HIGH-AFFINITY RECEPTOR–MEDIATED INTERNALIZATION AND DEGRADATION OF INTERLEUKIN 2 IN HUMAN T CELLS

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Interleukin 2 is a single polypeptide that functions as a growth factor for T and B cells (1–7). The cell growth signal induced by IL-2 is transduced via specific receptors on the cell surface (8). Recently, two distinct IL-2-R with high and low affinities for IL-2 were identified in human T and B cells, as well as murine T cells (9, 10). The mitogenic activity of IL-2 is transmitted via the high-affinity receptor (11–13), but little information is available about the mechanism of signal transmission from the IL-2-R. Hormones and other peptides are internalized into cells by endocytosis after binding to specific receptors on the cell surface (14–21). Internalization is the way to transport ligands such as transferrin (21) and low density lipoprotein (LDL)[1] (17), into the cytoplasm for use in the cell. Internalization of epidermal growth factor (EGF) (16, 22), platelet-derived growth factor (14), transferrin (21), insulin (23, 24), and LDL (17) appears to be essential for degradation and recycling of these ligands or their receptors. Furthermore, internalization of EGF and insulin may be related to triggering of the growth signal of appropriate cells (25–28), although protein kinase activity associated with the receptors is also thought to be involved in the mechanism of signal transduction (29).

After binding to specific receptors, IL-2 is internalized and then degraded (11, 30), but the mechanism of its internalization is unknown. Herein, we examine in detail the internalization and metabolic fate of IL-2 in various human cells. For this study, we used human T cell leukemia/lymphoma (lymphotrophic) virus type I (HTLV-I)-carrying human cell lines and PHA-treated PBL. HTLV-I-carrying T and B cell lines express IL-2-R constitutively without stimulation from antigens and mitogens (31, 32), and we previously (33) showed that the constitutive expression of IL-2-R was induced by HTLV-I infection. Since these cell lines can be classified into at least three groups with respect to their IL-2 reactivities, e.g., those with IL-2-dependent growth, IL-2-independent growth, and IL-2-mediated inhibition of growth (34), they were thought to be useful for

studies on normal and abnormal transduction of growth signals, which are elicited with or without IL-2-binding to receptors, respectively. We report that IL-2 bound to high-affinity receptors was rapidly internalized and degraded dependent on temperature in these human cells.

Material and Methods

Cell Lines. The origins, surface phenotypes, and characterization of IL-2-R of the cell lines used here were described previously (32). The ILT-Yan cell line is an IL-2-dependent cell line carrying HTLV-I. The TL-Mor cell line is an immortalizing transformed T cell line induced by HTLV-I. The MT-1 cell line carrying HTLV-I was established by Miyoshi et al. (35). PBL from a normal donor were treated with 1.0% PHA-M for 3 d in vitro. TL-Mor and MT-1 cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics. ILT-Yan cells were maintained in RPMI 1640 medium supplemented with 20% FCS, 2 mM L-glutamine, antibiotics, and 250 Jurkat U/ml of human rIL-2 (Shionogi Co., Osaka, Japan).

Before treatment with IL-2, the cells were always washed twice with PBS and incubated in RPMI 1640 medium containing 20% FCS and 25 mM Hepes (RPMI-Hepes) for 1 h at 37°C.

Radiolabeling of IL-2. rIL-2 was iodinated by the chloramine T method as reported previously (33). The specific activity of ^{125}I-labeled IL-2 was 5.9–8.8 × 10^5 cpm/pmol. The bioactivity of the rIL-2 was not significantly different from Jurkat-derived IL-2, and little affected by iodination.

^{3}H-labeled rIL-2 was prepared in a cell-free system of rabbit reticulocyte lysate (Amersham Corp., Buckinghamshire, United Kingdom) with L-4,5-[^{3}H]leucine (Amersham Corp.). The plasmid used in the transcription-translation system contained a gene coding for human IL-2 (36). The polypeptide synthesized was confirmed to be IL-2 by SDS-PAGE. The specific activity of ^{3}H-labeled IL-2 was ~1.6 × 10^6 cpm/nmol.

