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LOW AND HIGH AFFINITY CELLULAR RECEPTORS FOR INTERLEUKIN 2

Implications for the Level of Tac Antigen

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Interleukin 2 (IL-2),[1] formerly called T cell growth factor, provides a necessary signal for the transition of activated T cells from the G_1 to the S phase of the cell cycle (1, 2). The mechanism of action of the factor involves binding to a high-affinity receptor on the surface of responsive cells (3, 4). Based on a number of criteria, the murine monoclonal antibody anti-Tac, originally prepared by Uchiyama and co-workers (5), recognizes the human IL-2 receptor. The antibody was capable of blocking high-affinity IL-2 binding to activated cells (6, 7) and all of the T cell responses attributed to interaction of such cells with IL-2 (8). Furthermore, a covalent complex of IL-2 and Tac protein was isolated from IL-2–treated cells that had been subjected to chemical cross-linking (9). Finally, affinity supports coupled with the antibody and with IL-2 bound the same 55,000 mol wt glycoprotein from a mixture of detergent-solubilized cellular molecules (7). The antibody-coupled support was capable of removing all cellular proteins reactive with the IL-2–coupled support, and the IL-2–coupled support was capable of removing all antibody-reactive molecules. Thus, the number of IL-2 receptors appeared equal to the number of Tac proteins. In contrast to the latter finding, quantitative binding assays with radiolabeled IL-2 and anti-Tac indicated that activated T cells had 10–20 times more binding sites for the antibody than high-affinity sites for IL-2(10). Resolution of this dilemma is essential if the anti-Tac reagent is to be used to monitor expression of the IL-2 receptor in the absence of direct binding data.

As earlier suggested (7), one potential explanation for the quantitative difference between the two binding assays is that a large portion of the Tac proteins possess a low affinity for IL-2. Such molecules would bind to IL-2–coupled supports but would not have appeared in the original radiolabeled IL-2 binding assays (3), which were conducted at very low IL-2 concentrations. In this paper, we describe a binding phenomenon for IL-2 on activated T cells and certain B cells that has an association constant >5,000 times lower than that originally

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[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CTLL, cytolytic T lymphocyte line; DME, Dulbecco's modified Eagles medium; FBS, fetal bovine serum; HCL, hairy cell leukemia; HTLV, human T cell leukemia virus; IgG, immunoglobulin G; IL-2, interleukin 2; K_d, dissociation constant; leu, leucine; lys, lysine; PBL, peripheral blood mononuclear lymphocyte; PHA, phytohemagglutinin; PMA, phorbol-12-myristate-13-acetate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TdR, thymidine.

described (3). Moreover, the number of such binding sites was many times the number of high-affinity sites. As with the high-affinity interaction, the low-affinity binding of radiolabeled IL-2 was blocked by addition of excess unlabeled factor and substantially blocked by the addition of the anti-Tac antibody. Considering the accuracy of the methodology, the levels of total binding sites measured using radiolabeled IL-2 and anti-Tac were quite similar, thus supporting the notion that the two assays detect coincident sets of molecules.

Materials and Methods

Cell Cultures. The three murine, IL-2–dependent cytotoxic T cell lines (CTLL-2, subclone 15H [11] and CTL, a gift from Dr. T. Malek, National Institutes of Health) and helper T cell lines (HT-2, a gift from Dr. T. Malek) were maintained in Iscove's modified Dulbecco's modified Eagle medium (Iscove's DME; Grand Island Biological Co., Grand Island, NY) supplemented with 15% heat-inactivated fetal bovine serum (FBS, M.A. Bioproducts, Walkersville, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 2.5×10^{-5} M 2-mercaptoethanol, and 1.0 U/ml purified JURKAT-derived IL-2. All human and primate cell lines were maintained in RPMI 1640 medium (Grand Island Biological) supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. All cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. The 1C9 cell line was obtained by transformation of Tac-positive, normal human B cells with Epstein-Barr virus and was a generous gift from Dr. T. Waldmann, National Institutes of Health. A sample of hairy cell leukemia (HCL) was a kind gift from Dr. S. Korsmeyer. T lymphocytes were first removed from this sample by sheep erythrocyte rosetting before receptor binding analysis. HSB₂ cells (1 $\times 10^6$ cells/ml) were stimulated with 50 ng/ml phorbol-12-myristate-13-acetate (PMA; Consolidated Midlands Corp., Brewster, NY) for 20 h before receptor analysis.

