

# MONOCLONAL ANTIBODY DEFINING A MOLECULE POSSIBLY IDENTICAL TO THE p75 SUBUNIT OF INTERLEUKIN 2 RECEPTOR

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IL-2 is a well-characterized lymphokine that is responsible for growth or differentiation of lymphocytes. Intracellular signalings induced by IL-2 are transmitted via the surface IL-2R with a high affinity to IL-2 (1-3). The high affinity IL-2R has been demonstrated to be composed of at least two distinct subunits, IL-2Rp55 and IL-2Rp75, each of which contains an IL-2 binding site (4-8). IL-2Rp55, with a molecular weight of  $55 \times 10^3$ , is a so-called Tac antigen that has been defined by mAbs (9-11) and by its gene (12, 13), and does not seem to contain any functional domain for signal transduction. IL-2Rp75, with molecular weights of  $7-75 \times 10^3$ , has been detected by chemical crosslinking with  $^{125}\text{I}$ -labeled IL-2 (4-8). There is increasing evidence that the function of signal transduction is associated with IL-2Rp75. IL-2 has induced intracellular signals for the stimulation of cell growth (14-16) and phosphorylation of cellular proteins (17) in cells expressing IL-2Rp75 but not in those expressing IL-2Rp55 alone (14-16). Furthermore, the internalization of IL-2 was mediated by the high-affinity IL-2R or IL-2Rp75 but not by the low affinity IL-2R, IL-2Rp55 (18-20). Therefore, it is obvious that IL-2Rp75 can induce intracellular signals. However, the molecular nature of IL-2Rp75 and the mechanisms of signal transduction from IL-2R are still unknown. For investigation of these subjects, therefore, preparation of an mAb specific for IL-2Rp75 is desirable.

## Materials and Methods

*Cell Lines and mAbs.* The cell lines used here were seven human IL-2R<sup>+</sup> T cell lines carrying human T cell leukemia virus type I (HTLV-I) (21); TL-Mor, MT-2, HUT102, ILT-Hir, ILT-Mat, TL-Hir, and MT-1, two nonhuman IL-2R<sup>+</sup> T cell lines; Gibbon ape MLA144 and murine CTLL-2, and three human IL-2R<sup>-</sup> cell lines; MOLT-4, Jurkat and HL-60. MT-2C41 is a subclone of MT-2, which expresses a large amount of the IL-2R. ILT-Hir, ILT-Mat, and CTLL-2 were maintained in RPMI-1640 medium supplemented with 10% FCS 2 mM L-glutamine, antibiotics, and 1 nM human rIL-2 (obtained from Shionogi Co., Osaka, Japan). The other cell lines were maintained in the RPMI-1640 medium without IL-2.

mAbs used as controls were two IgG1 mAbs specific for IL-2Rp55, H-31, and H-48 (10, 11), and a IgG1 mAb,  $\gamma$ -481, specific for the Sendai virus (provided by Dr. Tozawa, Kitasato University, Sagamihara, Japan).

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*Preparation of a Hybridoma Producing an mAb.* BALB/c mice were immunized intraperitoneally with  $10^7$  TL-Mor cells per mouse four times at weekly intervals. The TL-Mor cells used for immunization were pretreated with 1.0 ml sera of BALB/c mice hyperimmunized with three human cell lines negative for IL-2R: MOLT-4, HPB-Null, and HL-60. The sensitized spleen cells were fused with a mouse myeloma cell line, SP2/0-Ag14, and hybridoma cell clones were obtained as described previously (22). More than 75,000 clones were screened for production of antibody by IL-2 binding blocking assays firstly with TL-Mor cells bearing IL-2Rp55 and IL-2Rp75 on microplates with vigorous washing, and secondly with MT-1 cells bearing only IL-2Rp55 in microtubes with sucrose cushion as reported previously (11, 18). One clone producing an mAb that blocked the IL-2 binding to TL-Mor cells, but not to MT-1 cells, was isolated and recloned. Finally, the hybridoma cell line, derived from a single cell, was established and named TU27. The class of antibody produced by TU27 is IgG1.

