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The recombinant immunotoxin anti-Tac(Fv)–*Pseudomonas* exotoxin 40 is cytotoxic toward peripheral blood malignant cells from patients with adult T-cell leukemia

(immunotoxins/human T-lymphotropic virus type 1/leukemia/anti-Tac)

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ABSTRACT **Anti-Tac(Fv)–PE40 is a recombinant single-chain immunotoxin containing the heavy and light variable regions of the anti-Tac monoclonal antibody fused to a mutant form of *Pseudomonas* exotoxin (PE). Anti-Tac binds to the p55 subunit of the human interleukin 2 (IL-2) receptor, and anti-Tac(Fv)–PE40 kills human or monkey cell lines that contain either the intact IL-2 receptor or its p55 subunit alone. To assess the usefulness of anti-Tac(Fv)–PE40 in treatment of IL-2 receptor-positive leukemia, we tested peripheral blood mononuclear cells from six patients with adult T-cell leukemia. In each of the six patients, anti-Tac(Fv)–PE40 was extremely cytotoxic to the malignant cells. Metabolic activity and sensitivity of the fresh cells improved when a small amount of IL-2 (10 units per ml) was present during incubation. The toxin concentration necessary to inhibit protein synthesis by 50% after 16-hr incubation of cells with immunotoxin varied from 1.6 to 16 ng/ml ($2.5–25 \times 10^{-11}$ M). In every case, binding was by means of the Tac antigen because anti-Tac(Fv)–PE40 cytotoxicity was prevented by adding excess anti-Tac antibody. Moreover, anti-Tac alone or an inactive mutant of anti-Tac(Fv)–PE40 without ADP-ribosylation activity had very little cytotoxic activity. Peripheral blood mononuclear cells from normal controls, from a patient with Tac-negative leukemia, and from adult T-cell leukemia patients without significant peripheral blood involvement were not sensitive to anti-Tac(Fv)–PE40. These results indicate that anti-Tac(Fv)–PE40 is a potent cytotoxin against adult T-cell leukemia cells *in vitro* and warrants clinical testing.**

Human T-cell leukemia (or T-lymphotropic) virus type 1 (HTLV-1) has been etiologically associated with adult T-cell leukemia (ATL) (1, 2), which is poorly responsive to conventional chemotherapy and carries a median survival of only a few months after diagnosis (3). The fact that these cells frequently express high levels of the interleukin 2 (IL-2) receptor is of particular interest because comparable numbers of such receptors have not been found on nonmalignant human tissues, except for lymphoid cells, such as activated T lymphocytes (4). Therapy with monoclonal antibodies or portions of antibodies linked to toxins that target cells expressing IL-2 receptors may be appropriate for a disease such as ATL, where exposure of intravascular and bone marrow tumor cells to large proteins would be optimal.

We have recently reported the production and *in vitro* activity of several oncotoxins active against malignant cell lines containing the IL-2 receptor. The toxin portion is composed of three functional domains (5). To construct oncotoxins, domain 1a of *Pseudomonas* exotoxin (PE), which is responsible for binding to the PE receptor, is usually removed or inactivated. However, both domain II, which is needed for translocation across cell membranes into the cytosol, and domain III, which catalyzes the ADP-ribosylation of elongation factor 2, are retained. As nonbinding mutants we have used *Pseudomonas* endotoxin 40 (PE40), which is devoid of domain 1a, or *Pseudomonas* endotoxin wherein four basic amino acids in domain 1a are converted to glutamates (PE^{4E}). Chimeric toxins have included IL-2–PE40 and IL-2–PE^{4E} (6, 7), and immunotoxin chemical conjugates have consisted of anti-Tac coupled to either PE40 (anti-Tac–PE40) or PE40 containing an extra lysine at its amino terminus (anti-Tac–Lys–PE40) to facilitate conjugation (8, 9).