IL-2 Binding Assay. IL-2 binding was measured by a modification of the ^{3}H-labeled anti-Tac antibody binding assay reported by Depper et al. (37). Samples of 5–10 × 10^5 cells were suspended in 120 μl of chilled ^{125}I–IL-2 samples, which were serially diluted with RPMI-Hepes containing 0.02% sodium azide in wells of microplates (3911; Falcon Labware, Oxnard CA). The microplates were shaken for 30 min at 4°C and further incubated at 4°C for 1 h. The supernatant (A) was harvested, and the cell pellet was gently resuspended in 100 μl of PBS. The cell suspension was then layered on 750 μl of RPMI 1640 medium containing 1 M sucrose and 0.02% BSA in a microtube (IEDA Trading Corp., Tokyo, Japan). After centrifugation at 12,000 rpm for 3 min at 4°C, the supernatant (B) and cell precipitate were harvested. The radioactivities of the supernatant (A + B) and precipitated cells were counted. The specific binding of ^{125}I–IL-2 to cells was calculated by subtracting the count of samples added with excess unlabeled IL-2 (>2 μM) for binding competition from the observed count. The count of samples with unlabeled IL-2 was always <30% of the observed count. Dissociation constants (K_d) and number of binding sites were calculated from Scatchard plots by the NONLIN program of Metzler and McEwen (38). The IL-2 binding assay was done in duplicate.

Acid Treatment of Cell-bound IL-2. Acid treatment was used to remove IL-2 attached to the cell surface. Cells (2 × 10^6) were incubated with 200 pM ^{125}I–IL-2 in RPMI-Hepes at 0°C on ice for 30 min. They were then washed three times with PBS containing 3% BSA, resuspended in 1.0 ml of glycine-HCl buffer or RPMI-Hepes, and incubated for 10 min at 0°C on ice. After centrifugation, the radioactivities of the acid-removable glycine buffer fraction and acid-unremovable cell precipitate fraction were each counted.

Determination of Internalized and Degraded IL-2. Cells (2 × 10^6) were incubated with 200 pM ^{125}I–IL-2 in RPMI-Hepes at 0°C on ice for 30 min. Then they were washed three times with PBS-BSA, resuspended in 1.0 ml RPMI-Hepes, and incubated at 37°C in a water bath for the indicated time. The suspension was centrifuged, the culture supernatant was harvested, and the cell pellet was then treated with chilled 0.2 M glycine buffer (pH 2.8) for 10 min at 0°C on ice. The radioactivities of the culture supernatant

fraction, acid-removable glycine buffer fraction, and acid-unremovable cell precipitate were each counted. For determination of degradation of ^{125}I–IL-2, these fractions were further treated with 10% TCA in the presence of 1 mg/ml BSA overnight at 4°C as described previously (11). The radioactivities of the TCA-soluble and -insoluble materials were counted separately.

Light-microscopic Detection of Internalized IL-2. For light-microscopic autoradiography, cells were treated with 1 nM [3H]IL-2 at 0°C for 30 min. They were then washed, resuspended in 1.0 ml RPMI-Hepes, and incubated at 37°C for the indicated time. Subsequently, they were fixed in 2% glutaraldehyde in PBS for 1 h, washed three times with PBS, and postfixed in 1% osmium tetraoxide for 1 h. All these steps were carried out at 4°C. The materials were then dehydrated in a graded ethanol series and embedded in Epon-Araldite. Thick (2 μm) sections were cut on a Porter-Blum MT-5000 ultramicrotome with glass knives. The sections were placed on glass slides and were coated by dipping in Sakura NR-M2 emulsion. The sections were exposed at 4°C for 2 wk. After development with Kodak D19 for 4 min, the sections were stained with toluidine blue and examined by a light microscopy (Nikon, Tokyo, Japan).

Silver grains in sections were counted under light microscope. Silver grains in three portions, the cell surface, the cytoplasm, and the nucleus, were counted separately.