*Binding Assay of IL-2 and TU27 mAb.* Human rIL-2 and purified TU27 mAb were radio-labeled with  $\text{Na}^{125}\text{I}$  (Amersham Corp., Arlington Heights, IL) by the chloramine T method, and their binding assays for various cells were performed as described previously (11, 18). In brief,  $10^6$  cells were incubated with  $^{125}\text{I}$ -IL-2 ( $1.5 \times 10^6$  dpm/pmol) or  $^{125}\text{I}$ -TU27 mAb ( $4 \times 10^6$  dpm/pmol) for 1.5 h at  $4^\circ\text{C}$ . The radioactivities in the supernatant and cells were measured separately. Their bindings were analyzed by Scatchard plots.

For  $^{125}\text{I}$ -IL-2 binding blocking assays with mAbs, the cells were pretreated with  $50 \mu\text{g}/\text{ml}$  of mAb in RPMI-1640 medium containing 1% BSA, 25 mM Hepes and 0.02% sodium azide for 1 h at  $4^\circ\text{C}$  before addition of IL-2.

*Inhibition of TU27 mAb Binding by IL-2.*  $10^6$  MT-2C41 cells were incubated with various concentrations of IL-2 in  $50 \mu\text{l}$  of RPMI-1640 medium containing 1% BSA, 25 mM Hepes, and 0.02% sodium azide at  $4^\circ\text{C}$  for 30 min, and then treated with  $50 \mu\text{l}$  of 1 nM  $^{125}\text{I}$ -TU27 mAb at  $4^\circ\text{C}$  for 1 h. The cells were precipitated in microtubes with 1 M sucrose cushion by centrifugation. The radioactivity associated with cells was counted and the number of binding sites of TU27 mAb was calculated from its specific radioactivity.

*Affinity Labeling of Cells with  $^{125}\text{I}$ -IL-2.* Affinity labeling of cells with  $^{125}\text{I}$ -IL-2 was performed as reported previously (18). In brief,  $10^7$  cells were preincubated for 1 h in the presence of  $50 \mu\text{g}/\text{ml}$  of mAbs or 180 nM unlabeled IL-2 and further incubated with 0.1 nM or 5 nM  $^{125}\text{I}$ -IL-2 for 1 h on ice. The cells were then treated with a chemical crosslinker, disuccinimidyl suberate (DSS Pierce Chemical Co., Rockford, IL), as reported by Sharon et al. (4). The DSS-treated cells were solubilized in lysis buffer (300 mM NaCl, 50 mM Tris-HCl [pH 7.4] and 0.5% NP-40). Then the cell lysates were subjected to 7.5% SDS-PAGE.

*Radioimmunoprecipitation.*  $2-4 \times 10^7$  cells were radiolabeled with  $\text{Na}^{125}\text{I}$  by using iodination reagent (IODO-GEN; Pierce Chemical Co.) as described previously (23). The radiolabeled cells were solubilized in 2 ml of buffer (25 mM Tris HCl [pH 7.5], 140 mM NaCl, 1 mM EDTA, 2 mM PMSF, 0.1% aprotinin, 0.5% NP-40), and appropriate aliquots were incubated with mAbs for 6 h. Then they were incubated with protein A-Sepharose pretreated with anti-mouse IgG for 4 h at  $4^\circ\text{C}$ . Immunoprecipitates bound to protein A-Sepharose were analyzed by 10% SDS-PAGE as described previously (23).

*Incorporation of [ $^3\text{H}$ ]Thymidine.* IL-2-dependent ILT-Mat cells were washed three times with PBS, then preincubated for 8 h in the medium without IL-2, and delivered into 96-well microplates. Subsequently, mAb was added at a concentration of  $50 \mu\text{g}/\text{ml}$ , and then IL-2 diluted serially was added to the wells. The cells were incubated for 24 h at  $37^\circ\text{C}$  under 7%  $\text{CO}_2$  in air. During the last 4 h of incubation  $1 \mu\text{Ci}$  of [ $^3\text{H}$ ]TdR was added to each well. The incorporated [ $^3\text{H}$ ]TdR was counted as described elsewhere (24).

