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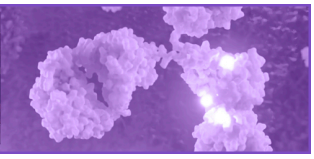

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INTERNALIZATION OF INTERLEUKIN 2 (IL-2) BY HIGH AFFINITY IL-2 RECEPTORS IS REQUIRED FOR THE GROWTH OF IL-2-DEPENDENT T CELL LINES

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During the growth of interleukin 2 (IL-2)-dependent T cells IL-2 binding is followed by internalization of the complex between IL-2 and the high affinity IL-2 receptor (HA-IL-2R). The respective role of IL-2 binding to HA-IL-2R and internalization of the complex has been examined. Monoclonal antibody 7D4 (IgM) blocks IL-2-dependent T cell growth although it does not affect IL-2 binding to HA-IL-2R. We show here that 7D4 inhibits T cell growth by blocking IL-2 internalization by HA-IL-2R. In contrast, Fab fragments prepared from 7D4 neither block IL-2 internalization nor inhibit T cell growth. Monoclonal 5A2, that recognizes an epitope related to the IL-2 binding site as well as its Fab fragment, inhibits T cell growth and IL-2 internalization. Monoclonal antibody 7D4, because of its pentameric structure, probably aggregates the IL-2R at the T cell surface and therefore prevents its internalization. The data presented in this paper suggest that simple occupancy of HA-IL-2R by IL-2 is not sufficient to transduce the T cell growth signal; this signal is transmitted only after internalization of the IL-2/HA-IL-2R complex.

The lymphokine interleukin 2 (IL-2),[2] a polypeptide hormone of 133 amino acids, plays a critical role in the proliferative expansion of T lymphocyte effector cells. IL-2 exerts its growth-promoting activity via specific cell surface receptors (IL-2R) (1) which exist in high and low affinity forms (2). All the positive biologic effects of IL-2 appear to be mediated by high affinity IL-2R (HA-IL-2R) (1, 2).

The existence of two types of receptors p55 (3) and p70 (4–7) that separately bind IL-2 with low or intermediate affinity has been demonstrated. The results suggest that the association between these two receptors constitute the HA-IL-2R (4–7). The mechanism and control of signal transduction after binding of IL-2 to HA-IL-2R is poorly understood. IL-2 binding is followed by internalization of the HA-IL-2R/IL-2 complex in human T cells and a chain of intracellular reactions resulting in T cell growth (8). During this complex process the respective roles of IL-2 binding to HA-IL-2R and the internalization of the HA-IL-2R/IL-2 complex are still unclear.

We have attempted to clarify this question using monoclonal antibodies (mAb) specific for the p55 subunit of the IL-2R (9, 10). The mAb 7D4 (IgM) has been shown to block IL-2-dependent T cell growth, although it is known to recognize an epitope distinct from the IL-2 binding site on murine IL-2R (9, 10). We show here that 7D4 inhibits T cell growth by blocking IL-2 internalization by HA-IL-2R. In contrast, Fab fragments prepared from 7D4 neither block IL-2 internalization nor inhibit T cell growth. As expected, mAb 5A2 (10) as well as its Fab fragment which recognizes an epitope related to the IL-2 binding site, inhibit T cell growth and IL-2 internalization. The data presented in this paper suggest that simple occupancy of HA-IL-2R by IL-2 is not sufficient to transduce the T cell growth signal. The growth signal is transmitted only by the internalization of the HA-IL-2R/IL-2 complex.

MATERIALS AND METHODS

Culture media, reagents, and T cell lines. All cultures were performed in RPMI 1640 medium as described elsewhere (11). Recombinant human IL-2 (rIL-2) (lot LP-265B, 99% pure, endotoxin content 0.01 ng/ml) with a specific activity of 3 U/ng was kindly donated by Cetus Co. (Emeryville, CA). ^{125}I-Radiolabeled IL-2 (23.8 μCi/μg) was purchased from New England Nuclear (Boston, MA).