The PE-based oncotoxin with the highest cytotoxic activity against cell lines derived from human ATL has been the single-chain immunotoxin anti-Tac(Fv)–PE40 (10). In this recombinant protein, a peptide linker connects the variable domain of the heavy chain to the variable domain of the light chain of anti-Tac, and the carboxyl terminus of the light chain is connected to the amino terminus of PE40. Its ID_{50}, or concentration needed for 50% inhibition of protein synthesis, determined after 20-hr incubation with the ATL-derived cell line HUT 102 was 0.15 ng/ml, compared to 5 ng/ml, with IL-2–PE40 (6), 2 ng/ml with IL-2–PE^{4E} (7), and 2.5 ng/ml with anti-Tac–Lys–PE40 (9).

For several reasons, drugs active against established cell lines may lack clinical applicability. For example, HUT 102 cells have $>5 \times 10^5$ p55 subunits per cell, which differs significantly from receptor numbers in patients, which are 10,000–30,000 (ref. 6, and T.W., unpublished data). In addition, factors such as toxin processing and translocation into the cytosol might cause tumor cells to respond to immunotoxins differently *in vivo* than after prolonged culture. We therefore decided to carry out preclinical testing using fresh peripheral blood mononuclear cells (PBMCs) from patients. Results with anti-Tac(Fv)–PE40 were compared with other agents directed at the IL-2 receptor, as well as with control molecules. Controls included anti-Tac(Fv)–PE40D553, which because of a single amino acid deletion in domain III,

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Abbreviations: ATL, adult T-cell leukemia; PE, *Pseudomonas* exotoxin; IL-2, interleukin 2; PBMC, peripheral blood mononuclear cells; rIL-2, recombinant IL-2; HTLV-1, human T-cell leukemia (or T-lymphotropic) virus; ID_{50}, concentration of immunotoxin needed for 50% inhibition of protein synthesis.

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had no ADP-ribosylation activity but had all other functions of anti-Tac(Fv)–PE40.

MATERIALS AND METHODS

Patients. Six patients with ATL, based on clinical criteria and HTLV-1 positive by antibody analysis, were examined. Cases 1–3 and 6 were patients enrolled on a clinical trial awaiting, but not yet receiving, therapeutic anti-Tac monoclonal antibody. Cases 4 and 5 were patients attending a clinic in Jamaica. Case 2 was chronic with recent progression to the acute stage, and others were of the acute type. Another patient with HTLV-1-negative T-cell lymphoma (patient 9) was examined, in addition to Tac-negative cells from a patient with acute lymphocytic leukemia (patient 10). Normal cells from two patients with ATL without significant peripheral blood involvement (patients 7 and 8) and cells from normal volunteers were also tested. Other clinical information is listed in Table 1.

Toxins. Anti-Tac(Fv)–PE40 was expressed and purified as reported (10). IL-2–PE40 and IL-2–PE^{4E} were furnished by Hoffmann–La Roche (11).

Controls. Anti-Tac monoclonal antibody was purified by Hazleton Laboratories (Vienna, VA). The plasmid for anti-Tac(Fv)–PE40D553 was constructed by inserting a fragment from pVC45D f+T (12) that contained the Glu-553 deletion into the plasmid encoding anti-Tac(Fv)–PE40. *Escherichia coli* BL21 (λDE3) cells, which carry a T7 RNA polymerase gene in lysogenic and inducible form were used as a host for transformation and expression of the deletion mutant, which was then purified like anti-Tac(Fv)–PE40 (10).

Cells and Cell Culture. HUT 102 cells were maintained in RPMI 1640 medium/10% heat-inactivated fetal bovine serum (Hazleton Laboratories).

Anticoagulated venous blood was obtained from patients, and cells were used for experiments within 24 hr. Blood was diluted with phosphate-buffered saline containing magnesium and calcium and centrifuged over Ficoll (Organon Teknika or Pharmacia) at 1500 × *g* for 30 min. PBMCs were aspirated from the Ficoll–plasma interface, washed with RPMI 1640 medium, and resuspended in RPMI 1640 medium/20% fetal bovine serum at 1×10^6 cells per ml. Cells were incubated in plastic flasks (to remove monocytes) with or without recombinant IL-2 (rIL-2) for 30–60 min before incubation with toxins. Before toxin incubation, a cytospin of the nonadherent cells was prepared to allow morphologic analysis of cells after the purification steps.