## Results

Cell lines were examined for the numbers of sites of high- and low-affinity IL-2R for IL-2 binding, and then for the numbers of binding sites of TU27 mAb by Scatchard plot analyses. The results are shown in Table I. In all the cell lines bearing high-affinity IL-2R, the numbers of TU27 mAb binding sites were similar to those of high-affinity IL-2R. In MLA144 cells, which have been shown to express only IL-

TABLE I  
Binding Sites of TU27 mAb and Sites of High- and Low-affinity IL-2R

Cell lines	IL-2R (sites/cell)		Binding of TU27 mAb (sites/cell)
	Low-affinity	High-affinity	
TL-Mor	519,000	6,500	5,000
MT-2	620,000	6,300	4,800
MT-2C41	1,700,000	21,000	15,700
HUT102	457,000	3,500	1,900
MT-1	372,000	<100	UD
TL-Hir	208,000	<100	UD
ILT-Hir	140,000	2,800	1,400
ILT-Mat	128,000	3,300	1,700
MLA144	1,600	UD	900
MOLT-4	UD*	UD	UD
Jurkat	UD	UD	UD
HL-60	UD	UD	UD
CTLL-2	752,000	2,400	UD

Scatchard plot analyses for IL-2 and TU27 mAb bindings were performed as described in Materials and Methods.

\* UD, undefined.

2Rp75 with an intermediate affinity to IL-2 (5), the number of TU27 mAb binding sites was also similar to that of IL-2 binding sites. On the other hand, the TU27 mAb binding site was undetectable on MT-1 and TL-Hir cell lines, which bear only IL-2Rp55, and on three human T and monocytic cell lines, MOLT-4, Jurkat, and HL-60, which are negative for IL-2R. A murine IL-2-dependent T cell line, CTLL-2, bearing both high- and low-affinity IL-2R, did not react with TU27 mAb either. These results indicate that TU27 mAb recognizes the antigen expressed on surfaces of human and Gibbon ape T cell lines bearing IL-2Rp75.

The molecular mass of the antigen for TU27 mAb was determined by the SDS-PAGE of radioimmunoprecipitation (Fig. 1). TU27 mAb immunoprecipitated molecules with molecular masses of 71-81 kD (~75 kD) from lysates of <sup>125</sup>I-labeled ILT-Mat, PHA-treated PBL, TL-Mor, MLA144, and MT-2 cells but not TL-Hir, Jurkat, and CTLL-2 cells, whereas H-31 mAb detected a band with an approximate molecular mass of 55 kD in the lysates of cells positive for IL-2Rp55, such as ILT-Mat and PBL cells.

To define further specificity of TU27 mAb, its effects on the IL-2 binding to cells were examined by chemical crosslinking with <sup>125</sup>I-IL-2. MLA144, MT-2, and TL-Hir cells preincubated in the presence or absence of TU27 mAb, H-31 mAb,  $\gamma$ -481 mAb, or unlabeled IL-2 were further incubated with <sup>125</sup>I-IL-2, and then treated with a chemical crosslinker, DSS. The cell lysates were analyzed by the SDS-PAGE followed by autoradiography (Fig. 2). TU27 mAb completely blocked the bindings of <sup>125</sup>I-IL-2 to the IL-2Rp75 of MLA144 and to IL-2Rp75 and IL-2Rp55 of MT-2 cells, but not the bindings to the IL-2Rp55 of TL-Hir cells. On the other hand, H-31 mAb specific for IL-2Rp55 completely blocked the binding to the IL-2Rp55 but not the IL-2Rp75 of these cells. As a control, unlabeled IL-2 blocked all the bindings of <sup>125</sup>I-IL-2 to IL-2Rp55 and IL-2Rp75.

