InterIeukin-2 binding requirements for entry of a diphtheria toxin related interleukin-2 fusion protein into cells

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Interleukin 2 receptor-targeted cytotoxicity. Receptor binding requirements for entry of a diphtheria toxin-related interleukin 2 fusion protein into cells*

The receptor binding requirements for entry of the NAD^+ ADP-ribosyltransferase component of DAB_{486}-IL 2 into target cells were examined. Experiments utilizing cell lines bearing either high-affinity or individual subunits of the interleukin 2 receptor (IL 2R) as well as human peripheral blood mononuclear cells with natural killer activity demonstrate that the high-affinity receptor facilitates delivery of fragment A from DAB_{486}-IL 2 to the cytosol approximately 1000 times more efficiently than either the intermediate-(p75) or low-affinity (p55) forms of the IL 2R. We show that elongation factor 2 (EF-2) in these cells is not quantitatively or qualitatively altered indicating that the relative resistance to intoxication displayed by IL 2R variant cell lines cannot be attributed to an altered intracellular target of the hybrid toxin. We also demonstrate that an alteration in the binding of DAB_{486}-IL 2 to the p75 subunit of the IL 2R may account for the selective cytotoxicity of DAB_{486}-IL 2 for cells bearing the heterodimeric high-affinity IL 2R.

1 Introduction

Because they combine selectivity and lethality, conjugate toxins have long been considered attractive vehicles for specific targeting of neoplastic cells. Traditionally, these hybrid molecules were constructed by chemically coupling portions of bacterial or plant toxins to various cell surface-active ligands, including mAb, hormones, growth factors and lectins [1–5]. Recently, recombinant DNA methodologies have been applied to the genetic assembly of several hybrid toxins. In these cases, α-melanocyte-stimulating hormone and IL 2 sequences have been used to replace the diphtheria toxin receptor-binding domain [6, 7], and transforming growth factor α, IL 2, CD4 and interleukin 6 have been used to replace the receptor-binding domain of *Pseudomonas* exotoxin A [8–11]. In all cases, the resulting gene fusion products were shown to be selectively cytotoxic for eukaryotic cells which express surface receptors for the respective ligands. For example, the diphtheria toxin-related IL 2 fusion protein, IL 2-toxin (hereafter designated DAB_{486}-IL 2), was found to selectively inhibit protein synthesis in IL 2R bearing T cells, including mitogen-activated human T lymphocytes (Waters, manuscript in preparation) and a number of IL $2R^+$ leukemic cell lines [12]. In contrast, cell lines which lack IL 2R were found to be uniformly resistant to the action of this fusion protein.

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In the present report, we further investigate the nature of the IL 2R and DAB_{486}-IL 2 interaction required for entry of the NAD^+ADP-ribosyltransferase (EC 2.4.2.30) component of the fusion protein into target cells. Using cells bearing high-affinity or structural variants of the IL 2R, we demonstrate that high-affinity IL 2R facilitate the delivery of fragment A from DAB_{486}-IL 2 to the cell cytosol 500–1000 times more efficiently than either the low-(p55) or intermediate-(p75) affinity forms of the IL 2R. Because the cytotoxic action of DAB_{486}-IL 2 is dependent upon passage through an acidic endosome [12], the present observations strongly suggest that DAB_{486}-IL 2 bound to the high-affinity form of the IL 2R is rapidly internalized into an acidified vesicle. We also present evidence that the relative resistance of cells that express solely p75 intermediate-affinity IL 2R is largely due to altered binding of the hybrid toxin to the p75 subunit.

2 Materials and methods

2.1 Reagents and buffers

DAB_{486}-IL 2 was purified from extracts of *Escherichia coli* (pABI6508) as previously described [7]. Purified rIL 2 for certain studies was kindly supplied by Dr. M. Gately (Hoffman-La Roche, Nutley, NJ), Dr. K. Koths (Cetus, Emeryville, CA) and K. S. (Takeda Chemical Industries Ltd., Osaka, Japan). Anti-p55 (Tac) was a gift of Dr. T. Waldmann (National Institutes of Health, Bethesda, MD). mAb to the p75 subunit of the IL 2R, anti-Tic*, was used as a clarified ascites fluid.