Two helper cell lines L-14 and HT-2 were used in the present studies. L-14 is specific for the synthetic random terpolymer poly (Glu^{60}, Ala^{30}, Tyr^{10}) and is H-2^{b}-restricted (12). L-14 cells were progressively adapted to grow in IL-2-containing medium alone (12, 13). HT-2 cells, initially described by J. Watson (14) also grow in the presence of IL-2-containing medium alone. All the cell lines are maintained by culture in complete medium supplemented with supernatant from concanavalin A-activated spleen cells prepared as described previously (11). Both cell lines were shown to be Fc receptor negative (data not shown).

mAb: purification, preparation of Fab fragments, radiolabeling, and specificity. mAb 5A2 and 7D4 were purified and labeled with ^{125}I (Amersham, UK) as already described (15). Fab fragments from 5A2 and 7D4 were prepared by digestion with mercuropapain and pepsin, respectively (15). Fab fragments were checked for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under both reducing and nonreducing conditions.

To check the immunochemical activity of Fab fragments, antibody-binding inhibition assays were performed. L-14 cells were washed twice and incubated in RPMI 1640 medium (pH 7) containing 0.1% sodium azide, 10^{-2} M HEPES, and 1% bovine serum albumin (RPMI-HEPES) for 2 hr at 37°C in order to remove membrane-bound IL-2. Cells (5×10^5/well) were incubated in RPMI-HEPES, with various concentrations of cold anti-IL-2R mAb or their Fab fragments for 90 min at 4°C in a total volume of 100 μl in 96-well V-formed plates (Greiner, Labortechnick, Bischwiller, France) in the presence of 400 μg/ml of normal rat immunoglobulins. This was followed without washing by a second 90-min incubation at 4°C with 5 ng/well of ^{125}I-labeled anti-IL-2R mAb 5A2 or 7D4. This concentration results in around 15% maximal binding under the

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[2] Abbreviations used in this paper: IL-2, interleukin 2; HA-IL-2R, high affinity interleukin 2 receptor; IL-2R, interleukin 2 receptor; mAb, monoclonal antibody; rIL-2, recombinant interleukin 2; EGF, epidermal growth factor.

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experimental conditions described. The cells were washed three times in RPMI-HEPES and the bound radioactivity was measured in a Beckman gamma scintillation counter. Results are expressed in terms of percent inhibition.

To check the specificity of 7D4, 5A2, and their Fab fragments, inhibition of IL-2 binding was performed as already described (10).

Inhibition of the IL-2-driven proliferation assay. This assay was performed as previously described (15). Briefly, cells were seeded in 96-well microtiter flat-bottomed plates (Corning, New York, ref. 25680) with limiting concentrations of rIL-2 giving approximately half-maximal proliferation. When indicated, different concentrations of purified mAb or their Fab fragments were added.

IL-2 internalization and inhibition of internalization by anti-IL-2R mAb or their Fab fragments. Internalized ^{125}I-labeled IL-2 was distinguished from surface-bound IL-2 by acid treatment. Exposure of cells for 2 min at 4°C to RPMI 1640, containing 25 mM sodium acetate, and 10^{-3} M HEPES adjusted to pH 4 and followed by two centrifugations in RPMI-HEPES completely removes surface-bound IL-2 without affecting cell viability.

For inhibition of internalization, the cell (5×10^5/well) were incubated in the presence of indicated concentrations of mAb 5A2, 7D4, or their Fab fragments at 4°C for 1 hr in a total volume of 100 μl of RPMI-HEPES in Eppendorf tubes. After incubation and without washing, 0.5 ng of ^{125}I-labeled IL-2 was added to each tube for another 20 min at 37°C. The cells were then immediately centrifuged in cold RPMI-HEPES to stop the reaction. The pellet of half of the samples was treated with RPMI (pH 4) followed by two successive washings in RPMI-HEPES. The other samples were washed with RPMI-HEPES only. The radioactivity of the cells was then determined as described above. The radioactivity left after acid treatment was taken as a measure of IL-2 internalization.