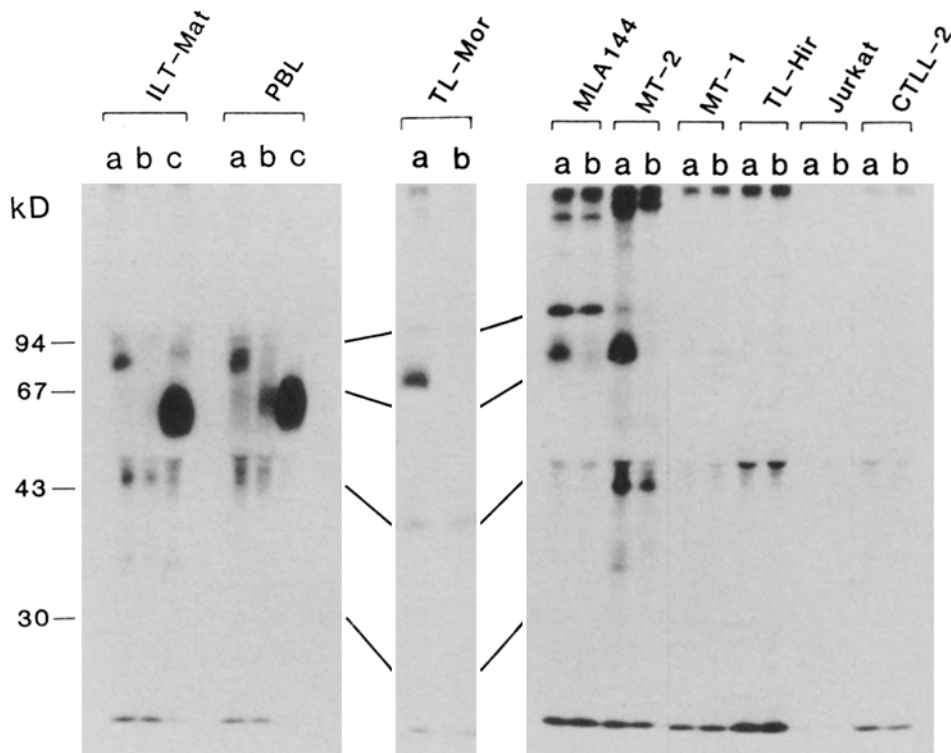


FIGURE 1. Immunoprecipitation of  $^{125}\text{I}$ -labeled cell lysates with mAbs. Surfaces of cells indicated was labeled with  $\text{Na}^{125}\text{I}$ , and their lysates were indirectly precipitated by TU27 mAb (*a*),  $\gamma$ -481 mAb as a negative control (*b*), and H-31 mAb (*c*). Immunoprecipitates were analyzed by 10% SDS-PAGE. PBL were stimulated with PHA for 1 d and then incubated with IL-2 for 2 d.

Furthermore, a Scatchard plot analysis of the IL-2 binding was performed in the presence of TU27 mAb, H-31 mAb, or H-48 mAb (Fig. 3). MT-2C41 cells expressed both high affinity IL-2R ( $K_d = 57 \text{ pM}$ ,  $2.1 \times 10^4$  sites/cell) and low-affinity IL-2R ( $K_d = 38 \text{ nM}$ ,  $1.7 \times 10^6$  sites/cell). TU27 mAb completely blocked IL-2 binding to the high-affinity IL-2R but not to the low-affinity IL-2R. The low-affinity IL-2R detected in the presence of TU27 mAb was calculated at  $2.1 \times 10^6$  sites/cell and  $47 \text{ nM}$  of  $K_d$ , which were not significantly different from those detected in the presence of control mAb. H-31 mAb, on the other hand, completely blocked IL-2 binding to both the high- and low-affinity IL-2R, but the intermediate-affinity IL-2R ( $K_d = 3.4 \text{ nM}$ ,  $1.7 \times 10^4$  sites/cell) became detectable in the presence of H-31 mAb. Furthermore, the effect of IL-2 on  $^{125}\text{I}$ -TU27 binding to MT-2C41 cells was also examined (Fig. 4). When MT-2C41 cells were preincubated with IL-2, the  $^{125}\text{I}$ -TU27 mAb binding to the cells was blocked dose dependently by IL-2.  $1.0 \text{ nM}$  IL-2 almost completely inhibited the  $^{125}\text{I}$ -TU27 mAb binding. These observations suggest that TU27 mAb recognizes an epitope located near to the IL-2 binding site of IL-2R $\text{p}75$ , resulting in blocking of  $^{125}\text{I}$ -IL-2 binding to IL-2R $\text{p}75$  and impeding the composition of the high-affinity IL-2R.