2.2 Cell culture

The eukaryotic cell lines used in this study are listed in Table 1 (Sect. 3.1) C91/PL cells [13], derived from HTLV-I transfected human cord blood T lymphocytes, were subcul-

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Abbreviations: **EF-2:** Elongation factor 2 **IC_{50}:** Concentration required for 50% inhibition of response

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tured at 3- to 4-day intervals in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 25 mM Hepes, pH 7.4, 2 mM L-glutamine, and 15% FBS. All other cell lines were maintained on a similar subculture schedule in RPMI 1640 medium supplemented with 25 mM Hepes, pH 7.4, 2 mM L-glutamine, and 10% FBS.

2.3 Cytotoxicity assay

Cells were seeded in 96-well V-bottom plates (Nunc, Roskilde, Denmark) at a concentration of 10^5/well in 100 µl complete medium. Prior to assay, MLA-144 cells, which produce IL 2 [14], were first washed in cold, acidified (pH 5) RPMI medium to remove bound IL 2. DAB_{486}-IL 2 was added at varying concentrations (10^{-12} M to 10^{-6} M) in complete medium. Cells cultured with medium alone were included as the control. Following 18 h incubation at 37 °C in a 5% CO_2 atmosphere, the plates were centrifuged for 5 min at 230 × g, and the medium was removed and replaced with 100 µl leucine-free medium (DMEM Selectamine, Gibco) containing 2.5 µCi/ml = 92.5 kBq/ml [^{14}C]leucine (New England Nuclear, Boston, MA). Cells were then incubated at 37 °C for 90 min and collected on glass fiber filters using a cell harvester (Skatron, Sterling, VA). Filters were washed, dried, and counted according to standard methods. All determinations were performed in pentuplicate.

2.4 NK cell activity

Human PBMC from healthy volunteers were incubated for 18 h in RPMI 1640 medium containing 10% FBS in the presence or absence of various concentrations of DAB_{486}-IL 2 and/or 200 U/ml human rIL 2. After washing, PBMC were tested for their cytotoxicity against K-562 target cells to measure NK cell activity, and anti-CD3 mAb-producing hybridomas to measure T cell-mediated cytotoxicity using a 4-h ^{51}Cr-release assay as reported previously [15].

2.5 Determination of elongation factor 2 (EF-2) levels available for ADP-ribosylation

The ADP ribosylation of EF-2 was measured as described by Moynihan and Pappenheimer [16]. Cells were seeded in 24-well Linbro plates (Flow Laboratories, McLean, VA) at a concentration of 5 × 10^5 cells/1.3 ml/well. Assay media consisted of RPMI 1640 with 2 mM L-glutamine and 15% FBS for C91/PL cells and 10% FBS for all other cell lines. For each cell line, DAB_{486}-IL 2 was added to triplicate wells at a final concentration of 10^{-9} M. Two triplicate sets of control cells were incubated in medium alone. Following incubation for 24 h at 37 °C under 5% CO_2, each cell suspension was transferred to a microcentrifuge tube and centrifuged for 5 min. Cell pellets were lysed and assayed for the level of EF-2 available for ADP ribosylation by the addition of purified diphtheria toxin fragment A [17] and [^{32}P]NAD to a final concentration of 2 µg/ml and 1 µCi/ml, respectively. After a 13-min incubation at 40 °C, the reaction was terminated by the addition of 10% trichloroacetic acid. Precipitated protein was collected on Whatman (Clinton, NJ) GF-A glass fiber filters which were then washed, dried, and counted in a liquid scintillation counter (Beckman Instruments, Irvine, CA).