RESULTS

Immunochemical activity of Fab fragments from 5A2 and 7D4 anti-IL-2R mAb. Figure 1 shows the inhibition of binding of ^{125}I-labeled mAb 5A2 (Fig. 1*A*) and 7D4 (Fig. 1*B*) to L-14 T helper cells by different concentrations of cold mAb and their corresponding Fab fragments. These inhibitions are specific and concentration-dependent; inhibition by the 5A2 Fab fragment was found to be 2 to 3 times less efficient than inhibition by cold 5A2. Inhibition by the 7D4 Fab fragment is around 10 times less efficient that inhibition by the whole cold molecule. These figures are derived from the quantity, in terms of weight, of cold inhibitor giving 50% inhibition. In the experiments described below we have taken into account the reduced efficiency of 5A2 and 7D4 Fab fragments. The K_d (mol/liter) of mAb 7D4 and 5A2 has been measured and found to be 0.49×10^{-8} and 0.94×10^{-8}, respectively. The K_d of 7D4 and 5A2 Fab fragments were found to be 8.2×10^{-8} and 4.2×10^{-8}, respectively. Furthermore, ^{125}I-labeled Fab fragments from 5A2 and 7D4 only bind to cells expressing IL-2R molecules (data not shown).

Fab fragments prepared from 5A2 and 7D4 retain their specificities (Table I). mAb 5A2 and its Fab fragment dramatically inhibit IL-2 binding, whereas 7D4 and its Fab fragment have no significant effect. This is in agreement with previous studies where we have shown that the epitope recognized by 5A2 is intimately related to the IL-2 binding site, whereas the epitope recognized by 7D4 is distant from the IL-2 binding site of the IL-2R (10).

Effect of Fab fragments from 5A2 and 7D4 on IL-2-dependent T cell growth. Figure 2 shows the ability of mAb 5A2 and 7D4 and their corresponding Fab fragments to inhibit IL-2-induced proliferation of L-14 (Fig. 2*A*) and HT-2 (Fig. 2*B*) cells. In agreement with previous results, mAb 5A2 inhibits the proliferation of both L-14 and HT-2 cells (10, 15). Its Fab fragment also efficiently inhibits this proliferation. Efficiency of inhibition by the 5A2 Fab was 4 to 6 times less (in terms of inhibitor weight) than that of the intact IgG molecule, thus correlating with results described in Figure 1A). This result was confirmed with both L-14 and HT-2 cells. As a control, mAb GK1.5 (anti-L3T4) does not affect the proliferation of the L3T4$^+$ L-14 cells, whereas mAb H-35.89-9 (anti-lymphocyte function-associated antigen (LFA)-1)