Human phytohemagglutinin(PHA)-activated lymphoblasts were prepared by culturing peripheral blood mononuclear cells isolated on Ficoll-Hypaque with 1.5 μg/ml PHA (HA-16; Wellcome Reagents, England) in supplemented RPMI 1640 medium for 72 h. The proportion of blast cells was enriched to >95% by discontinuous Percoll (Pharmacia, Inc., Uppsala, Sweden) gradient centrifugation (12). For short-term culture of PHA-lymphoblasts, the 72-h blast cells were washed and resuspended at 1×10^5 cells/ml in supplemented RPMI 1640 with 1 U/ml IL-2. Every 48 h, the cells were pelleted and resuspended in fresh medium containing IL-2. At 9 d after the original lectin stimulation, the cells were restimulated with 1.0 μg/ml PHA in the presence of IL-2. Receptor expression was measured after 16 h.

Interleukin 2. Biosynthetically radiolabeled and unlabeled IL-2 were prepared from the cell supernatant of a high-producing subclone of the JURKAT cell line (J6.8.9.15.32) after induction of the cells with PHA and PMA (13). The cells were suspended at 4 × 10⁶/ml in serum-free DME for the preparation of unlabeled IL-2 and in leucine, lysine-free DME (Grand Island Biological Co.), to which was added 40 μM unlabeled leucine and 65 μM unlabeled lysine, for the radiolabeled preparations. To the latter cultures were added 0.1–0.5 mCi/ml [³H]leucine (50 Ci/mmol; ICN, Irvine, CA) and 0.1–0.5 mCi/ml [³H]lysine (45 Ci/mmol; ICN). IL-2 secretion was induced by inclusion of 1.5 μg/ml PHA and 50 ng/ml PMA. The cell supernatant was harvested after 16 h at 37°C. Bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) at 1 mg/ml was added to the radiolabeled preparations as a carrier.

IL-2 was purified from the JURKAT cell supernatant using an immunoaffinity column coupled with a murine monoclonal antibody (46C8-A2) specific for the N-acetyl galactosamine derivative of human IL-2 (14, 15). This form of IL-2 represents ~60% of the factor released by stimulated JURKAT cells (16). Separate experiments have demonstrated that the affinity of IL-2 for its cellular receptor is not affected by the extent of glycosylation of the factor (3, 15). Bound IL-2 was recovered in >95% yield by elution of the affinity column with 1.5% HOAc. The recovered unlabeled factor was judged to be >99% pure

by two-dimensional gel electrophoresis (16), reverse-phase HPLC (unpublished observation), and N-terminal sequence analysis (16). The material was free of PHA and PMA, based on the gel electrophoretic data and [^{3}H]PMA (New England Nuclear, Boston, MA) tracer analysis (16). The radiolabeled IL-2 was eluted from the affinity column in 1.5% HOAc with 1 mg/ml BSA and equilibrated with RPMI 1640, 25 mM Hepes, pH 7.2 (Grand Island Biological Co.) on a YM-5 membrane (Amicon Corp., Lexington, MA). The specific radioactivity of [^{3}H]leu,lys IL-2, in terms of dpm ^{3}H per picomole IL-2, was estimated by measuring the bioactivity of the preparation in the T cell proliferation assay (see next section) and converting this value to the weight of IL-2 using the previously determined specific activity of the factor (310,000 units of bioactivity per milligram of IL-2 protein; reference 16) and its molecular weight (15,500 pg/pmol).