Result

Scatchard Plot Analysis of IL-2-R. ^{125}I–IL-2 binding assays were performed to determine the density and affinity of IL-2-R expressed on various human cells, as described in Materials and Methods. Both high- and low-affinity receptors were detected on two HTLV-I-carrying T cell lines, ILT-Yan and TL-Mor, and on PHA-treated normal PBL (Fig. 1). In contrast, HTLV-I-carrying MT-1 cell line expressed predominantly low-affinity receptors, with very few high-affinity
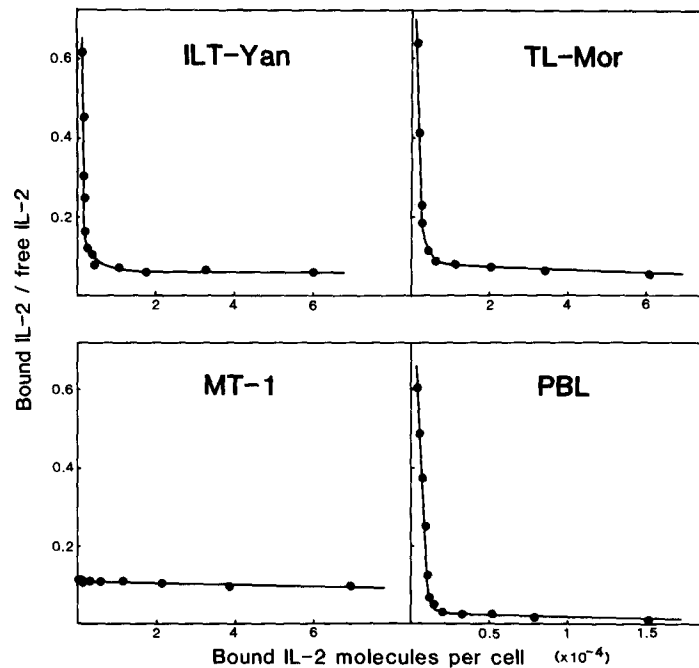

FIGURE 1. Scatchard plot analysis of binding of ^{125}I-labeled IL-2. ^{125}I–IL-2 binding assays were performed as described in Materials and Methods.

receptors in the culture conditions used (Fig. 1). The K_d of high- and low-affinity receptors were estimated by computer analysis as 7.6–16.8 pM and 20.9–64.2 nM, respectively (Table I). The densities of high-affinity receptors ranged from 1,240 to 1,630 sites/cell, and were not significantly different on HTLV-I-carrying T cells and PHA-treated PBL (Table I), but the densities of low-affinity receptors on the three HTLV-I-carrying T cell lines were 5–10 times higher (201,000–560,000 sites/cell) than on the PHA-treated PBL (49,000 sites/cell; Table I).

Effect of Acid Treatment on Cell-bound IL-2. We used acid treatment to distinguish surface-bound IL-2 from intracellular IL-2. ILT-Yan cells were incubated with 200 pM ^{125}I–IL-2 at 0°C, washed, and treated with glycine buffers of pH 2.8, 3.3, 3.8, and 4.8, or with RPMI-Hepes (pH 7.4). The radioactivities of the acid-unremovable cell precipitate fractions and acid-removable buffer fractions were then measured. Treatment at pH 2.8, 3.3, or 3.8 removed >90% of the surface-bound IL-2, whereas treatment at pH 4.8 or 7.4 removed <10% (Fig. 2). Acid treatment removed IL-2 from TL-Mor cells and PHA-treated PBL in the same way (data not shown).

Internalization and Degradation of IL-2. Cell-bound IL-2 was rapidly internalized to be acid-unremovable when it was incubated at above 0°C. ILT-Yan cells treated with 200 pM ^{125}I–IL-2 at 0°C were washed and incubated at various temperatures for the times indicated in Fig. 3, centrifuged, and the cell pellet was treated with acid buffer (pH 2.8). At 37°C, a rapid increase in internalized IL-2 was detected during the first 30 min of incubation, and >50% of the cell-bound IL-2 was internalized within the first 10 min, whereas the amount of surface-bound acid-removable IL-2 decreased proportionately. At lower temperatures, the appearance of internalized IL-2 was slower, and at 0°C, no internalized IL-2 appeared within 120 min. The maximum levels of internalized IL-2 at 37 and 27°C were similar, and were observed after incubation for 30 and 120 min, respectively. After reaching a maximum, the level decreased rapidly at 37°C. At 17°C, the internalized IL-2 increased linearly, and conversely, surface-bound IL-2 decreased for at least 120 min. Radioactivity was also detected in the culture supernatant: it increased rapidly after at least 60 min at 37°C, but little at the lower temperatures (Fig. 3). This increase in radioactivity in the culture supernatant at 37°C correlated quantitatively with decrease in internalized IL-2. The $t_{1/2}$ of IL-2 internalization was estimated as <10 min in the cells at 37°C. Similar kinetics of IL-2 internalization at 37°C was observed in TL-Mor