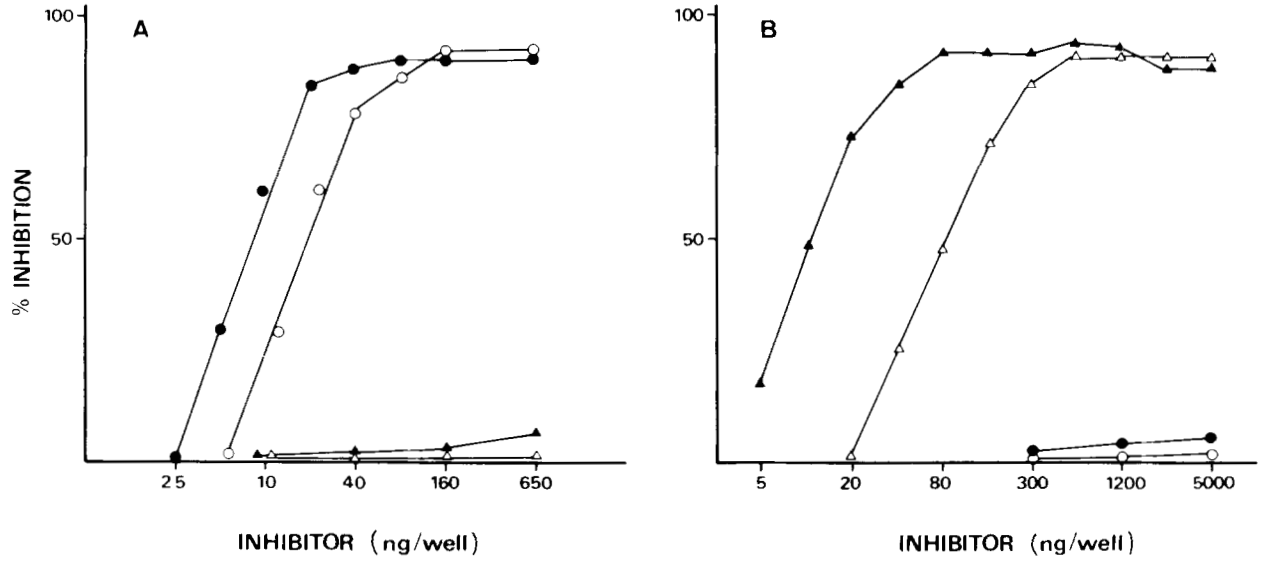

Figure 1. Inhibition of binding of ^{125}I-labeled mAb 5A2 (*A*) and ^{125}I-labeled mAb 7D4 (*B*) to the p55 subunit of the IL-2R by unlabeled mAb 5A2, 7D4, and their respective Fab fragments. L-14 T helper cells (5×10^5/100 μl/well) were incubated with the indicated concentrations of unlabeled mAb 5A2 (●——●), 5A2 Fab (○——○), 7D4 (▲——▲) and 7D4 Fab (△——△) at 4°C for 90 min followed by a second incubation at 4°C for 90 min with 5 ng of ^{125}I-labeled mAb. This concentration results in 15% maximal binding under the experimental conditions described and corresponds to around 4000 and 5500 cpm for 7D4 and 5A2, respectively. Results are expressed as per cent inhibition.

$$\left(1 - \frac{\text{cpm bound in the presence of inhibitor} - \text{background}}{\text{cpm bound in the absence of inhibitor} - \text{background}}\right) \times 100.$$

A, Eight nanograms of cold 5A2 and 18 ng of cold 5A2 Fab, respectively, are required to obtain 50% inhibition of ^{125}I-labeled 5A2 binding. *B*, Ten nanograms of cold 7D4 and 95 ng of cold 7D4 Fab, respectively, are required to obtain 50% inhibition of ^{125}I-labeled 7D4 binding.

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TABLE I

Effect of 5A2, 7D4, and their Fab fragments on IL-2 binding

T Cell	Inhibitor	% Inhibition at Different Concentrations of Inhibitor (in μg/well)[a]				
		60	30	15	8	4
L-14	5A2		90	85	75	82
	5A2(Fab)		79	75	83	81
	7D4		10	16	14	11
	7D4(Fab)		22	22	22	21
HT-2	5A2	82	75	80	20	
	5A2(Fab)	79	81	82	23	
	7D4	3	5	6	8	
	7D4(Fab)	18	15	15	15	

[a] Indicated concentrations of mAb 5A2, Fab 5A2, 7D4, and Fab 7D4 were added to L-14 (5×10^5 cells/100 μl/well) or HT-2 (10^5 cells/100 μl/well) for 90 min at 4°C followed by a second incubation with 0.5 ng of ^{125}I-labeled IL-2 for another 90 min at 4°C. Determination of bound ^{125}I-labeled IL-2 was measured as described elsewhere (10). The radioactivity of ^{125}I-labeled IL-2 bound in the absence of inhibitors was 300 and 1000 cpm on L-14 and HT-2 cells, respectively. Background binding never exceeded 50 cpm. The percent of inhibition of IL-2 binding was calculated as follows:

$$\left(1 - \frac{\text{cpm bound in the presence of inhibitor} - \text{background}}{\text{cpm bound in the absence of inhibitor} - \text{background}}\right) \times 100.$$

does not affect the proliferation of the L3T4$^-$ LFA-1$^+$ HT-2 cells.

