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 55×10^3, 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}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$^+$ 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$^+$ T cell lines; Gibbon ape MLA144 and murine CTLL-2, and three human IL-2R$^-$ 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, γ-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 radiolabeled with $Na^{125}I$ (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}I-IL-2 (1.5×10^6 dpm/pmol) or ^{125}I-TU27 mAb (4×10^6 dpm/pmol) for 1.5 h at 4°C. The radioactivities in the supernatant and cells were measured separately. Their bindings were analyzed by Scatchard plots.

For ^{125}I-IL-2 binding blocking assays with mAbs, the cells were pretreated with 50 μg/ml of mAb in RPMI-1640 medium containing 1% BSA, 25 mM Hepes and 0.02% sodium azide for 1 h at 4°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 μl of RPMI-1640 medium containing 1% BSA, 25 mM Hepes, and 0.02% sodium azide at 4°C for 30 min, and then treated with 50 μl of 1 nM ^{125}I-TU27 mAb at 4°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}I-IL-2. Affinity labeling of cells with ^{125}I-IL-2 was performed as reported previously (18). In brief, 10^7 cells were preincubated for 1 h in the presence of 50 μg/ml of mAbs or 180 nM unlabeled IL-2 and further incubated with 0.1 nM or 5 nM ^{125}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 nM NaCl, 50 mM Tris-HCl [pH 7.4] and 0.5% NP-40). Then the cell lysates were sujected to 7.5% SDS-PAGE.

Radioimmunoprecipitation. $2–4 \times 10^7$ cells were radiolabeled with $Na^{125}I$ 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°C. Immunoprecipitates bound to protein A-Sepharose were analyzed by 10% SDS-PAGE as described previously (23).

Incorporation of [^{3}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 μg/ml, and then IL-2 diluted serially was added to the wells. The cells were incubated for 24 h at 37°C under 7% CO_2 in air. During the last 4 h of incubation 1 μCi of [^{3}H]TdR was added to each well. The incorporated [^{3}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 ^{125}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 ^{125}I-IL-2. MLA144, MT-2, and TL-Hir cells preincubated in the presence or absence of TU27 mAb, H-31 mAb, γ-481 mAb, or unlabeled IL-2 were further incubated with ^{125}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 ^{125}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 ^{125}I-IL-2 to IL-2Rp55 and IL-2Rp75.