Interleukin 2 Bioassay. IL-2 bioactivity was determined by the concentration-dependent stimulation of proliferation, as indicated by [^{3}H]thymidine (TdR) incorporation, of a cloned, IL-2–dependent murine T cell line (CTLL-2, subclone 15H) (17). Serial twofold dilutions of the samples and a standard IL-2 preparation were incubated with 4,000 CTLL cells (200 μl total volume) for a period of 16–18 h. At that time, the cultures were pulsed with 50 μl of [^{3}H]TdR, 10 μCi/ml (New England Nuclear). After 4 h, the cultures were harvested on glass fiber paper using a Titertek Cell Harvester (Flow Laboratories, Rockville, MD). The dilutions yielding half-maximal [^{3}H]TdR incorporation were determined by probit analysis (17) and the dilution of the sample was divided by that of the standard to yield the IL-2 concentration in units per milliliter. The standard IL-2 preparation was arbitrarily assigned a value of 1 U/ml and induced 50% of the maximum [^{3}H]TdR incorporation at a final dilution of 1:20 to 1:40 (the final dilution after addition of 100 μl of the CTLL suspension) (13). As used here, 1 U of bioactivity was equivalent to ~41.5 IL-2 Reference Reagent "units" as defined by the Biological Response Modifiers Program of the National Cancer Institute.

Anti-Tac Monoclonal Antibody. The anti-Tac antibody (5) was purified from the ascites fluid of hybridoma-inoculated BALB/c mice using protein A-Sepharose (Sigma). The antibody was radiolabeled by reductive methylation with formaldehyde and sodium [^{3}H]borohydride (New England Nuclear) (9). The specific radioactivity was calculated using an average of the protein content as determined by the Lowry assay (18) and quantitative amino acid compositional analysis.

Radiolabeled IL-2 and Anti-Tac Binding Assays. Before determining the level of binding, all cell types were incubated twice for 1 h at 37°C in 50 ml RPMI 1640 with extensive washing between and after the incubations in order to remove endogenous IL-2. Previous studies (3, 19) have indicated that receptor-bound IL-2 will either dissociate or be internalized during this time period. In some cases, the washed cells were also incubated for 20 s in 10 mM sodium citrate, 0.14 M NaCl, pH 4.0 followed by centrifugation at 9,000 g for 15 s in a Microfuge 12 (Beckman Instruments, Palo Alto, CA), a procedure shown to remove intact cell surface-bound IL-2 (19). After washing, the cells were resuspended in RPMI 1640, 25 mM Hepes, pH 7.3, containing 10 mg/ml BSA (RPMI-BSA). Identical binding results were obtained using RPMI 1640 with 10% fetal calf serum in place of the BSA.

To determine the level of binding, serial dilutions of [^{3}H]leu,lys IL-2 and [^{3}H]anti-Tac IgG were incubated at 37°C with 0.3–2 × 10^6 cells in a total volume of 100 μl RPMI-BSA using 1.5-ml Eppendorf micro test tubes (Brinkman Instruments, Westbury, NY). For the anti-Tac binding assay, a control murine myeloma IgG2$_a$ antibody, designated UPC-10 (Litton Bionetics Inc., Kensington, MD), was included at 200 μg/ml to minimize potential interactions of the [^{3}H]anti-Tac with Fc receptors. The tubes were mechanically rotated about their longest axis (horizontal position) at 20 rpm in a 37°C water bath for 20–30 min. In separate experiments (unpublished observations), it was determined that this period of incubation was sufficient to allow maximum high (20 min) and low-affinity (30 min) binding to occur. After incubation with the radiolabeled probe, the cells were washed two to four times, depending upon the amount of radiolabel added, with 1 ml ice-cold RPMI-BSA. The supernatant from the first wash was counted by liquid scintillation to determine the level of unbound IL-2 or anti-Tac antibody. The cells were resuspended