Cytotoxicity Assays. Because the patient cells were metabolically less active than HUT 102 cells, assay conditions were adjusted to increase the amount of radioactive leucine incorporation into the cells. Changes from conditions recently reported (10) included adding rIL-2 at 10 units per ml to the cells before toxin exposure, increasing cell density from 3×10^5 to 1×10^6 cells per ml, increasing [^{3}H]leucine from 2 to 10 μCi per well (1 Ci = 37 GBq), and increasing pulsing time from 1.5 to 4 hr. Cells were exposed to toxins for 16 hr before pulsing. HUT 102 cells, with these adjustments, showed increased counts per minute (cpm) incorporated into protein from 5×10^3 to 1.5×10^5.

Cell Marker Analysis. Marker studies using fluorescein-activated cell sorter analysis of peripheral uncentrifuged blood were done either in our laboratory (T.W.) or by Fast Systems (Gaithersburg, MD).

Table 1. Clinical characteristics of patients

No.	Age/sex	Diagnosis	Leukocytes, cells/mm^3
1	62/M	Acute ATL	29,700
2	42/F	Chronic → acute ATL	14,200
3	32/F	Acute ATL	17,300
4	31/F	Acute ATL	81,000
5	33/F	Acute ATL	6,000
6	29/F	Acute ATL	26,100
7	35/M	Acute ATL, nonleukemic	3,400
8	67/F	Acute ATL, nonleukemic	6,000
9	65/M	T-cell lymphoma	86,500
10	7/F	Childhood ALL	65,000

ALL, acute lymphocytic leukemia.

RESULTS

To test the effect of anti-Tac(Fv)–PE40, cells were obtained from eight HTLV-1-positive ATL patients, six of whom had significant peripheral blood involvement and two of whom did not. Cells from HTLV-1-negative patients with T-cell leukemia and childhood acute lymphocytic leukemia and from normal volunteers were also studied; the characteristics of the patients appear in Table 1.

Cells from various patients were incubated with different concentrations of several chimeric toxins. Fig. 1 *A–F* and Table 2 show that anti-Tac(Fv)–PE40 was very active against the cells of ATL patients with HTLV-1 positive disease. The ID_{50} values range from 1.6 to 16 ng/ml. All six of these samples came from patients with a significant percent of Tac-positive cells. Under these assay conditions, the ID_{50} for HUT 102 cells was 0.2–0.7 ng/ml (Fig. 1*G*). Normal cells were not sensitive to anti-Tac(Fv)–PE40 even at 1000 ng/ml

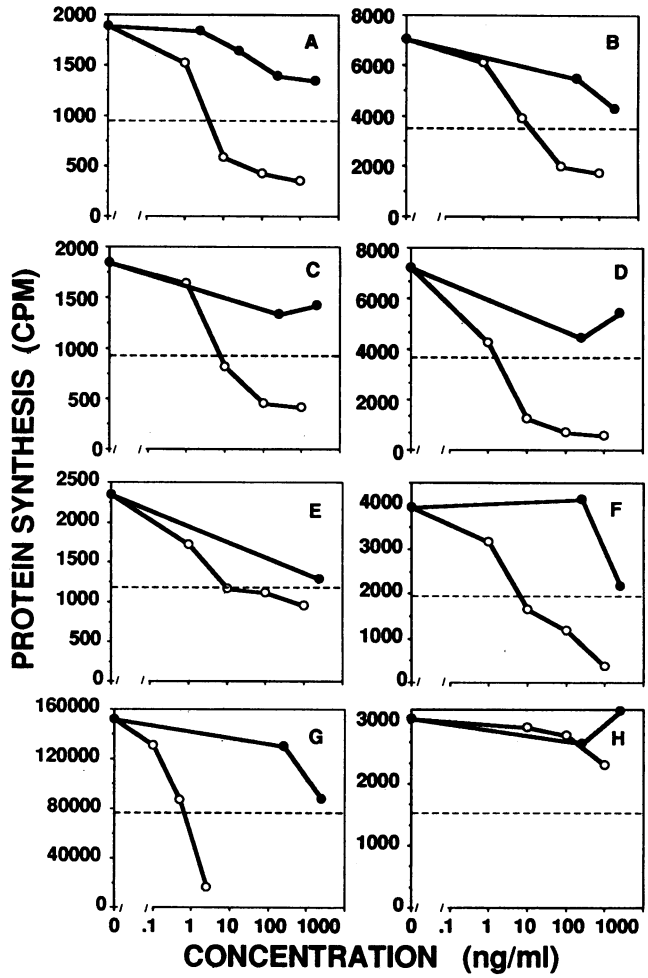

FIG. 1. Inhibition of [^{3}H]leucine incorporation by anti-Tac(Fv)–PE40 (○) compared with anti-Tac (●). (*A–F*) PBMCs from ATL patients (cases 1–6, respectively). (*G*) HUT 102 cells. (*H*) PBMC from normal control.