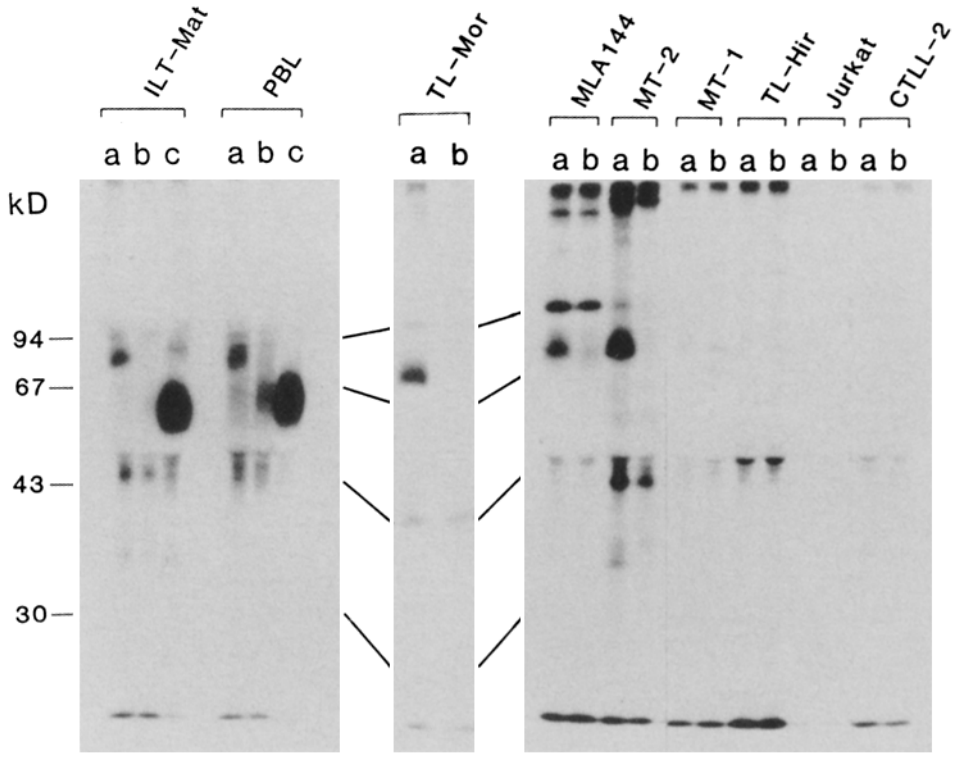

FIGURE 1. Immunoprecipitation of ^{125}I-labeled cell lysates with mAbs. Surfaces of cells indicated was labeled with Na^{125}I, and their lysates were indirectly precipitated by TU27 mAb (*a*), γ-481 mAb as a negative control (*b*), and H-31 mAb (*c*). Immunoprecipitates were analyzed by 10% SDS-PAGE. 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 pM, 2.1 × 10^4 sites/cell) and low-affinity IL-2R (K_d = 38 nM, 1.7 × 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 × 10^6 sites/cell and 47 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 nM, 1.7 × 10^4 sites/cell) became detectable in the presence of H-31 mAb. Furthermore, the effect of IL-2 on ^{125}I-TU27 binding to MT-2C41 cells was also examined (Fig. 4). When MT-2C41 cells were preincubated with IL-2, the ^{125}I-TU27 mAb binding to the cells was blocked dose dependently by IL-2. 1.0 nM IL-2 almost completely inhibited the ^{125}I-TU27 mAb binding. These observations suggest that TU27 mAb recognizes an epitope located near to the IL-2 binding site of IL-2Rp75, resulting in blocking of ^{125}I-IL-2 binding to IL-2Rp75 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

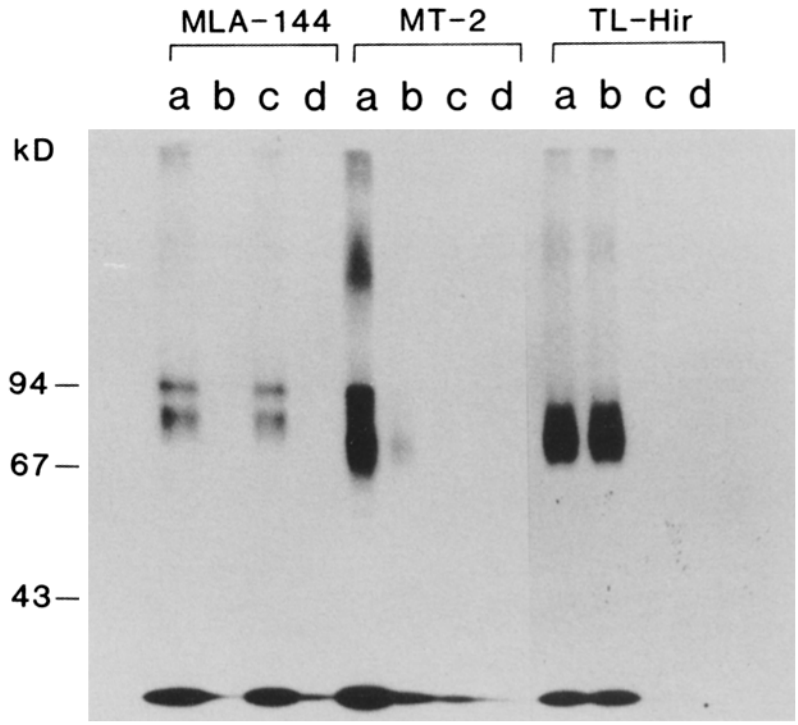

FIGURE 2. Effects of TU27 mAb on affinity-labeling of ^{125}I-IL-2. Cells were incubated for 1 h in the presence of γ-481 mAb (*a*), TU27 mAb (*b*), H-31 mAb (*c*), or unlabeled IL-2 (*d*). Subsequently, MLA144 and TL-Hir cells were incubated with 5 nM ^{125}I-IL-2 and MT-2 cells with 0.1 nM ^{125}I-IL-2.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