Next, the effects of TU27 mAb on growth of IL-2-dependent ILT-Mat cells were

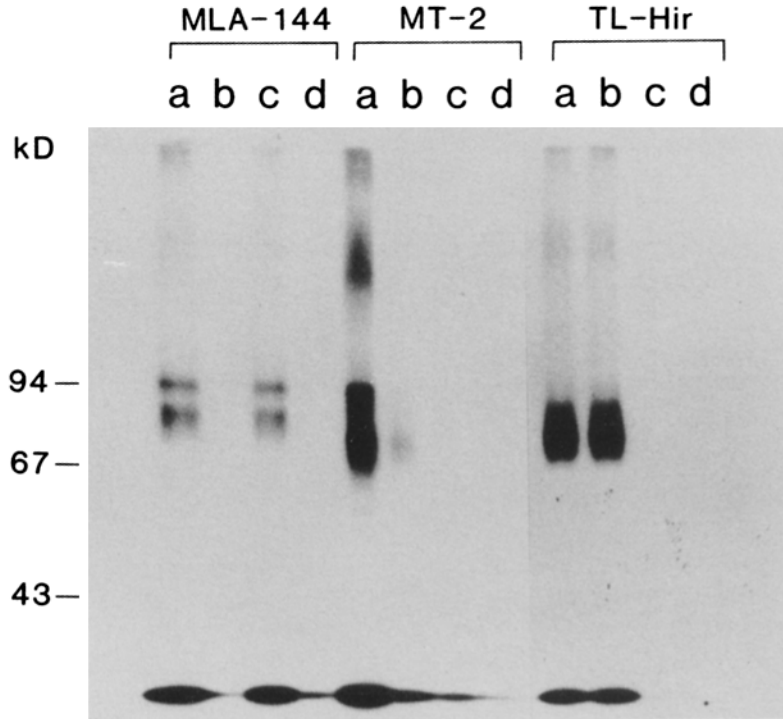


FIGURE 2. Effects of TU27 mAb on affinity-labeling of  $^{125}\text{I}$ -IL-2. Cells were incubated for 1 h in the presence of  $\gamma$ -481 mAb (a), TU27 mAb (b), H-31 mAb (c), or unlabeled IL-2 (d). Subsequently, MLA144 and TL-Hir cells were incubated with 5 nM  $^{125}\text{I}$ -IL-2 and MT-2 cells with 0.1 nM  $^{125}\text{I}$ -IL-2. Their lysates were analyzed by 7.5% SDS-PAGE.

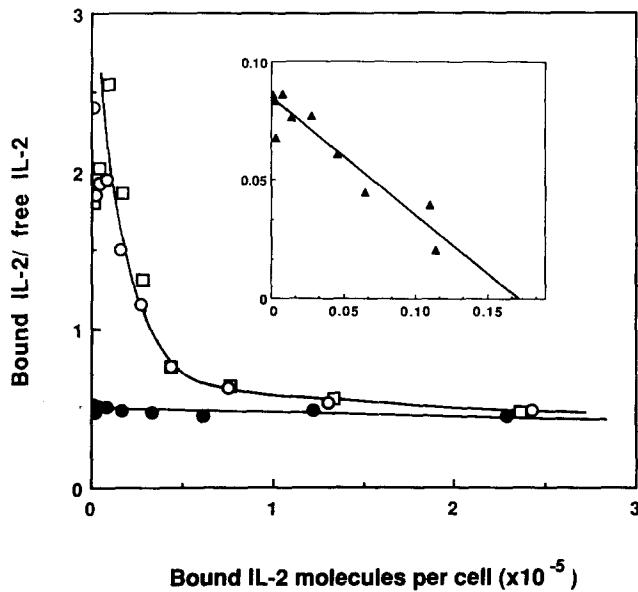


FIGURE 3. Effects of TU27 mAb on Scatchard plot analysis of  $^{125}\text{I}$ -IL-2 binding. MT-2C41 cells were treated with TU27 mAb (●), H-31 mAb (▲), or H-48 mAb (□) and untreated (○) for 1 h at 4°C. They were then incubated with  $^{125}\text{I}$ -IL-2 for 1.5 h at 4°C. Bindings of  $^{125}\text{I}$ -IL-2 were analyzed by Scatchard plot.

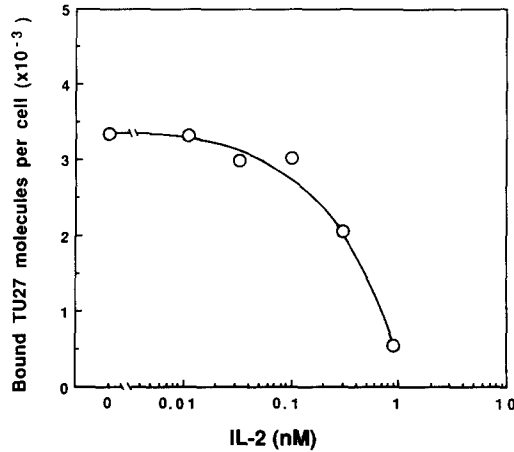


FIGURE 4. Inhibition of  $^{125}\text{I}$ -TU27 mAb binding by IL-2. MT2C41 cells were incubated with indicated doses of IL-2 in 50  $\mu\text{l}$  medium at 4°C for 30 min, and then volumes of 50  $\mu\text{l}$  of 1 nM  $^{125}\text{I}$ -TU27 mAb were added to each well, and cells were incubated at 4°C for 1 h. The number of cells bound  $^{125}\text{I}$ -TU27 mAb molecules was determined as described in Materials and Methods.