in 100 µl RPMI-BSA and centrifuged (9,000 g for 90 s) through a 200 µl layer of a mixture of 84% silicone oil (550 fluid; Contour Chemical Co., North Reading, MA) and 16% paraffin oil (Fisher Scientific Co., Philadelphia, PA) to remove the small amount of residual unbound radioactivity. The tips of the tubes (400-µl polyethylene; Bio-Rad Laboratories, Richmond, CA) containing the cell pellet were cut off and placed in 20-ml glass scintillation vials. The cells were expelled from the tips using 200 µl phosphate-buffered saline (PBS) and solubilized by the addition of 200 µl 1% SDS, followed by 10 ml Liquiscint (National Diagnostics, Somerville, NJ). The radioactivity was counted by liquid scintillation on a Tri-Carb 4640 (Packard Instrument Co., Downers Grove, IL). The results were converted to dpm before applying graphical analysis. The average counting efficiency for the bound fraction was 39% and, for the unbound fraction, 32%.

Results

Purity of Radiolabeled Probes. The radiolabeled IL-2 and anti-Tac reagents were purified by affinity chromatography on anti-IL-2 monoclonal antibody and protein A-coupled columns, respectively. The [^{3}H]IL-2 was prepared at several different levels of specific radioactivity in order to measure binding over a wide range of concentrations. SDS-polyacrylamide gel electrophoretic (SDS-PAGE) analysis of the various radiolabeled probes indicated that each of the IL-2 preparations consisted of a single labeled component of 15,500 mol wt and that the [^{3}H]anti-Tac preparation contained only the 50,000 and 25,000 mol wt bands expected of heavy and light IgG chains (Fig. 1).

Extension of the [^{3}H]IL-2 Binding Assay to High IL-2 Concentrations. The radiolabeled IL-2 binding assays originally described by Robb et al. (3) used IL-2 concentrations between 1 and 200 pM. At these IL-2 levels, the authors
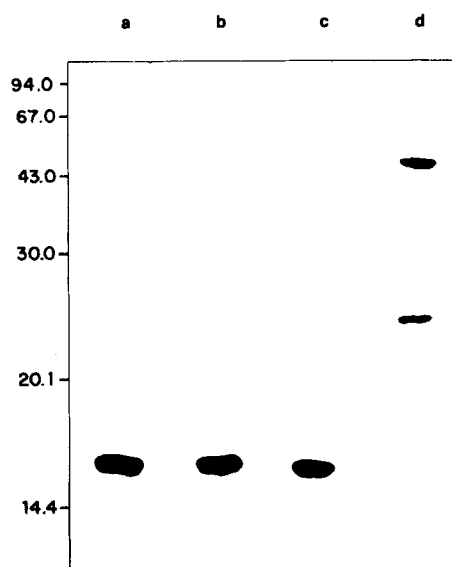

FIGURE 1. SDS-PAGE analysis (12% acrylamide, samples treated with 2-mercaptoethanol, reference 16) of three preparations of [^{3}H]IL-2 with specific radioactivities of 3.82 × 10^5 dpm/pmol (lane a), 2.42 × 10^5 dpm/pmol (lane b), and 3.09 × 10^4 dpm/pmol (lane c) and of [^{3}H] anti-Tac antibody (8.33 × 10^4 dpm/pmol, lane d). About 20,000 dpm of each of the IL-2 radiolabeled probes and 10,000 dpm of the anti-Tac probe were applied to the gel. The radioactivity was visualized by fluorography (36).