Table 2. Activity of various oncotoxins against human cells

No.	Diagnosis	ID_{50}, ng/ml: Anti-Tac(Fv)–PE40	IL-2–PE40	IL-2–PE^{4E}
1	ATL	4	>1000	250
2	ATL	16	>1000	610
3	ATL	7.5	>1000	1000
4	ATL	1.6		
5	ATL	10		
6	ATL	6		
7	ATL, nonleukemic	>1000	>1000	>1000
8	ATL, nonleukemic	>1000		
9	T-cell lymphoma	170		
10	Childhood ALL	>1000		
11	Normal	>1000	>1000	>1000
12	Normal	>1000	>1000	>1000
13	HUT 102	0.2	17	0.8

ALL, acute lymphocytic leukemia.

(Fig. 1*H*). Tac-negative malignant cells from a patient (case 10) with childhood acute lymphocytic leukemia also were not affected by anti-Tac(Fv)–PE40, confirming the specificity of its action. The Tac-expressing cells from a patient (case 9) with HTLV-1-negative T-cell leukemia/lymphoma were somewhat sensitive to anti-Tac(Fv)–PE40 (ID_{50} = 170 ng/ml) (Table 2).

To show that the effect of anti-Tac(Fv)–PE40 was not simply due to binding to the Tac antigen and displacing rIL-2, we compared the action of anti-Tac alone to that of anti-Tac(Fv)–PE40 and found in each ATL patient that anti-Tac alone was much less active and even at 2.5 μg/ml did not inhibit protein synthesis by >50% of control (Fig. 1). Anti-Tac at 2.5 μg/ml and anti-Tac(Fv)–PE40 at 1000 ng/ml both approximate 1.5×10^{-8} M.

To prove further that the ATL cells were targets because of the presence of Tac antigen and not some other factor, we used the monoclonal antibody anti-Tac to compete in the protein synthesis inhibition assay and showed competition in each case. One example appears in Fig. 2, where the anti-Tac antibody, but not a control antibody (OVB3), prevented anti-Tac(Fv)–PE40-mediated cytotoxicity.

To assist in the interpretation of the sensitivity of each patient's PBMCs to anti-Tac(Fv)–PE40, we used fluorescein-activated cell sorting to obtain receptor data on each patient. The data of Table 3 indicate the percent of lymphocytes positive for the p55 subunit of the IL-2 receptor and other antigens. p55 or Tac positivity ranged between 40% and 94% for leukemic ATL patients and 86% for the HTLV-1-negative T-cell lymphoma patient. Because of low intensity, Tac percentage data for the two nonleukemic ATL patients (cases 7 and 8) were considered normal.

Table 3. Surface marker data

No.	Surface marker, %: CD25 Tac	CD2 T11	CD4 Leu3	CD8 Leu2a	Mikβ1
1	73	86	84	0	0
2	70	85	81	12	0
3	71	77	89	2	1
4	94	98	98	3	14
5	40	90	57	21	32
6	72	90	84	16	6
7	22	81	36	39	
8	21	31	35	11	0
9	86	94	91	1	1
10	<10	<10	<10	<10	

Having shown that the cytotoxicity of anti-Tac(Fv)–PE40 depended on binding to the Tac antigen but that such binding alone was insufficient, we wished to rule out the possibility that cell death was from a factor other than ADP-ribosylation. Therefore, we prepared a molecule exactly like anti-Tac(Fv)–PE40, except lacking ADP-ribosylation activity due to deletion of Glu-553 (13). The purified protein was not cytotoxic to HUT 102 cells, even at 1 μg/ml, which is nearly five orders of magnitude above the ID_{50} of anti-Tac(Fv)–PE40. Furthermore, the protein blocked the cytotoxic effect of anti-Tac(Fv)–PE40 on HUT 102 cells, showing that it bound to the IL-2 receptor (data not shown). We therefore added anti-Tac(Fv)–PE40D553 alone to cells from all but the first patient and found no more inhibition of protein synthesis than by anti-Tac itself (Fig. 3).