The results obtained with the 7D4 Fab fragment are different. Although 7D4 does inhibit the IL-2-induced proliferation of L-14 and HT-2, its Fab fragment does not affect this response. Fab fragments were tested at very high concentrations in order to compensate for the decreased efficiency of 7D4 Fab (Fig. 1*B*). mAb 7D4 at a concentration of 0.02 μg/100 μl/well inhibits IL-2-induced proliferation of L-14 T cells by 50%, whereas 10 μg/100 μl/well of 7D4 Fab do not affect this response. With HT-2 cells, 0.8 μg/100 μl/well of 7D4 inhibits proliferation by 50%, whereas 40 μg/100 μl/well of 7D4 Fab do not affect this response.

Effect of 5A2 and 7D4 Fab fragments on HA-IL-2R internalization. L-14 cells were incubated with ^{125}I-labeled IL-2 (Fig. 3) and the IL-2 internalized was measured as indicated in *Materials and Methods*. The process is very fast and a significant amount of ^{125}I-labeled IL-2 is internalized after only 5 min. After 15 to 20 min the process reaches a plateau, representing around 50% of the cell surface-bound IL-2. Similar results were obtained with the HT-2 cell line (data not shown). In the following experiment, a period of 20 min was chosen to study the effects of mAb on IL-2 internalization.

Figure 4 shows the inhibition of IL-2 internalization by 5A2 and 7D4 and their Fab fragments by using L-14 (Fig. 4*A*) and HT-2 cells (Fig. 4*B*). mAb 5A2 and its Fab inhibit IL-2 internalization very efficiency. This effect is probably the consequence of the inhibition of IL-2 binding which may be responsible for IL-2R internalization. However, mAb 7D4 which does not inhibit IL-2 binding (Table I) strongly inhibits IL-2 internalization, paralleling its inhibition of IL-2-dependent proliferation. In contrast, 7D4 Fab blocks neither IL-2-induced proliferation (Fig. 2) nor IL-2 internalization, even when tested at concentrations up to 400 μg/100 μl/well to compensate for its decreased efficiency (Fig. 1).

We have verified that the IL-2 bound to the cell surface of HT-2 in the presence of mAb 7D4 does not undergo any significant biochemical modification. It can be removed from the surface either by acidic pH treatment (Fig. 4) or by trypsin treatment (data not shown). Furthermore the IL-2 removed from the surface by acidic pH treatment migrates as a single band of around 15,000 in sodium dodecyl sulfate gels (data not shown).

DISCUSSION

The data presented in this paper indicate that: 1) binding of IL-2 to HA-IL-2R alone is inadequate to induce