demonstrated a saturable binding phenomenon with a dissociation constant (K_d) of 5–6 pM for activated human T cells and 20 pM for activated murine T cells. The number of binding sites detected on the human cells, however, was far below that estimated using the anti-Tac monoclonal antibody (6, 8, 10). A similar discrepancy was suspected for the murine T cells based on the intensity of fluorescence staining with a rat monoclonal antibody (20) specific for the murine IL-2 receptor (unpublished observation). Since it was possible that IL-2 might also interact with a distinct set of sites with a markedly lower affinity (7), the binding curve analysis was extended up to IL-2 concentrations 1,000-fold higher than originally used. The results of such a binding experiment, as shown in Fig. 2, confirmed that high-affinity saturable binding occurred at IL-2 concentrations <1 nM. At IL-2 concentrations above this level, however, additional factor binding was detected. In this experiment, the K_d of the binding at high IL-2 concentrations for the human T cell leukemia/lymphoma (HTLV$_I$)-infected line, HUT 102B2, for human PHA-activated lymphoblasts, and for the murine, IL-2–dependent CTLL-2 line was estimated to be 15–30 nM, or ~5,000 times higher than that of the saturable binding at picomolar IL-2 levels. Furthermore, the number of such low-affinity binding sites was at least 5–10 times the number of high-affinity sites. Addition of 10 μM unlabeled IL-2 reduced the binding of [^{3}H]IL-2 by 90% or more for each of the concentrations tested (Fig. 2). Thus, the low-affinity binding consisted predominately of a saturable component.
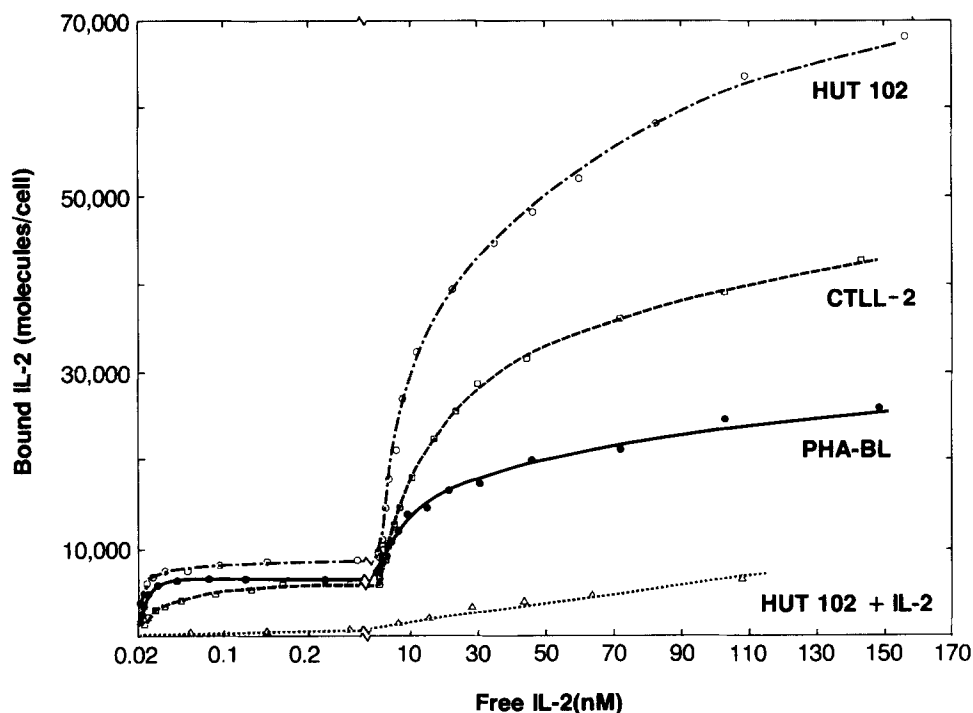

FIGURE 2.   Typical curve for the binding of [^{3}H]leu,lys IL-2 (2.42 × 10^5 dpm/pmol) to HUT 102B2 cells, PHA-activated (72 h) human lymphoblasts, and murine CTLL-2, subclone 15H cells over a 100,000-fold range of IL-2 concentration. Nonsaturable binding on the HUT 102B2 cells was estimated by including 10 μM unlabeled IL-2.