rIL-2 was added to the freshly isolated cells in an attempt to maintain their metabolic activity during short-term culture. This approach was useful because protein synthesis rates increased up to 2-fold by rIL-2 at 10 units per ml (Table 4, Fig. 4). However, 100 units per ml was less effective. Fig. 4 shows that at maximum concentration of anti-Tac(Fv)–PE40 used, the residual amount of protein synthesis did not change with

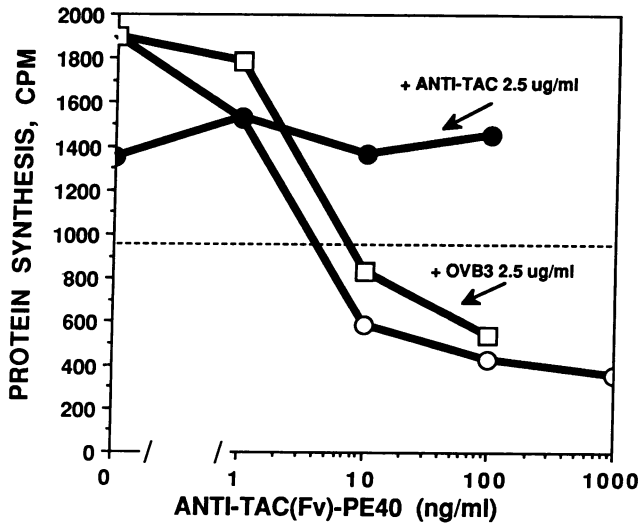

FIG. 2. Competition of anti-Tac(Fv)–PE40-mediated cytotoxicity of PBMCs from case 1, using a constant concentration of anti-Tac (2.5 μg/ml) (●) or control antibody OVB3 (2.5 μg/ml) (□) and various concentrations of immunotoxin (○).

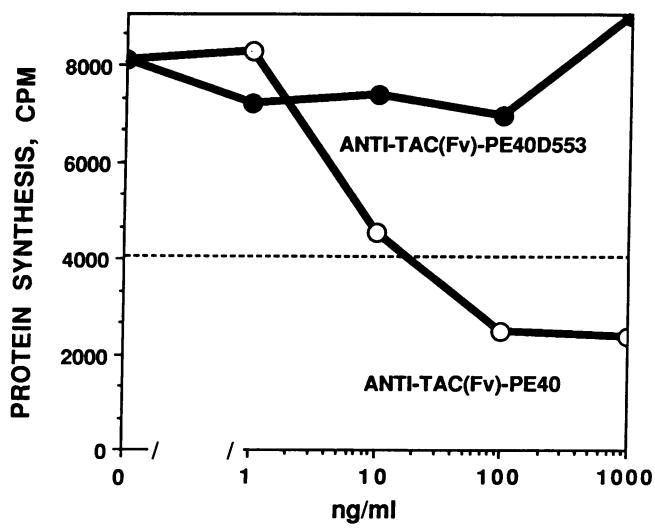

FIG. 3. Comparison of the cytotoxic activity of anti-Tac(Fv)–PE40 (○) with anti-Tac(Fv)–PE40D553 (●) in cells from patient 2.

Table 4. Sensitivity to anti-Tac(Fv)–PE40 with and without rIL-2

Case	Without rIL-2		With rIL-2 (10 units/ml)	
	Control, cpm	ID_{50}, ng/ml	Control, cpm	ID_{50}, ng/ml
1	950	20	1900	4
2	4100	90	7100	16
3	1500	20	1800	7.5
4	4200	<10	7200	1.6
6	2200	6	4000	6

rIL-2 concentration. This residual protein synthesis was most likely from cells without or with too few IL-2 receptors that would not be killed by anti-Tac(Fv)–PE40 nor be stimulated by rIL-2. Table 4 also shows reduction in ID_{50} values in most cases, with rIL-2 addition.