In experiments involving pre-incubation of cell lysates with fragment A, the latter was added to a final concentration of 2.7 × 10^{-6} M in lysis buffer without [^{32}P]NAD. The mixture was incubated at 40 °C for 30 min, after which additional fragment A was added to 2 µg/ml in lysis buffer containing 1 µCi/ml [^{32}P]NAD. The lysates were then processed as described above.

2.6 Iodination of rIL 2

rIL 2 was enzymatically iodinated with enzymobeads (Bio-Rad Laboratories, Richmond, CA) according to the instructions of the manufacturer. One mCi Na^{125}I (DuPont-NEN, Boston, MA) was used in the reaction with 25 µg rIL 2. The reaction was allowed to proceed for seven minutes at room temperature and was quenched by the addition of NaN_3 and NaI. FBS was added to a final concentration of 10% and the mixture was chromatographed on a 2-ml Excellulose GF-5 column (Pierce, Rockford, IL) which had been equilibrated with RPMI 1640 medium supplemented with 25 mM Hepes, pH 7.4, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. The sp. act. of iodinated rIL 2 was approximately 26 µCi/µg (0.4 µCi/pmol).

2.7 Competitive displacement of ^{125}I-labeled rIL 2 ([^{125}I]rIL 2) by rIL 2 or DAB_{486}-IL 2

The radiolabeled rIL-2-binding assay was performed essentially as described by Wang and Smith [18]. Cells were harvested and washed three times with RPMI 1640 medium containing 25 mM Hepes, pH 7.4, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FBS. Cells were added at 5 × 10^6 – 10 × 10^6/ml to [^{125}I]rIL 2 in the presence or absence of increasing concentrations of unlabeled rIL 2 or DAB_{486}-IL 2. The cell suspension was then incubated for 30–120 min at 37 °C under 5% CO_2. In some experiments cells were pretreated at 37 °C for 60 min in Dulbecco's PBS containing 1 mg/ml BSA (Sigma, St. Louis, MO), 15 mM NaN_3, and 50 mM 2-deoxy-D-glucose, pH 7.2, to inhibit internalization of radiolabeled ligand [19]. The reaction was then overlayed on a mixture of 80% 550 fluid (Accumetric Inc., Elizabethtown, KN): 20% paraffin oil (ϱ = 1.03 g/ml) and microcentrifuged, after which the cell pellet was excised. The aqueous phase and pellet of each sample, representing free and bound ligand, respectively, was then counted in a Nuclear Chicago gamma counter.

3 Results

3.1 Facilitated delivery of fragment A from DAB_{486}-IL 2 by high-affinity IL 2R

We have previously demonstrated that immunoaffinity purified DAB_{486}-IL 2 inhibits protein synthesis in several high-affinity IL $2R^+$ cell lines, while IL $2R^-$ cells are resistant to the hybrid toxin [12]. Recently, the high-affinity IL 2R has been shown to be composed of at least two subunits: a 55-kDa glycoprotein (p55, Tac antigen) and a

Table 1. Native and variant IL 2R+ cell lines

Cell line	Source	IL 2R status p75	IL 2R status p55	K_d (nM)	Ref.
C91/PL	HTLV-I-transformed, human T cell	+	+	0.005 (high)	[13]
HUT 102/6TG	HTLV-I-infected, adult T cell leukemia	+	+	0.006–0.019 (high)	[21, 24, 26]
YT2C2	Human immature T cell	+	–	1.5	[22]
SKW6.4	Human B cell leukemia	+	–	1.0–3.0	[30]
MLA-144	Gibbon-T cell leukemia	+	–	0.4–0.9	[14, 26, 31]
MT-1	HTLV-I-infected, adult T cell leukemia	–	+	6–14	[26, 29]

75-kDa (p75) glycoprotein [20–25]. The p75, but not the p55, glycoprotein has been shown to undergo accelerated internalization after binding IL 2 [26–29]. With the availability of a number of cell lines which express either the p55 or p75 subunit (Table 1), we have now examined the IL 2R structural requirements for DAB_{486}-IL 2 action, particularly focusing upon whether the presence of the high-affinity form of the IL 2R is an absolute requirement for intoxication.