To further evaluate the proportion of saturable binding, serial dilutions of unlabeled IL-2 were added to [^{3}H]leu,lys IL-2 at three different concentrations of the radiolabeled probe. The concentrations of radiolabeled IL-2 were chosen to examine high-affinity binding, low-affinity binding, and binding intermediate between these two extremes. As shown in Fig. 3, using HUT 102B2 and murine CTLL-2 cells, addition of increasing amounts of unlabeled IL-2 resulted in up to 99% inhibition of the binding of [^{3}H]leu,lys IL-2 at a low concentration of the probe. Similarly, unlabeled IL-2 competed for up to 95 and 87% of the binding at intermediate and high levels of labeled factor. Since nonspecific binding increases linearly with the concentration of labeled probe, it was not unexpected that the level of nonsaturable binding remaining after cold competition represented a larger fraction of the total binding for the low-affinity phenomenon than it did for the binding at picomolar concentrations of radiolabeled IL-2. The results demonstrate, however, that the low-affinity binding predominantly represents specific interaction of the factor with a cellular component(s).

Relationship of IL-2 Binding to the Tac Antigen. Previous studies (6, 7) indicated that the anti-Tac antibody could completely inhibit the binding of radiolabeled IL-2 at picomolar concentrations of the growth factor. To evaluate the affect of the antibody on low-affinity IL-2 binding, serial dilutions of anti-Tac were combined with different concentrations of the radiolabeled IL-2 probe. Anti-Tac inhibited >95% of the [^{3}H]leu,lys IL-2 binding to HUT 102B2 cells at the lowest concentration of IL-2 (high-affinity binding). At progressively higher IL-2 levels, a decreasing proportion of the binding was blocked (Fig. 4). Nevertheless, at the highest IL-2 concentration, at which >90% of the binding should
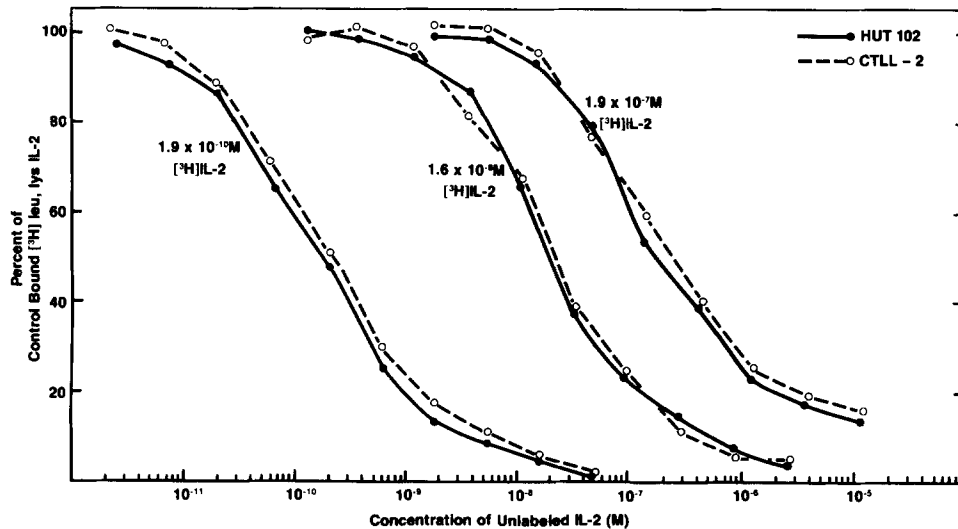