TABLE I
Affinity and Number of IL-2-R

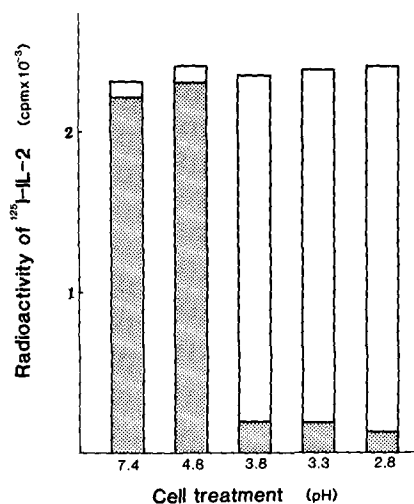| Cell line	High affinity		Low affinity	
	K_d (pM)	Sites/cell	K_d (nM)	Sites/cell
ILT-Yan	7.6 ± 2.0	1,460 ± 160	20.9 ± 15.1	201,000 ± 105,000
TL-Mor	11.1 ± 1.9	1,630 ± 440	38.1 ± 11.9	326,000 ± 61,000
MT-1	UD[‡]	UD	64.2 ± 4.7	560,000 ± 283,000
PBL[*]	16.8 ± 8.0	1,240 ± 460	41.0 ± 37.0	49,000 ± 18,000

[*] PBL were treated with PHA for 3 d.
[‡] UD, undetectable.

FIGURE 2.   Removal of surface-bound IL-2 by acid treatment. ILT-Yan cells were incubated with 200 pM ^{125}I–IL-2 at 0°C for 30 min, and washed. They were then treated with chilled 0.2 M glycine-HCl buffer at the indicated pH, or with RPMI-Hepes (pH 7.4). The radioactivities of acid-removable buffer fractions (open bars) and acid-unremovable cell precipitate fractions (shaded bars) were counted.

cells and PHA-treated PBL, but too little IL-2 bound to MT-1 cells for examination of IL-2 internalization (Fig. 3). The reason for the small number of IL-2 bound to MT-1 cells is that the procedure used for treatment of cells with IL-2 permitted IL-2 to bind to high-affinity receptors but not low-affinity receptors, because three washings of cells with PBS-BSA after treatment with IL-2 resulted in dissociation of IL-2 from low-affinity receptors, as shown in Fig. 4.

For examination of the degradation of IL-2, the culture supernatant, acid-removable, and acid-unremovable fractions of ILT-Yan cells obtained in the experiment at 37°C in Fig. 3 were treated with 10% TCA (Fig. 5). Treatment with TCA resulted in scarcely any precipitation of radioactivity from the culture supernatant fraction but almost complete precipitation of the radioactivity in the acid-removable fraction. Most acid-unremovable radioactivity was also precipitable from cells within 30 min of incubation, but subsequently became partially resistant to precipitation. These findings suggest that ^{125}I–IL-2 was internalized in the cells, becoming acid-unremovable, and was then degraded to small TCA-soluble fragments that were released from the cells, resulting in radioactivity in the culture supernatant. Similar degradation of IL-2 was detected with TL-Mor cells and PHA-treated PBL (data not shown). The $t_{1/2}$ of IL-2 degradation was estimated as 60–80 min in these cells.