As shown in Fig. 1, dose-response analysis of high-, intermediate-, and low-affinity IL 2R-bearing cells demonstrates that cell lines which bear only p 55 or p75 monomeric IL 2R proteins are relatively resistant to DAB_{486}-IL 2. These cells characteristically require concentrations of DAB_{486}-IL 2 in excess of 5×10^{-8} M to achieve 50% inhibition of [^{14}C]leucine incorporation ($IC_{50} \geq 5 \times 10^{-8}$ M). On the other hand, cell lines such as C91/PL and HUT 102/6TG, which bear heterodimeric high-affinity IL 2R, are 500–1000-fold more sensitive to the hybrid toxin ($IC_{50} \leq 1.0 \times 10^{-10}$ M). Thus, nanomolar concentrations of DAB_{486}-IL 2 are sufficient to cause virtually complete inhibition of protein synthesis in high-affinity IL 2R cells with essentially no effect on cells expressing either p75 or p55 subunits alone. Even at DAB_{486}-IL 2 concentrations as high as 10 nanomolar, only a 10%–30% reduction in protein synthesis is observed in p75 or p55 monomer-bearing cells. These results are surprising in view of the reported ability of the p75 subunit to rapidly internalize native IL 2.

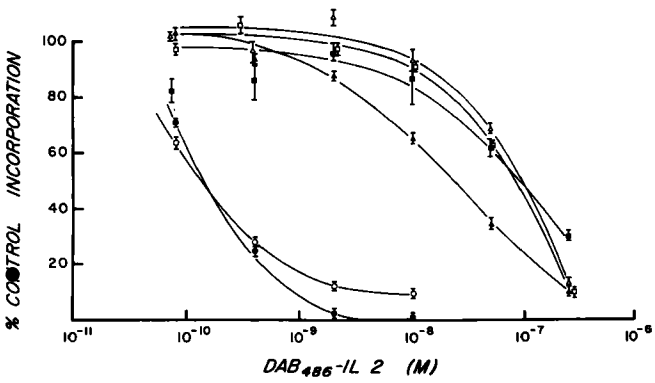

Figure 1. Comparison of sensitivity of IL 2R+ and IL 2R variant cell lines. C91/PL (●) HUT 102/6TG (○) and variant cell lines were incubated with DAB_{486}-IL 2 as described earlier. IC_{50} for C91/PL and HUT 102/6TG is ca. 1×10^{-10} M. Receptor-defective variants YT2C2 (▲), SKW 6.4 (□), MLA-144 (△), and MT-1 (■) are resistant to the action of DAB_{486}-IL 2.

3.2 IL 2R-mediated uptake of DAB_{486}-IL 2 toxin by IL 2R variant cells

Because of the relatively high DAB_{486}-IL 2 concentrations required to inhibit protein synthesis in p75+ p55−-bearing cells, we sought to determine whether the observed cytotoxicity was IL 2R mediated by using free rIL 2, anti-p75 (Tic) and anti-p55 (Tac) mAb to competitively inhibit DAB_{486}-IL 2 action. Table 2 shows that incubation of YT2C2 cells with 2×10^{-7} M DAB_{486}-IL 2 inhibits [^{14}C]leucine incorporation to 57% of control levels. In the presence of a 100-fold molar excess of rIL 2 (2×10^{-5} M) DAB_{486}-IL 2-mediated inhibition of [^{14}C]leucine incorporation is blocked in YT2C2 cells. Similarly, the addition of a 100-fold molar excess of rIL 2 (10^{-7} M) to cultures also blocked DAB_{486}-IL 2 (10^{-9} M)-mediated inhibition of [^{14}C]leucine incorporation in high-affinity IL 2R C91/PL cells. Anti-p55 (Tac) added at essentially equimolar concentrations with the hybrid toxin blocked DAB_{486}-IL 2-mediated protein synthesis inhibition in C91/PL cell cultures but, as expected, had no effect on p55− YT2C2 cells. The addition of anti-p75 (Tic) to these cultures, however, resulted in complete inhibition of DAB_{486}-IL 2 action. Similar results were obtained when anti-p75 (Tic) was added to cultures containing high-affinity IL 2R cells and DAB_{486}-IL 2 (data not shown). These experiments suggest that the action of DAB_{486}-IL 2 on the p75+ p55− cell lines is IL 2R specific, and not merely the result of fluid phase uptake of the hybrid toxin into endocytic vesicles.