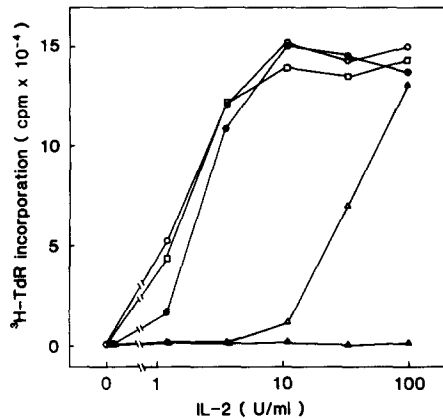


FIGURE 5. Effects of TU27 and H-31 mAbs on  $^3\text{H}$ -TdR incorporation of IL-2-dependent cells. IL-2-induced incorporation of  $^3\text{H}$ -TdR was assayed for IL-2 dependent ILT-Mat cells in the absence (O) or presence of 50  $\mu\text{l}/\text{ml}$  of TU27 mAb (●), H-31 mAb (Δ),  $\gamma$ -481 mAb (□), and a combination of TU27 and H-31 mAbs (▲).

examined by the  $^3\text{H}$ -TdR incorporation assay in the presence of various concentrations of IL-2 from 0 to 100 U/ml, and were compared with those of H-31 mAb (Fig. 5). TU27 mAb partially inhibited the growth of ILT-Mat cells at low concentrations of IL-2, <11 U/ml. At a concentration of 1 U/ml of IL-2, ILT-Mat growth was inhibited up to 80% in the presence of concentrations of TU27 mAb from 11 to 100  $\mu\text{g}/\text{ml}$  (Fig. 6). Growth inhibition of 50% was seen at 2  $\mu\text{g}/\text{ml}$  of TU27. Whereas H-31 mAb completely inhibited the growth at the low concentrations of IL-2, and partially inhibited at 11 and 33 U/ml of IL-2, but little inhibited it at 100 U/ml of IL-2. However, combination of TU27 and H-31 mAbs induced almost complete inhibition of growth in the whole range of IL-2 doses (Fig. 5).

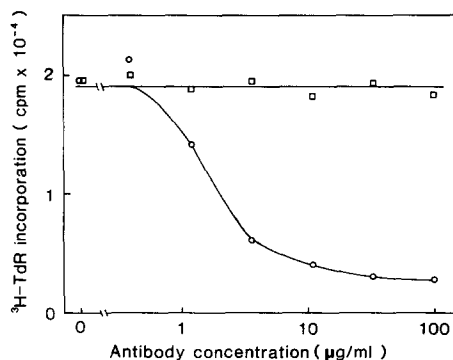


FIGURE 6. Dose-dependent effect of TU27 mAb on IL-2-induced [ $^3\text{H}$ ]TdR incorporation of ILT-Mat cells. IL-2-induced incorporation of [ $^3\text{H}$ ]TdR of IL-2-dependent ILT-Mat cells was determined in the presence of indicated doses of TU27 mAb (○) or  $\gamma$ -481 mAb (□). Cells were cultured in the presence of IL-2 at a concentration of 1 U/ml.

### Discussion

We have just established a mouse hybridoma cell line, TU27, producing an mAb that specifically recognizes human and Gibbon ape IL-2Rp75 as defined by the followings. TU27 mAb reacted with all the cell lines bearing IL-2Rp75 tested, except a murine T cell line, CTLL-2. The number of TU27 mAb binding sites was similar to that of high-affinity IL-2 binding sites. In SDS-PAGE analysis, TU27 mAb detected a cell surface molecule with an approximate molecular mass of 75 kD. Furthermore, TU27 mAb completely blocked the binding of  $^{125}\text{I}$ -IL-2 to IL-2Rp75 but not IL-2Rp55. Vice versa, IL-2 also completely blocked the binding of  $^{125}\text{I}$ -TU27 mAb to cells bearing IL-2Rp75.