Light-microscopic autoradiography confirmed that the acid-unremovable cell-associated IL-2 is intracellular IL-2 internalized by the cells. ILT-Yan cells were treated with [^{3}H]IL-2 at 0°C for 30 min, washed, and incubated at 37°C for the indicated times, and then subjected to microscopic autoradiography (Fig. 6). Localization of the grains observed in Fig. 6 was graphed in Fig. 7, and nonspecific binding of [^{3}H]IL-2 was calculated to be <10% of the total binding by adding unlabeled IL-2 (data not shown). Most of the grains of [^{3}H]IL-2 were
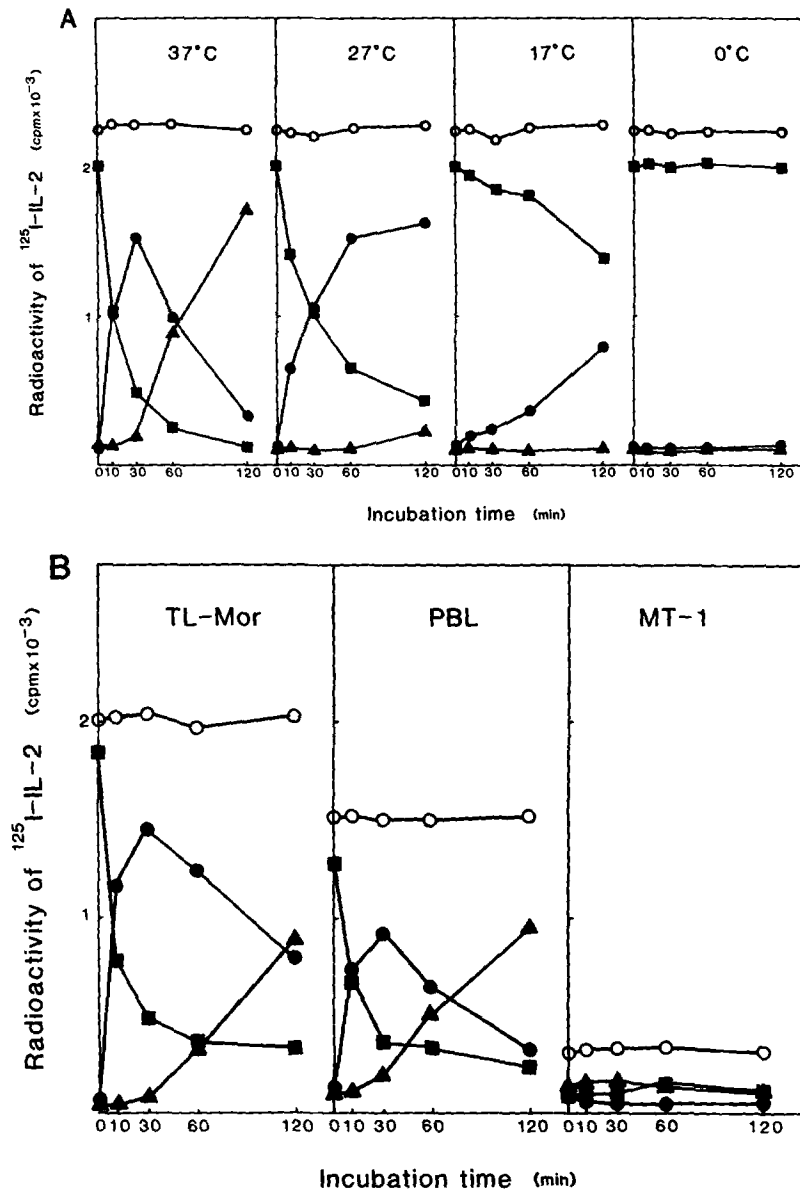
FIGURE 3. Temperature-dependent appearance of internalized IL-2. (A) ILT-Yan cells were incubated with 200 pM ^{125}I-IL-2 at 0°C for 30 min, washed, then incubated at various temperatures for the indicated times. They were then centrifuged and were treated with chilled 0.2 M glycine buffer, pH 2.8, for 10 min. The radioactivities of culture supernatants (▲), acid-removable glycine buffer fractions (■) and acid-unremovable cell precipitate fractions (●) were counted. The sum of the counts in these three fractions is shown as the total radioactivity (○). (B) TL-Mor, PHA-treated PBL and MT-1 cells were examined similarly at 37°C.
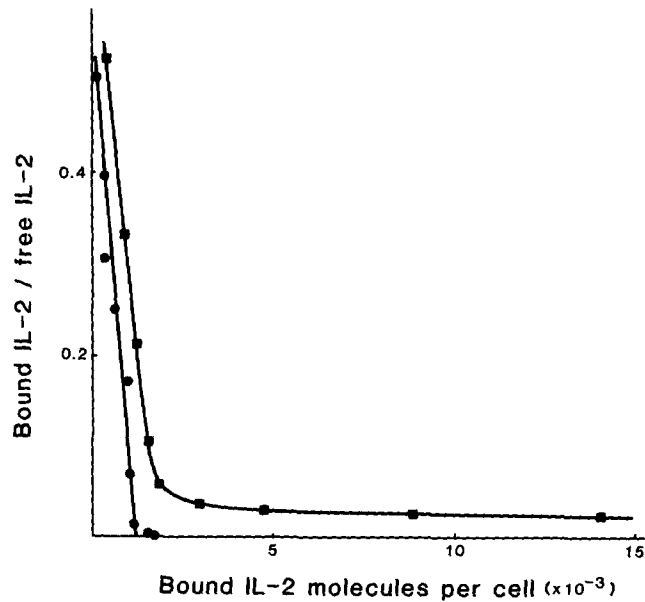
FIGURE 4. Scatchard plot analysis of ^{125}I-IL-2-binding after washing cells. ILT-Yan cells were treated with ^{125}I-IL-2 at 0°C, then washed three times with PBS-BSA (●). ILT-Yan cells were treated with ^{125}I-IL-2 at 0°C, and the cell-associated ^{125}I-IL-2 was separated by centrifugation with 1 M sucrose cushion without washing, as described in the IL-2 binding assay of Materials and Methods (■). Specific binding of ^{125}I-IL-2 to cells were calculated by subtracting nonspecific binding of ^{125}I-IL-2 from observed binding.
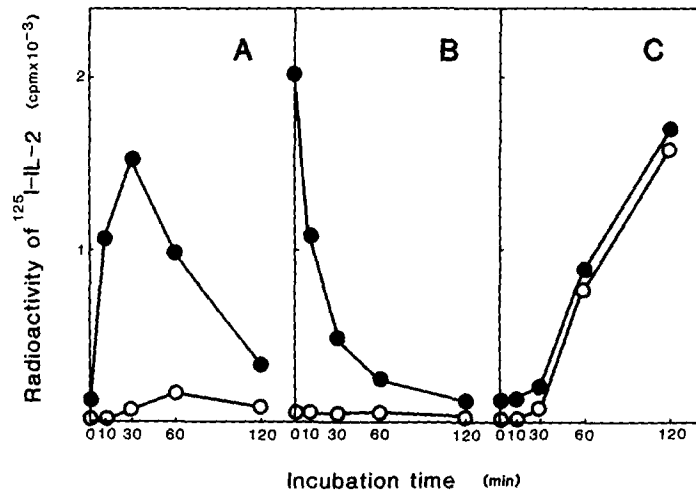