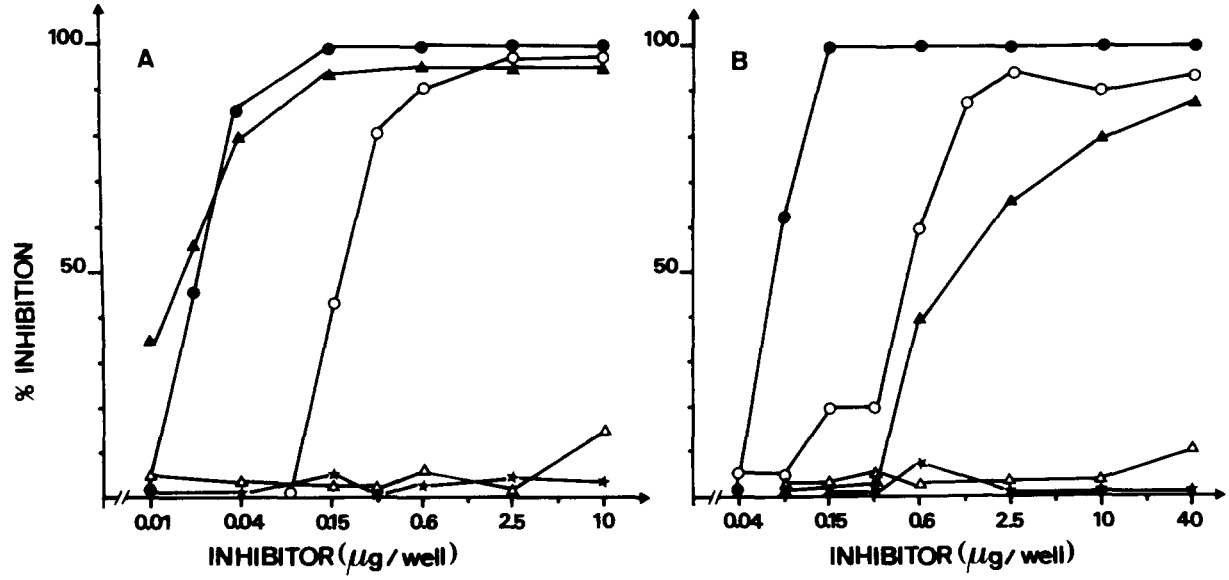

Figure 2. Effect of mAb 5A2 and 7D4 and their Fab fragments on IL-2-induced proliferation of L-14 (*A*) and HT-2 (*B*) cells. L-14 (2×10^4/200 μl/well) and HT-2 (10^3 cells/200 μl/well) were cultured with various amounts of 5A2 (●——●), 5A2 Fab (○——○), 7D4 (▲——▲), or 7D4 Fab (△——△) in the presence of 0.6 ng/well of rIL-2. This concentration of rIL-2 was found to give around 50% maximal proliferation. After 48 hr of culture, the cells were pulsed and [^{3}H]TdR incorporation measured 16 hr later. [^{3}H]TdR incorporation in control wells was 15,000 and 25,000 cpm for L-14 and HT-2, respectively. Anti-L3T4 mAb GK1.5 (*——*) and anti-LFA-1 mAb H-35.89.9 (*——*) were used as controls for L-14 and HT-2 cells, respectively. Percent inhibition was calculated as follows:

$$\left(1 - \frac{\text{cpm bound in the presence of inhibitor} - \text{background}}{\text{cpm bound in the absence of inhibitor} - \text{background}}\right) \times 100.$$

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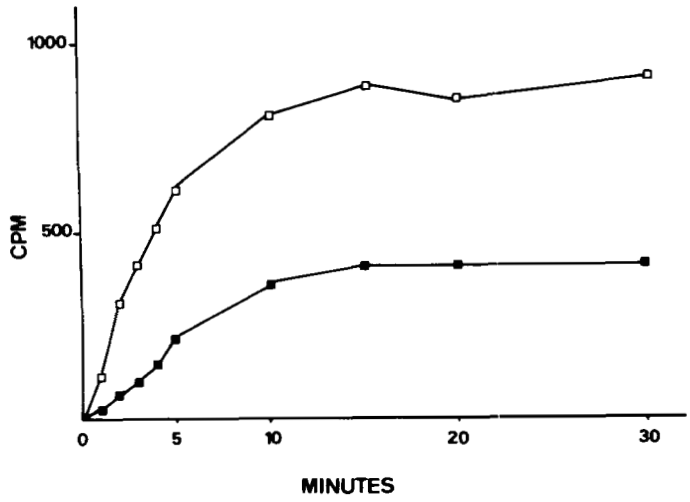