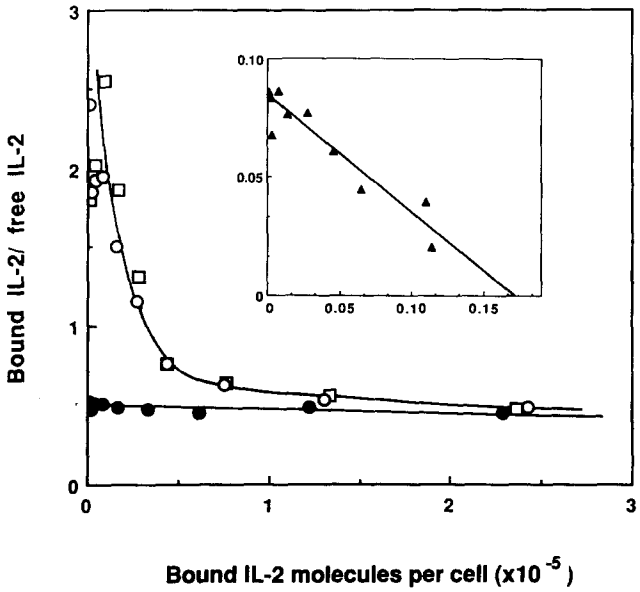

FIGURE 3. Effects of TU27 mAb on Scatchard plot analysis of ^{125}I-IL-2 binding. MT-2C41 cells were treated with TU27 mAb (●), H-31 mAb (▲), or H-48 mAb (□) and untreated (○) for 1 h at 4°C. They were then incubated with ^{125}I-IL-2 for 1.5 h at 4°C. Bindings of ^{125}I-IL-2 were analyzed by Scatchard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

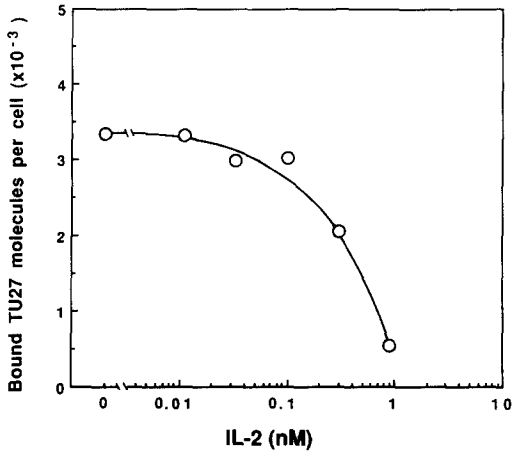

FIGURE 4. Inhibition of ^{125}I-TU27 mAb binding by Il-2. MT2C41 cells were incubated with indicated doses of IL-2 in 50 μl medium at 4°C for 30 min, and then volumes of 50 μl of 1 nM ^{125}I-TU27 mAb were added to each well, and cells were incubated at 4°C for 1 h. The number of cells bound ^{125}I-TU27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

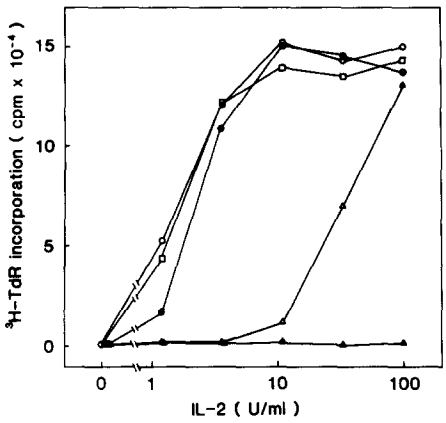

FIGURE 5. Effects of TU27 and H-31 mAbs on [^{3}H]TdR incorporation of IL-2-dependent cells. IL-2-induced incorporation of [^{3}H]TdR was assayed for IL-2 dependent ILT-Mat cells in the absence (○) or presence of 50 μl/ml of TU27 mAb (●), H-31 mAb (△), γ-481 mAb (□), and a combination of TU27 and H-31 mAbs (▲).

examined by the [^{3}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 μg/ml (Fig. 6). Growth inhibition of 50% was seen at 2 μg/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).

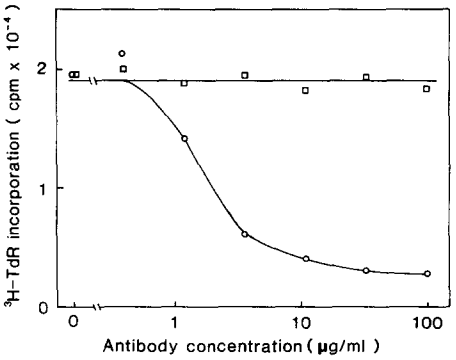

FIGURE 6. Dose-dependent effect of TU27 mAb on IL-2-induced [^{3}H]TdR incorporation of ILT-Mat cells. IL-2-induced incorporation of [^{3}H]TdR of IL-2-dependent ILT-Mat cells was determined in the presence of indicated doses of TU27 mAb (O) or γ-481 mAb (□). Cells were cultured in the presence of IL-2 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 sitse 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}I-IL-2 to IL-2Rp75 but not IL-2Rp55. Vice versa, IL-2 also completely blocked the binding of ^{125}I-TU27 mAb to cells bearing IL-2Rp75.

There are at least tnree bands that can be detected by the ^{125}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 [^{3}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 [^{3}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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