FIGURE 5. Degradation of ^{125}I-IL-2. The acid-unremovable (A), acid-removable (B) fractions, and culture supernatant (C), of ILT-Yan cells obtained in the experiment in Fig. 3 at 37°C were treated with 10% TCA overnight at 4°C. The TCA-soluble (○) and total (TCA-soluble + TCA-insoluble) (●) radioactivities were counted.

seen near the cell surface at 0 min of incubation. After incubation for 5 min, about half the grains had moved to the cytoplasm, and 10% of the grains were seen in the nucleus. After 60 min incubation, the numbers of cell surface–
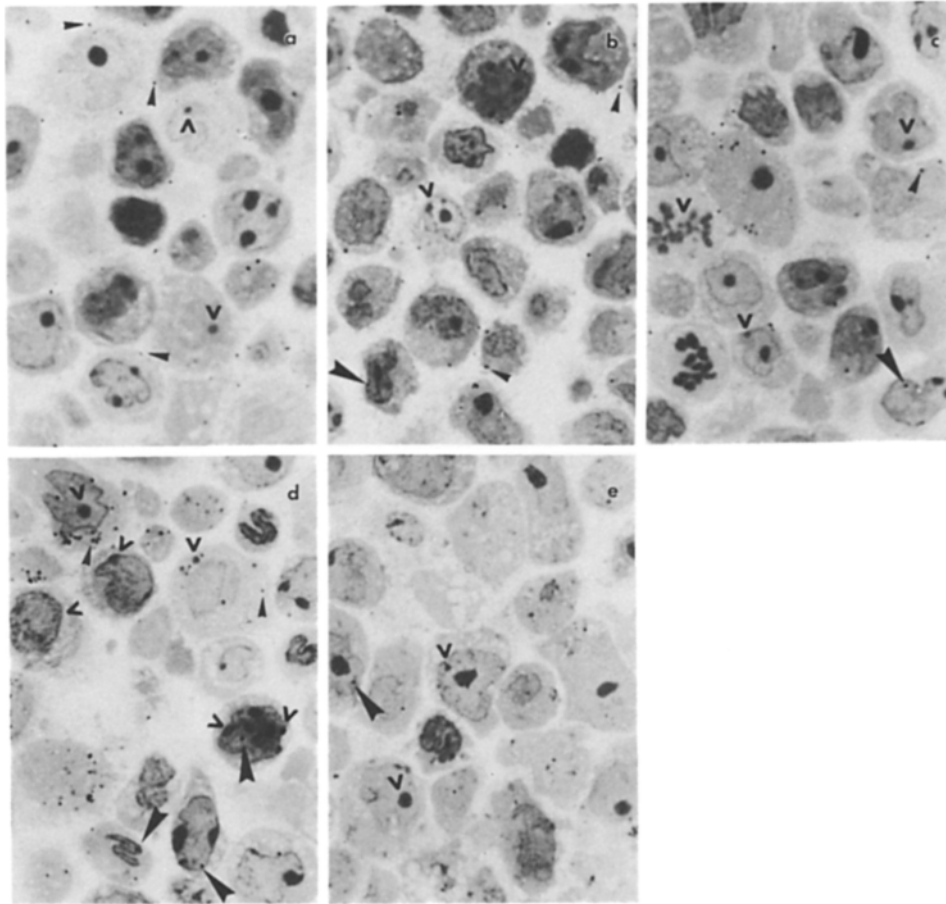
FIGURE 6.   Light-microscopic autoradiographs of IL-2 internalized by cells. ILT-Yan cells were treated with ^{3}H-labeled IL-2 at 0°C for 30 min. They were then washed and incubated at 37°C for 0 min (a), 5 min (b), 30 min (c), 60 min (d), and 120 min (e), and prepared for light-microscopic autoradiography. Arrowheads indicate silver grains in the nucleus (➤) and cytoplasm or cell surface (➤), and the intracellular dense organelles (>).