3.3 Resistance of human NK cell activity to DAB_{486}-IL 2-mediated cytotoxicity

PBMC with NK activity have been reported to bear only the p75 subunit of the IL 2R on the cell surface [32–34]. Since cells of this phenotype are clearly responsive to IL 2 and appear to be precursors of LAK cell activity [35, 36], we wanted to determine the extent to which DAB_{486}-IL 2 may have an impact on their function. We therefore cultured PBMC from healthy donors in the presence or absence of rIL 2 and DAB_{486}-IL 2, and measured subsequent NK cell

Table 2. Receptor-specific cytotoxicity is mediated by the p75 subunit of the IL 2R in the presence of high concentrations of DAB_{486}-IL 2

Exp. no.	Addition	% Control [^{14}C]leucine incorporation by high- and intermediate-affinity IL 2R-bearing cells: p55/p75	p75
1	–	100	100
	DAB_{486}-IL 2[a]	23	57
	rIL 2[b]	117	89
	DAB_{486}-IL 2 + rIL 2	102	95
2	–	100	100
	DAB_{486}-IL 2[a]	28	55
	anti-p75 (Tic)[c]	134	122
	DAB_{486}-IL 2 + anti-p75 (Tic)	104	145
3	–	100	100
	DAB_{486}-IL 2[a]	24	51
	anti-p55 (Tac)[b]	76	96
	DAB_{486}-IL 2 + anti-p55 (Tac)	73	54

a) Concentration of DAB_{486}-IL 2 used: expts. 1 and 3: p55/p75, 1×10^{-9} M; p75, 2×10^{-7} M. Exp. 2: p55/p75, 8×10^{-10} M; p75, 1×10^{-6} M.
b) rIL 2 added at 100-fold molar excess, anti-p55 (Tac) mAb at 40% molar excess.
c) Anti-p75 (Tic) mAb at 1/10 dilution of clarified ascites fluid.

Table 3. Effect of DAB_{486}-IL 2 on human NK cell activity[a]

Culture conditions	NK cell activity (% specific lysis) E/T ratio: 40	20	10
Medium	26	16	9
rIL 2	43	31	27
DAB_{486}-IL 2 (10^{-7} M)	12[b]	9[b]	7
DAB_{486}-IL 2 (10^{-7} M) + rIL 2	20	14	10
DAB_{486}-IL 2 (10^{-8} M)	27	18	14
DAB_{486}-IL 2 (10^{-8} M) + rIL 2	42	31	19
DAB_{486}-IL 2 (10^{-9} M)	24	20	14
DAB_{486}-IL 2 (10^{-9} M) + rIL 2	38	32	22
DAB_{486}-IL 2 (10^{-10} M)	27	22	14

a) Freshly isolated PBMC from healthy donors ($n = 4$; 2×10^6 cells/ml) were incubated for 18 h in RPMI 1640 medium and 10% FBS in the presence or absence of various concentrations of DAB_{486}-IL 2 and/or 200 U/ml (3×10^{-9} M) human rIL 2. PBMC were washed and tested for their cytotoxicity against K-562 target cells in a 4 h ^{51}Cr-release assay. Results represent means of % specific lysis from 4 different donors at 3 different E/T ratios.
b) $p < 0.002$ (Student's two-tailed t-test).