DISCUSSION

Our main objective was to show that anti-Tac(Fv)–PE40, a potent immunotoxin for leukemic cell lines expressing the human Tac antigen (10), would also be cytotoxic for ATL cells obtained directly from patients and maintained in short-term culture. It seemed possible that cells directly isolated from patients might be much less sensitive than continuous cell lines. Previously, two other reagents directed against cells with IL-2 receptors had been tested against freshly isolated cells from ATL patients. One of those was a recombinant fusion protein of diphtheria toxin and IL-2 (DT–IL-2, ref. 14) and the other was a chemical conjugate of the anti-Tac antibody with ricin A chain (15). In the DT–IL-2 study, protein synthesis of cells from lymph nodes of 3 of 3 ATL patients and PBMCs from 2 of 11 ATL patients was inhibited >50% after 6-hr incubation with DT–IL-2 at ≈600 ng/ml. In the anti-Tac–ricin study, ID_{50} values against PBMCs from two patients with ATL were 60–120 ng/ml after 48-hr incubation with toxin. DT–IL-2 and anti-Tac ricin killed HUT 102 cells with ID_{50} values of 1–1.5 and 40 ng/ml, respectively.

Sensitivity of ATL Patients' Cells. In this study using partially purified mononuclear cells isolated from the blood of ATL patients, all six samples were very sensitive to the cytotoxic action of anti-Tac(Fv)–PE40; ID_{50} values ranged from 1.6–16 ng/ml. Furthermore, the cytotoxic effect was specifically prevented by excess anti-Tac and not produced by a mutant form of anti-Tac(Fv)–PE40 that lacks ADP-ribosylating activity. These data indicate not only that anti-Tac(Fv)–PE40 is binding to patient cells but that cell death is mainly from the ADP-ribosylating function of the toxin.

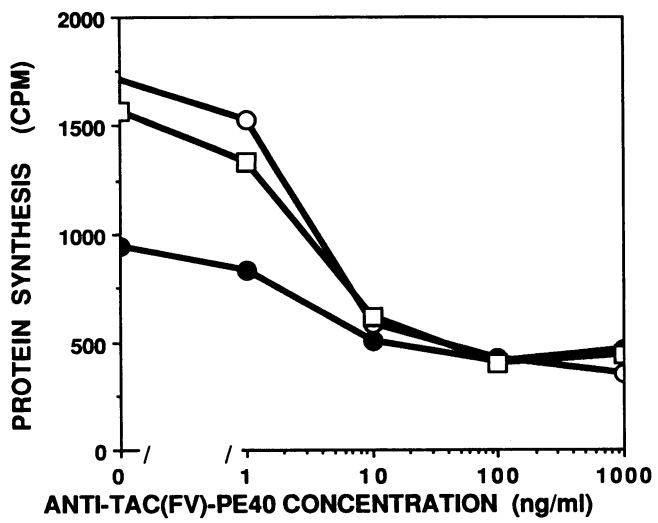

FIG. 4. Effect of pretreatment of PBMC without (●), with 10 units per ml (○), or with 100 units per ml (□) of rIL-2 on the response to anti-Tac(Fv)–PE40 in cells from patient 1.

The HTLV-1-negative T-cell lymphoma patient (case 9) had 86% Tac-positive lymphocytes but the highest ID_{50} value (170 ng/ml) of the sensitive patients. Possible explanations include low numbers of receptors per cell or differences in toxin internalization or processing; whether such factors are disease-related may be answered by experiments on more of these Tac-positive HTLV-1-negative patients.

Comparison with IL-2 Recombinant Toxins. We also measured the activity of two other agents directed at the IL-2 receptor, IL-2–PE40 and IL-2–PE^{4E}. Although quite active on HUT 102 cells, neither agent was very active against the cells from the ATL patients (Table 2). Based on *in vitro* data from cases 1–3, we conclude that IL-2–PE40 and IL-2–PE^{4E} are unlikely to be clinically useful in ATL.