FIGURE 3.   Competitive inhibition of the binding of [^{3}H]leu,lys IL-2 (3 different concentrations) by unlabeled IL-2 using HUT 102B2 and murine CTLL-2, subclone 15H cells. [^{3}H]-leu,lys IL-2 at the concentrations indicated in the figure was combined with serial dilutions of unlabeled IL-2, followed by addition of the cells. The concentrations of radiolabeled IL-2 were chosen to give saturation of the high-affinity binding sites (1.9 × 10^{-10} M total concentration), half-saturation of the low-affinity binding sites (1.6 × 10^{-8} M total concentration) and saturation of the low-affinity binding sites (1.9 × 10^{-7} M total concentration).
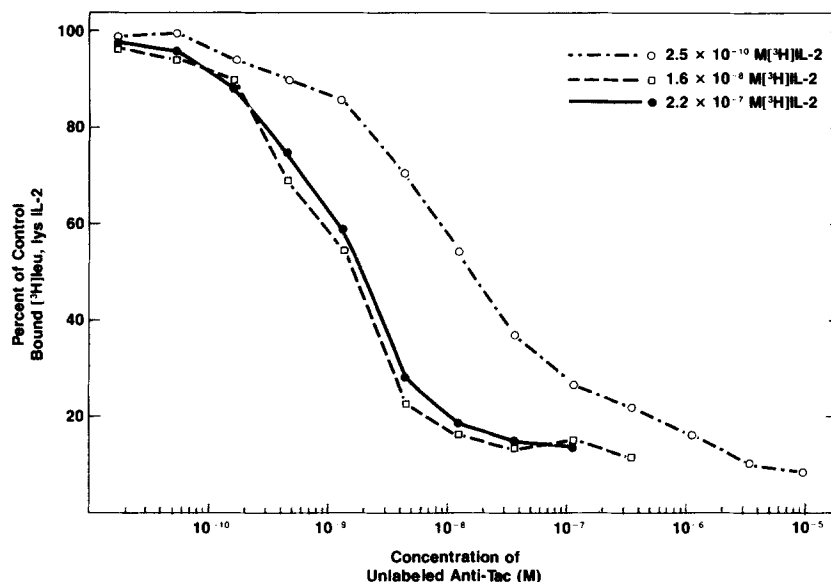
FIGURE 4. Inhibition of the specific binding of [^{3}H]leu,lys IL-2 (3 different concentrations) by unlabeled anti-Tac antibody using HUT 102B2 cells. [^{3}H]leu,lys IL-2 at the concentrations indicated in the figure was combined with serial dilutions of anti-Tac antibody, followed by addition of the cells. Control murine IgG had no effect on the level of bound [^{3}H]IL-2 even when present at 10^{-5} M. The number of molecules of [^{3}H]IL-2 bound per cell for each of the IL-2 concentrations was 4,720 (2.5 × 10^{-10} M), 17,010 (1.6 × 10^{-8} M), and 64,400 (2.2 × 10^{-7} M). The results were first corrected for the levels of nonsaturable binding determined in Fig. 3.

have been of the low-affinity type, the antibody still inhibited 80–90% of the specific IL-2 binding. Thus, at least the majority of the low-affinity interaction of IL-2 with the HUT 102B2 cells appeared to involve the Tac protein. At the lowest concentration of [^{3}H]IL-2, where the binding was entirely of the high-affinity type, a 55-fold molar excess of antibody over growth factor was required to reduce the receptor-association by half. In contrast, at the highest concentration of [^{3}H]IL-2, where most of the binding was low-affinity, the molar ratio of antibody to IL-2 at 50% inhibition was 1:130. Thus, anti-Tac was far more efficient in blocking the low-affinity phenomenon.