activity using K-562 target cells in the 4-h ^{51}Cr-release assay. Anti-CD3-induced T cell cytotoxicity was measured in the same assay using an anti-CD3 mAb-producing target cell line [15]. Results shown in Table 3 demonstrate that concentrations of DAB_{486}-IL 2 at or in excess of 10^{-7} M are required to inhibit NK cell activity. Since inhibition is completely blocked by rIL 2, the action of the fusion protein appears to be mediated by the IL 2R. DAB_{486}-IL 2 had no effect on the level of T cell-mediated lysis against anti-CD3 mAb-producing hybridoma target cells (data not shown). The effector cell in this instance is $CD3^+$ and $CD8^+$ with large granular lymphocyte morphology and lacks the IL 2R [15].

3.4 Sensitivity of EF-2 in IL 2R variant cells to diphtheria toxin and DAB_{486}-IL 2

Taken collectively, the above data suggest that either the intracellular target of toxicity is altered in $p75^+$ $p55^-$ cells, or that binding, internalization and processing of DAB_{486}-IL 2 is 500–1000-fold less efficient. In order to examine the former possibility, we measured the level and sensitivity of EF-2 in high-, intermediate-, and low-affinity IL 2R-bearing cell lines.

Table 4. EF-2 available for ADP ribosylation in cell lines bearing high-, intermediate-, and low-affinity IL 2R following exposure to DAB_{486}-IL 2

Cell line	IL 2R	EF-2 available for ADP ribosylation[a] (cpm [^{32}P]-NAD bound): Control	DAB_{486}-IL 2[b]	Fragment A[c]
C91/PL	p55, p75	13 454 ± 986	500	1007 ± 411
MLA-144	p75	11 110 ± 1780	11 178 ± 1717	500
SKW6.4	p75	18 891 ± 2723	18 839 ± 2625	500
MT-1	p55	11 351 ± 947	10 434 ± 1367	500

a) From 5×10^5 cells. Reported values are means ± SE.
b) Eighteen-hour pre-incubation *in vitro* with 1×10^{-9} M DAB_{486}-IL 2.
c) Thirty-minute, 37 °C, pre-incubation of control lysate with 2.7×10^{-6} M purified diphtheria toxin fragment A prior to addition of [^{32}P]NAD.

The data presented in Table 4 indicate that all of the IL 2R variant cell lines contain comparable levels of EF-2 available for ADP ribosylation (control column). However, YT2C2 cells contain somewhat lower levels of EF-2 as measured by this assay. As anticipated, incubation of high-affinity IL 2R-bearing cells with DAB_{486}-IL 2 results in a reduction of EF-2 available for ADP ribosylation (DAB_{486}-IL 2 column). In marked contrast, cells bearing only the p75 or p55 subunit of the IL 2R are fully resistant to the action of 1×10^{-9} M DAB_{486}-IL 2. As shown in Table 4 (fragment A column), preincubation of control cell lysate EF-2 with fragment A purified from diphtheria toxin results in complete loss of EF-2 in the lysate as a target for subsequent toxin action. These results indicate that the sensitivity of EF-2 to fragment A-catalyzed ADP-ribosylation does not vary between cell types. Thus, resistance of the IL 2R variant cell lines to intoxication by DAB_{486}-IL 2 cannot be attributed to an EF-2 which is insensitive to ADP ribosylation.