There are at least three bands that can be detected by the  $^{125}\text{I}$ -IL-2 affinity labeling and crosslinking method; they are the 55-kD molecule of IL-2Rp55, and the 75- and 70-kD molecules of IL-2Rp75 (7, 25). MLA144 and YT cells are known to express the 75- and 70-kD molecules but not the 55-kD molecule, although the 75- and 70-kD molecules have often been detected as one band (4-6). TU27 mAb immunoprecipitated a 75-kD molecule but not 70-kD molecule, which suggested a possibility that the 75-kD molecule is antigenically different from the 70-kD molecule, although it is still unclear whether these two molecules are related to each other in any way. The present study also suggested that the 75-kD molecule of IL-2Rp75 has an IL-2 binding site. However, we have not obtained any direct evidence that the 75-kD molecule precipitated with TU27 mAb was autophosphorylated (data not shown), suggesting that the 75-kD IL-2R molecule could be distinct from the class of receptors containing a tyrosine kinase.

It has been considered that the IL-2-mediated growth signal is transduced from the high-affinity IL-2R or the intermediate-affinity IL-2R but not from the low-affinity IL-2R, and that the IL-2Rp75 composing the high- and intermediate-affinity IL-2R is crucial for signal transduction (14-18, 20). As TU27 mAb blocked IL-2 binding to IL-2Rp75 and to high-affinity IL-2R but not to low-affinity IL-2R, it was suspected that TU27 mAb inhibits IL-2-dependent growth of cells or that it stimulates cell growth as an agonist of IL-2. The IL-2-dependent growth of ILT-Mat cells was significantly inhibited by TU27 mAb at low concentrations of IL-2, <11 U/ml, but not at high concentrations of IL-2, although the combination of TU27 and H-31

mAbs specific for IL-2Rp55 induced complete inhibition of it in the whole range of IL-2 doses up to 100 U/ml. In spite of the complete blocking of IL-2 binding to high-affinity IL-2R by TU27 mAb in the Scatchard plot analysis, TU27mAb did not completely inhibit the IL-2-dependent cell growth. This suggests two possibilities. One is that a difference exists in the ability of TU27 mAb to block IL-2 binding between the Scatchard plot analysis at 4°C and the [<sup>3</sup>H]TdR incorporation assay at 37°C. Under the latter condition, IL-2Rp75, which can be newly synthesized, may bind IL-2 associated with IL-2Rp55 to compose the high-affinity IL-2R. In such a case, TU27 mAb would not be able to block the IL-2 binding because of its lower binding affinity ( $K_d = 2.1$  nM) to the high-affinity IL-2R ( $K_d = 28$  pM) which could be efficiently composed of a large amount of IL-2 associated with IL-2Rp55 expressed on cell surface. The other possibility is that the IL-2-mediated growth signal could be transduced not only from the high-affinity IL-2R but also from the low-affinity IL-2R of ILT-Mat cells in the presence of TU27 mAb. There is a report describing that L cells transfected with the IL-2Rp55 gene were stimulated to incorporate [<sup>3</sup>H]TdR by IL-2 (26), although IL-2 has not been found to induce any intracellular signaling in cell lines expressing only the low-affinity IL-2R, IL-2Rp55, in other reports (17, 27, 28). To determine which of the above possibilities for signal transduction from IL-2R, further characterization of the molecular nature of IL-2Rp75 is required. Our new mAb, TU27 mAb, should be useful for such studies.

### Summary

A mouse hybridoma cell line, TU27, producing an mAb was established. TU27 mAb reacted with various human and Gibbon ape T cell lines bearing the IL-2R p75 (IL-2Rp75), but not with cell lines expressing only Tac antigen, IL-2Rp55, and numbers of its binding sites on cell surfaces were similar to those of high-affinity IL-2R. Radioimmunoprecipitation with TU27 mAb defined a molecule with a molecular mass of 75 kD on the surface of IL-2Rp75 bearing cells. TU27 mAb completely blocked IL-2 binding to IL-2Rp75 and to the high-affinity IL-2R but not to IL-2Rp55 composing the low-affinity IL-2R. The IL-2-dependent growth of a human T cell line, ILT-Mat, was significantly inhibited by TU27 mAb only at low concentrations of IL-2, and combination of TU27 mAb and H-31 mAb specific for IL-2Rp55 completely inhibited the cell growth even at high concentrations of IL-2. These data strongly suggest that TU27 mAb is specific for the human IL-2Rp75.

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