associated grains had decreased further, while grains in the cytoplasm and nucleus had increased to 64 and 18%, respectively. After 120 min incubation, grains in the nucleus had increased further to 24%, while those in the cytoplasm had slightly decreased. Grains on the cell surface had ceased to decrease, and had increased slightly at 120 min of incubation. These changes of cell-surface IL-2 and intracellular IL-2 demonstrated by autoradiography correlated well with changes of acid-removable and acid-unremovable IL-2, respectively.

Discussion

We (39) and others (9) examined the affinities and densities of IL-2-R on various HTLV-I-carrying human cell lines. As shown in Table I, the cell line MT-1 is unique in having abundant low-affinity IL-2-R but very few high-affinity receptors. IL-2-dependent T cell lines, such as ILT-Yan, and immortalizing
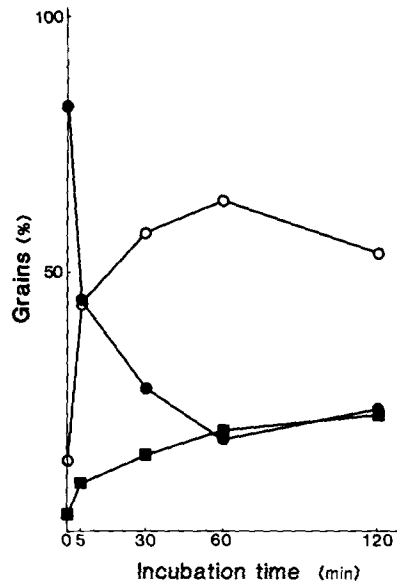
FIGURE 7. Subcellular localization of internalized IL-2. >500 silver grains of [³H]IL-2 in Fig. 6 were counted at each time, and their subcellular locations were classified as cell surface (●), cytoplasmic (○) and nuclear (■).

transformed T cell lines, such as TL-Mor, were established from HTLV-I-infected T cells, and these cell lines, like other HTLV-I-carrying cell lines, express normal ranges of high-affinity IL-2-R, but more low-affinity IL-2-R than PHA-treated normal PBL (39). Using these HTLV-I-carrying cell lines and PHA-treated normal PBL, we examined the internalization and degradation of IL-2. First, we confirmed that surface-bound ^{125}I-IL-2, but not internalized ^{125}I-IL-2, can be removed from cells by acid treatment. >90% of the cell-bound IL-2 was dissociated by treatment with acid buffer pH 3.8 or below at 0°C, but IL-2 internalized into cells at 37°C became acid unremovable. A kinetic study by microscopic autoradiography on [³H]IL-2-treated cells clearly showed that the acid-unremovable IL-2 is internalized, intracellular IL-2. IL-2 internalization is a rapid reaction that is dependent on temperature. >50% of the IL-2 bound to high-affinity receptors was internalized within 10 min when the cells were shifted from 0 to 37°C. Since Robb and Lin (30) reported that IL-2 internalization by murine T cells has a $t_{1/2}$ of 27 min, IL-2 internalization by human T cells seems to be slightly rapid.

