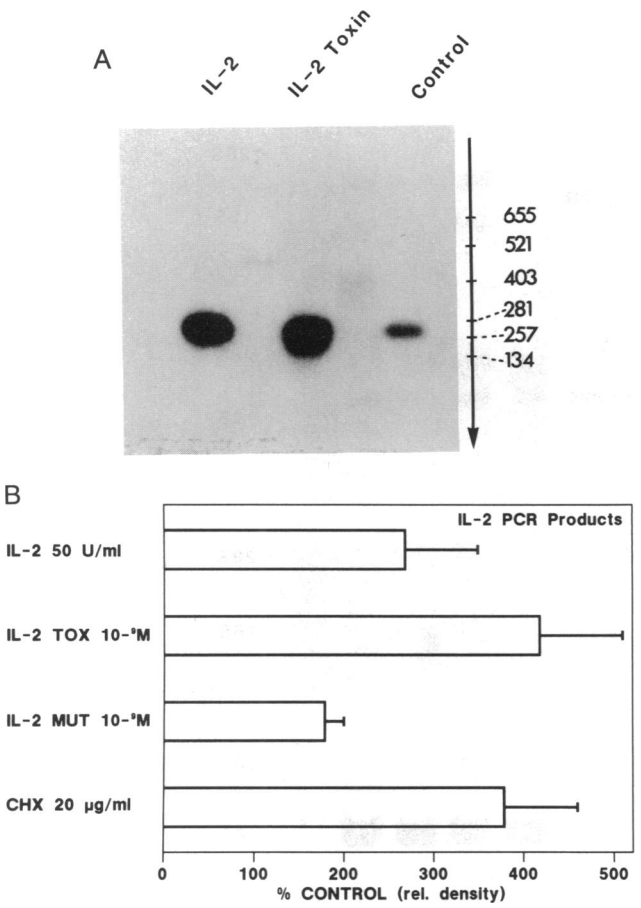

FIG. 3. (*A*) Amplification of IL-2 mRNA sequences results in a 279-base-pair cDNA fragment; specificity was verified by DNA size markers and hybridization to full-length IL-2 cDNA under stringent conditions. (*B*) Slot blot analysis and densitometry of IL-2 PCR products. At 7 hr of culture, rIL-2-, IL-2–toxin-, A197/IL-2–toxin (IL-2 MUT)- and CHX-treated cell cultures contain elevated IL-2 mRNA levels, when compared to untreated cells (mean ± SEM of four experiments; $P < 0.05$). Maximal accumulation of cytoplasmic IL-2 mRNA is detected in IL-2–toxin-treated T cells.

mechanism distinct from CHX-mediated effects. For example, it is possible that IL-2-mediated cell cycle progression is linked to *de novo* IL-2 gene transcription. In fact, IL-2–toxin seems to synergistically combine the distinct effects of recombinant IL-2 and CHX on IL-2 *de novo* transcription and/or IL-2 mRNA stabilization (Fig. 3).

Chimeric toxins, constructed from bacterial toxin genes by replacing the DNA sequences coding for the toxin receptor binding domain with sequences coding for cytokines, are being produced in several laboratories (1, 10, 21, 22). The fusion proteins encoded by such gene fusions are exceptionally powerful and selective cytotoxins *in vitro* and are, in many cases, under development as potential therapeutic agents. We have probed the complex time-related effects of IL-2–toxin. Whereas the ultimate effect of this protein results in death of high-affinity IL-2R-bearing cells, the early (<7 hr) effects of IL-2–toxin upon PHA-activated T lymphoblasts are mediated by the IL-2R binding domain. Subsequently, the inhibitory effects of the ADP-ribosyltransferase upon cellular protein synthesis are exerted. Because transcripts generated from several T-cell activation genes are readily degraded by newly synthesized proteins, not all of the cellular effects resulting from inhibition of protein synthesis are "inhibitory." Inhibition of protein synthesis actually results in a transient increase in steady-state levels of certain activation-related transcripts. Eventually though, intoxication leads to death of IL-2R$^+$ target cells. Hence, the effects of such chimeric toxins upon cellular function are highly complex. The IL-2R binding domain produces stimulatory effects. The initial effects produced by inhibition of protein synthesis result in elevated cytoplasmic levels of certain mRNAs. These events antedate cell death.

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