Figure 3. Kinetics of IL-2 internalization. L-14 (5×10^5 cells/well) in RPMI-HEPES were incubated with 0.5 ng of ^{125}I-labeled IL-2 at 4°C for 1 hr followed by incubation at 37°C for varying periods of time. At indicated times following incubation, the cells were immediately put in chilled RPMI-HEPES (pH 7.0) and centrifuged. The cells were then treated either with RPMI (pH 7) or RPMI (pH 4) for 2 min. ^{125}I-labeled IL-2 internalized inside the cells was determined as described in *Materials and Methods*.

proliferation; 2) IL-2 internalization by HA-IL-2R is an essential step in transduction of the growth signal; and 3) mAb can block IL-2-induced T cell growth either by blocking IL-2 binding or by preventing HA-IL-2R internalization, probably by aggregating these receptors at the T cell surface.

Most of the results of this report are derived from the study of the effects of mAb 7D4 and its Fab fragment which are specific for the p55 subunit of the IL-2R. mAb 7D4 does not recognize the IL-2 binding site and does not affect IL-2 binding but inhibits IL-2-induced T cell proliferation. This inhibition of proliferation appears to relate to its ability to inhibit internalization of IL-2 bound to the HA-IL-2R. Cleavage of 7D4 into Fab fragments abrogates these effects without affecting the specificity. mAb 7D4 because of its pentameric structure probably aggregates the IL-2R at the T cell surface and therefore prevents its internalization.

From our data, we cannot exclude that IL-2 binding transmits positive signals in the absence of internalization and that unknown inhibitory effects of mAb 7D4 are responsible for inhibition of T cell growth. However, this appears unlikely since 7D4 Fab does not affect T cell growth and neither does mAb 2E4 which is an IgG directed against an epitope identical or very close to the epitope recognized by 7D4 (9, 10).

The experimental model described here permits the dissociation of the events dependent on IL-2 binding from the events that follow internalization of the HA-IL-2R/IL-2 complex. Although IL-2 binding in the presence of 7D4 is not followed by T cell growth, other early events may occur such as RNA synthesis, IL-2R phosphorylation (16, 17), modulation of adenylate cyclase activity (18), or translocation of protein kinase C (19) as had been observed in other systems. In the epidermal growth factor (EGF) model, it has been shown that cross-linking of the membrane surface receptor by concanavalin A blocks receptor internalization and mitogenic effects of EGF but does not affect early events such as RNA synthesis (20).

The results obtained with mAb 7D4 and 5A2 delineate some specific features of the IL-2R model. During the process of signal transduction, surface aggregation of