Tac Positivity. As Table 3 indicates, lymphocytes in whole blood as assessed by fluorescein-activated cell sorter analysis were heterogenous for anti-Tac reactivity. Because our Ficoll centrifugation step was designed to isolate all the mononuclear cells from blood and the plastic-adhesion step removed monocytes, the data in Table 3 should closely approximate percentages of antigen positivity among the partially purified patient cells placed into short-term culture. Because a significant fraction of cells from each patient was Tac negative (presumably nonmalignant), we did not expect to inhibit protein synthesis completely in each sample. In cases 1–6 (Fig. 1 *A–F*), a leveling off of protein synthesis was reached at high anti-Tac(Fv)–PE40 concentrations. This level correlated inversely with the percent of Tac-positive lymphocytes indicated in Table 3. For example, the lowest residual cpm level was seen by using cells from patient 4 (Fig. 1*D*) whose lymphocytes were 94% Tac positive. However, protein synthesis from the PBMCs of patient 5, which were only 40% Tac positive, could be inhibited >50%, perhaps indicating that without any toxin, the malignant cells synthesized more protein per cell than did the nonmalignant cells. The level of residual protein synthesis appeared to correlate with the ID_{50} value. Therefore, in patients with low percent Tac positivity, the ID_{50} values determined may, indeed, be underestimates of the true sensitivity of leukemic cells to anti-Tac(Fv)–PE40.

p75 Expression. Table 3 lists percent of lymphocytes reacting with antibody Mik-β1, which reacts with p75, the IL-2 receptor subunit that alone has IL-2 affinities intermediate between that of the low-affinity Tac (p55) antigen and the intact p55–p75 complex (16). p75 expression is quite low in most ATL patients when compared with Tac expression. Because both anti-Tac and IL-2-based PE recombinant toxins are active on cells expressing p55 without p75 (6, 17), it is not surprising that cytotoxicity was demonstrated against cells from patients 1–3 by all three toxins (Table 2). Moreover, because the dissociation constants of anti-Tac and Il-2 for p55 are 10^{-9} and 10^{-8} M, respectively, anti-Tac-based toxins might be expected to be more cytotoxic against ATL cells than IL-2-based toxins.

Effect of rIL-2 Addition. We found that with rIL-2 (10 units per ml), PBMCs from the ATL patients incorporated up to 2-fold more leucine (Table 4). That rIL-2 was causing higher receptor expression and increased protein synthesis among resistant Tac-negative cells is unlikely because residual protein synthesis with anti-Tac(Fv)–PE40 at 1000 ng/ml did not change with rIL-2 concentration (Fig. 4). Table 4 shows that with rIL-2, cytotoxicity of anti-Tac(Fv)–PE40 was, in some cases, 5-fold greater than without rIL-2. The ID_{50} reduction with rIL-2 could be from enhanced IL-2-receptor expression among Tac-positive cells or enhanced internalization of toxin. If control cpm is the summation of protein synthesis

from IL-2 receptor-positive and -negative cells without toxin, and rIL-2 enhances only receptor-positive cells, the mixture of cells may appear more sensitive to anti-Tac(Fv)–PE40 with rIL-2.

Prolonged Incubation Times. When incubation time was extended to 2–3 days (patients 3, 4, 6, and 9), cytotoxic activity of anti-Tac(Fv)–PE40 increased as much as 10-fold. Normal cells remained resistant under these conditions (data not shown). We suspect that toxin internalization and inhibition of protein synthesis is a slow process in these freshly isolated patient cells. Whether this situation will be similar in patients or reflects metabolic changes that occur during short-term culture remains to be established. These data suggest that continuous infusion of anti-Tac(Fv)–PE40 for several days could be needed to obtain a maximum clinical response. Continuous infusion may also be required because in mice anti-Tac(Fv)–PE40 has a half-life of 20–40 min (17).

We have shown that anti-Tac(Fv)–PE40 is highly effective not only against ATL established cell lines and normal human lymphocytes activated by phytohemagglutinin or mixed lymphocyte reaction (10, 17) but also against malignant cells directly isolated from patients. ID_{50} values were several orders of magnitude less than the serum level safely obtained in mice, which exceeds 1500 ng/ml (17). We have no animal model of ATL at this time but suspect that intravascular tumor cells will be particularly susceptible to low concentrations of toxin *in vivo*.

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