3.5 Competitive displacement of [^{125}I]rIL 2 by DAB_{486}-IL 2 and rIL 2

The p55 subunit of the IL 2R does not mediate efficient internalization of bound IL 2 [28]. By comparison, IL 2 associated with the p75 subunit of the IL 2R is known to be internalized as rapidly as by the high-affinity IL 2R ($t_{1/2}$ = 15 min) [26]. Accordingly, we reasoned that the resistance to DAB_{486}-IL 2 intoxication displayed by $p75^+$ $p55^-$ cells may be attributable to altered binding rather than internalization. To investigate the nature of the DAB_{486}-IL 2: IL 2R interaction, competitive displacement experiments were performed using ^{125}I-labeled rIL 2, comparing cells expressing the high-affinity form of the IL 2R with those expressing each of the isolated subunits. As shown in Table 5, the concentration of DAB_{486}-IL 2 required to half-maximally displace [^{125}I]rIL 2 from the high-affinity p55 : p75 heterodimer is about 65-fold lower than the concentration required to achieve 50% displacement from either isolated p75 or p55 chains. Also it is noteworthy that equivalent concentrations of DAB_{486}-IL 2 are required to displace [^{125}I]rIL 2 from cells bearing either isolated p75 chains or p55 chains. By comparison, the concentrations of rIL 2 required to achieve 50% displacement of [^{125}I]rIL 2 from each of the three classes of binding sites reflected the characteristic high, intermediate and low affinities of IL 2 binding to these distinct receptor sites.

4 Discussion

The studies reported here demonstrate that the diphtheria toxin-related rIL 2 fusion protein, DAB_{486}-IL 2, is preferentially bound and internalized by cells expressing the heterodimeric high-affinity form of the IL 2R. Cells which bear only p55 or p75 subunits of the IL 2R are relatively resistant to the action of DAB_{486}-IL 2, typically requiring incubation in the presence of concentrations in excess of 5×10^{-8} M to achieve half-maximal inhibition of [^{14}C]leucine incorporation. In marked contrast, other HTLV-I-infected cell lines such as C91/PL and HUT 102/6TG which constitutively express functional high-affinity IL 2R are 500–1000-fold more sensitive to the action of DAB_{486}-IL 2 ($IC_{50} \leq 1 \times 10^{-10}$ M). These DAB_{486}-IL 2 concentrations are comparable to those required to inhibit protein synthesis in recently activated peripheral blood T cell blasts [Waters, manuscript in preparation], as well as leukemic cells freshly isolated from patients with acute and lymphoma-type adult T cell leukemia [37]. On the other hand, human peripheral blood monocytes with NK activity, which are reported to display only the p75 IL 2R subunit on the cell surface [32–34], are as resistant to DAB_{486}-IL 2 action as p75 only cell lines ($IC_{50} \cong 1 \times 10^{-7}$ M). At the elevated DAB_{486}-IL 2 concentrations required to inhibit protein synthesis in p75 IL 2R cells, intoxication is nevertheless receptor mediated; both rIL 2 and mAb directed at the p75 subunit block binding and internalization of the fusion toxin.

The relative inefficiency of DAB_{486}-IL 2 intoxication of p75 IL 2R cells would not have been predicted from a knowledge of the subunit's intrinsic IL 2 binding and internalization characteristics. The p75 subunit is reported to bind rIL 2 with intermediate affinity ($K_d = 10^{-9}$ M) and internalize bound ligand with the same efficiency as the high-affinity IL 2R ($t_{1/2}$ = 15 min) [18, 26]. These characteristics initially led us to consider the possibility that IL 2R variant cells had an EF-2 which is altered at the site where the catalytic subunit of the toxin normally binds. Cells with altered EF-2 have been reported and are considerably less sensitive to diphtherial intoxication [38]. We found, however, that the levels of EF-2 in cell lysates, as well as the intrinsic sensitivity of these EF-2 preparations to ADP ribosylation, are similar in DAB_{486}-IL 2-sensitive and -resistant cells.