At 37°C, decrease of internalized IL-2 was observed after 1 h of incubation, which could be mainly due to degradation of the IL-2 and excretion of its products into the culture supernatant, because the radioactivity in the culture supernatant that was not precipitated with 10% TCA increased inversely with decrease in the amount of internalized ^{125}I-IL-2. The $t_{1/2}$ of IL-2 degradation was estimated as 60–80 min in the T cells we observed. In murine cells, the $t_{1/2}$ of IL-2 degradation was reported to be 78 min (30), indicating that there is no significant difference in the $t_{1/2}$ of IL-2 degradation in human and murine T cells. Robb et al. (11, 30) suggested that, as IL-2 degradation is inhibited by chloro-

quine, which is known to inhibit lysosomal enzymes, the internalized IL-2 could be degraded in lysosomes. The degradations of other ligands, such as EGF and insulin in lysosomes has also been reported (16–20). Therefore, the IL-2 bound to high-affinity receptors was probably mainly internalized and then quickly degraded in lysosomes.

When cells treated with IL-2, even at high concentrations, were washed extensively, IL-2 bound to high-affinity receptors, but not appreciably to low-affinity receptors (Fig. 4). We also examined IL-2 internalization without washing the cells, to determine whether low-affinity receptors also mediate internalization of IL-2. In this experiment, ~5% of IL-2 bound to MT-1 cells, which express only low-affinity receptors, became acid-unremovable. This acid-unremovable IL-2 could be due to fluid-phase pinocytosis, but not due to internalization, because treatment of the cells with sodium azide in glucose-free medium did not inhibit appearance of the acid-unremovable IL-2. On the other hand, internalization mediated by high-affinity receptors was completely inhibited by the same treatment.[2] Therefore, internalization of IL-2 seems to be mediated by high-affinity receptors. Smith and Cantrell (12), and Depper et al. (13) suggested that the cell growth signal of IL-2 can be transduced from high-affinity, but not low-affinity receptors. [^{3}H]Thymidine uptake by PHA-treated PBL was maximal in the presence of 200 pM IL-2, which saturates high- but not low-affinity receptors, and decrease of low-affinity receptors did not affect [^{3}H]thymidine uptake of PHA-treated PBL. It is possible that IL-2 internalization mediated by the high-affinity receptors is essential for the transduction of growth signal induced by IL-2, although further study is necessary to obtain direct evidences for the contribution of IL-2 internalization to the signal transduction from IL-2-R.

Light-microscopic autoradiography confirmed the internalization of IL-2. Furthermore, some IL-2 was detected in the nucleus. Intranuclear accumulation of EGF (40), nerve growth factor (41), and insulin (42) have also been reported, although the biological significance of the intranuclear accumulation of these ligands or receptors is unknown. IL-2 or IL-2-R may be transferred to the nucleus, where they function directly to elicit the growth signal. We are now examining details of the localization of internalized IL-2 and IL-2-R and their fate in the cells.

Summary

Receptor-mediated internalization and degradation of IL-2 were investigated in cell lines carrying human T cell leukemia/lymphoma (lymphotrophic) virus type I (HTLV-I) and PHA-treated normal PBL. The HTLV-I-carrying cell lines ILT-Yan and TL-Mor, and the PBL expressed both high- and low-affinity IL-2-R. However, another HTLV-I-carrying T cell line, MT-1, expressed mainly low-affinity receptors. >50% of the IL-2 bound to high-affinity receptors was internalized within 10 min when these cells were incubated at 37°C. The internalized IL-2 was rapidly degraded and the products were excreted into the culture fluid. The $t_{1/2}$ of IL-2 degradation in these cells was estimated as 60–80 min at 37°C. The internalization and degradation of IL-2 were both temperature dependent.

[2] Fujii, M., K. Sugamura and Y. Hinuma. Internalization of IL-2 is mediated by high-affinity but not low-affinity IL-2 receptors. Manuscript in preparation.

Light-microscopic autoradiography with ^{3}H-labeled IL-2 confirmed the internalization of IL-2, and suggested that some IL-2 might be carried to the nucleus.

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