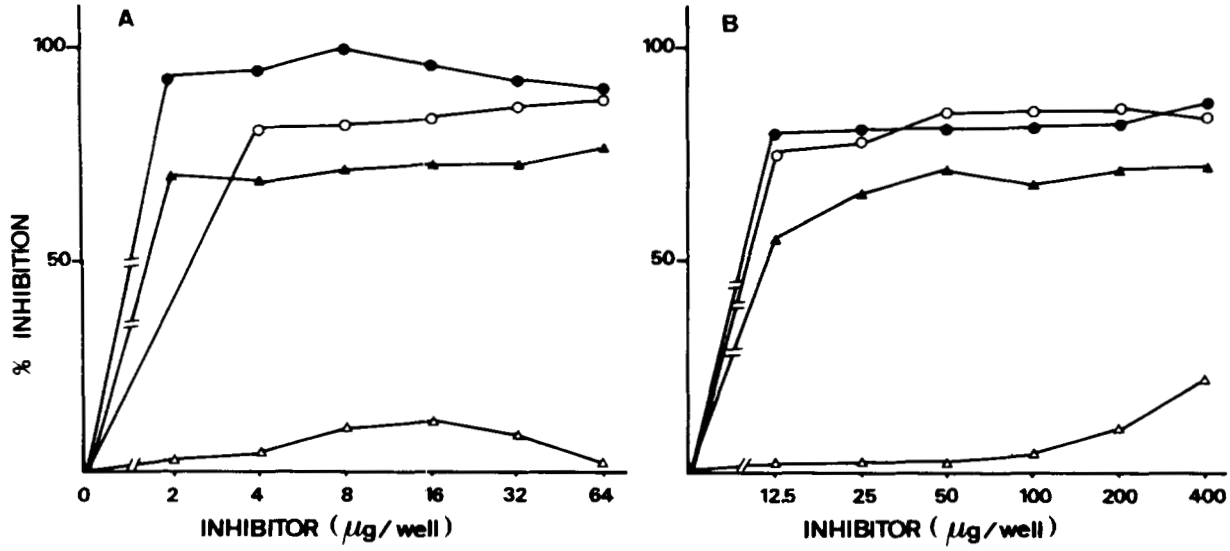

Figure 4. Influence of mAb 5A2 and 7D4 and their Fab fragments on the internalization of ^{125}I-IL-2 on L-14 (*A*) and HT-2 (*B*) cells. L-14 (5×10^5 cells/100 μl/well) and HT-2 (10^5 cells/100 μl/well) were incubated with indicated concentrations of 5A2 (●——●), 5A2 Fab (○——○), 7D4 (▲——▲), or 7D4 Fab (Δ——Δ) at 4°C for 1 hr. ^{125}I-labeled IL-2 (0.5 ng/100 μl/well) was added for another 20 min. The reaction was stopped by chilling the cells and the ^{125}I-labeled IL-2 internalized was determined by treating the cells with acidic RPMI, pH 4, for 2 min. ^{125}I-labeled IL-2 bound to the cell surface in the absence of mAb gave 910 and 2160 cpm for L-14 and HT-2 cells, respectively. For L-14 cells, the inhibition of IL-2 binding is from 78 to 85% with the different doses of mAb 5A2 and its Fab and 7 to 14% with mAb 7D4 and its Fab. For HT-2 cells the inhibition of IL-2 binding is 85 to 95% with the different doses of mAb 5A2 and its Fab and 9 to 13% with the different doses of mAb 7D4 and its Fab. ^{125}I-labeled IL-2 internalized in the absence of mAb gave around 500 and 1100 cpm for L-14 and HT-2 cells, respectively. Percent inhibition of internalization was calculated as follows:

$$\left(1 - \frac{\text{cpm internalized bound in the presence of inhibitor} - \text{background}}{\text{cpm internalized bound in the absence of inhibitor} - \text{background}}\right) \times 100.$$

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specific membrane receptors has been observed (21). In the EGF model, this intermediate stage before internalization seems essential. Fragments of EGF not able to induce aggregation of the receptor do not lead to any signal transduction. However, exposure of cells to EGF fragments in the presence of multivalent antibody specific for the EGF fragments restores both the capacity to aggregate the receptor and to mediate biologic effects (21). It is noteworthy that 7D4 probably causes some aggregation of the IL-2R because of its pentameric structure but this does not induce any mitogenic signal (15). On the contrary, by blocking IL-2 internalization, 7D4 affects the process of signal transduction. In various membrane receptor systems like EGF (22), specific biologic effects including mitogenesis can be mimicked successfully by binding of monoclonal or polyclonal antibodies under appropriate experimental conditions. In these systems, therefore, the receptor alone is responsible for signal transduction and the hormone is not absolutely required either at the surface or inside the cells. In the IL-2R system, none of the mAb studies to date, even those specific for epitopes related to the IL-2 binding site, can replace IL-2 (10, 15). Occupancy of the IL-2 binding site or of a related area by 5A2 or by its Fab fragment failed to induce any signal for the growth or the activation of T cells (15). Therefore, despite the fact that we have shown that internalization of IL-2 bound to HA-IL-2R is required for T cell growth, the respective roles of IL-2 and its receptor inside cells remain to be established. IL-2 may be itself necessary or may be involved only in receptor internalization and the latter may be the active molecule inside the cell.

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