An alternative explanation for the 500–1000-fold difference in DAB_{486}-IL 2 sensitivity observed between p75 and high-affinity IL 2R-bearing cells is an alteration in binding of the hybrid toxin to this subunit. Although both the p75 subunit and the high-affinity heterodimer share the common property of rapidly internalizing bound ligand, p75 binding is characterized by slow kinetics of association/dissociation. The high-affinity heterodimer, however, displays the fast on-rate of p55 and the slow off-rate of p75 [18, 31]. Thus, an alteration in DAB_{486}-IL 2 binding to the p75 subunit may more dramatically influence the kinetics of

Table 5. Relative ability of rIL 2 and DAB_{486}-IL 2 to displace [^{125}I]rIL 2 from high-, intermediate- and low-affinity IL 2R

Cell line	IL 2R	50% displacement DAB_{486}-IL 2	50% displacement rIL 2
HUT 102/6TG	p55, p75	$8.1 \pm 0.6 \times 10^{-9}$ M	$3.8 \pm 0.6 \times 10^{-11}$ M
YT2C2	p75	$5.3 \pm 0.2 \times 10^{-7}$ M	$4.4 \pm 0.6 \times 10^{-9}$ M
MT-1	p55	$4.0 \pm 0.5 \times 10^{-7}$ M	$2.2 \pm 0.6 \times 10^{-8}$ M

a) Concentration [^{125}I]rIL 2: HUT 102/6TG, 10 pM; YT2C2, 0.5 nM; MT-1, 1 nM.

DAB_{486}-IL 2 interaction with cells bearing the p75 subunit than with cells expressing the high-affinity heterodimer. The results of our competitive displacement experiments are consistent with this interpretation. As determined by the concentration of fusion protein required to inhibit 50% of specific [^{125}I]rIL 2 binding it is evident that DAB_{486}-IL 2 displays altered binding to both subunits of the IL 2R. However, relative to rIL 2 itself, binding of DAB_{486}-IL 2 to the p75 subunit appears to be more significantly affected than binding to p55.

Since Collins et al. [39] have reported that the N-terminal sequences of native IL 2, particularly Asp^{20}, are essential for binding to the p75 subunit of the IL 2R, we postulate that the more markedly altered binding of DAB_{486}-IL 2 to this subunit may result from steric constraints imposed on the fusion toxin : p75 interaction by the N-terminal fusion of human IL 2 sequences to the truncated C-terminal end of diphtheria toxin. In this instance the fusion junction between the toxin and growth factor components of the hybrid protein may place Asp^{20}, or other key p75 binding residues of IL 2, in internal or less favorable positions. Consistent with this interpretation are the results obtained by Lorberboum-Galski et al. [40] who have described the cytotoxic action of a *Pseudomonas* exotoxin A-based IL 2 fusion protein, IL 2-PE40. In this case, cells which express the high-affinity form of the IL 2R have been found to be only 8–20 times more sensitive to the action of IL 2-PE40 than cell lines expressing either the low or intermediate forms of the IL 2R. Furthermore, only 6-fold differences in p75 binding are reported for IL 2-PE40 binding relative to rIL 2, unlike the 120-fold difference we observe. In IL 2-PE40, the fusion junction is between the C-terminus of IL 2 and the N-terminus of PE40 [9]. Since the N-terminus of this fusion protein consists of IL 2 sequences, this structural difference may allow Asp^{20} a greater degree of freedom to interact with the p75 subunit. The observed cytotoxicity of IL 2-PE40 towards p75 only-bearing cells supports this hypothesis.

The 500–1000-fold difference in sensitivity of p75 and high-affinity IL 2R cells to DAB_{486}-IL 2 intoxication suggests the feasibility of tailoring therapy with this fusion toxin to spare both NK cells and other cells which bear only p75 IL 2R while retaining the ability to selectively target and inactivate cells with high-affinity IL 2 binding sites. In view of the emerging role that NK cells appear to play in both tumor and virus surveillance [41], the subtle, but significant, differences in cellular targeting exhibited by DAB_{486}-IL 2 and IL 2-PE40 may offer experimental therapeutic alternatives for selective intervention in disease states involving one or the other type(s) of IL 2R-expressing cells.

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