In a second approach to evaluating the relationship of IL-2 binding to the Tac protein, serial dilutions of unlabeled IL-2 were tested for their efficacy in blocking the cellular binding of [^{3}H]anti-Tac. The concentration of antibody used was sufficient for saturation of the anti-Tac binding sites. Furthermore, the number of antibody-binding sites detected in the controls exceeded by many fold the number of high-affinity IL-2 binding sites (see Table I). The results demonstrated that IL-2 could totally block the binding of labeled antibody to either HUT 102B2 or 1C9 cells (Fig. 5). Thus, all the Tac proteins on these cells appeared capable of interacting with IL-2. At the concentration of antibody chosen, a 100-fold molar excess of IL-2 over anti-Tac was necessary to cause a 50% reduction in antibody binding. Half-maximal inhibition of anti-Tac binding occurred at the same IL-2 concentration for both cell lines. Interestingly, ~8% of the anti-Tac binding on the HUT 102B2 cells was inhibited at very low IL-2
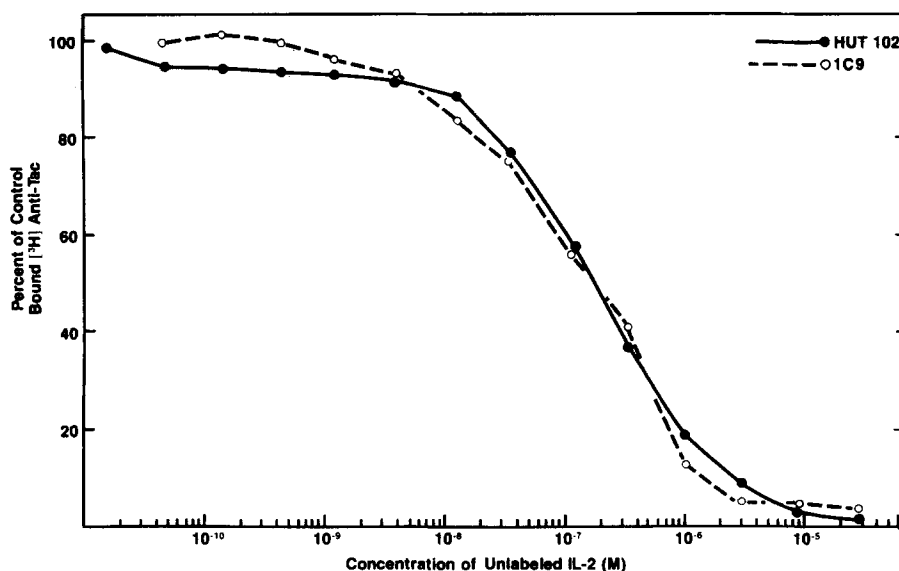
FIGURE 5. Inhibition of the binding of [^{3}H]anti-Tac antibody (1.8 × 10^{-9} M total concentration) by serial dilutions of unlabeled IL-2 using HUT 102B2 and 1C9 cells. The radiolabeled antibody and IL-2 were combined before addition of the cells. The level of radiolabeled antibody was chosen to give saturation of the anti-Tac binding sites.

levels. This amount is very similar to the fraction of high-affinity IL-2 binding sites present on this cell line (Table I).

Scatchard Analysis of High and Low Affinity IL-2 Binding and of Anti-Tac Binding. To evaluate the interaction of IL-2 with a variety of cell types, binding assays were separately performed at high and low ranges of IL-2 concentration. The results (Figs. 6–8, Table I) indicated that at IL-2 concentrations below 1 nM, saturable binding occurred to a variety of cell types that were positive for the IL-2 receptor as defined by monoclonal antibodies (6, 20). In addition, as was evident at IL-2 concentrations above 1 nM, each of these cell types also displayed many-fold higher levels of low-affinity IL-2 binding sites. In contrast, unstimulated cells (PBL) and a number of Tac-negative T cell lines, such as Molt 3 and JURKAT, and B cell lines, such as Raji and Daudi, lacked the capacity for significant high and low affinity IL-2 binding (Table I). The level of IL-2 binding on the receptor-positive cells was only slightly increased by elution of endogenous factor with a low pH buffer (19). Thus, following preparation of the cells for the assay, 90% or more of the high and low affinity binding sites remaining on the cell surface appeared unoccupied (data not shown).

PHA-activated lymphoblasts cultured in IL-2–containing medium for 10 d lost most of their high and low affinity receptors (Table I). Restimulation of these cells with PHA resulted in a dramatic reexpression of IL-2 binding capacity in parallel with an increase in their level of the Tac antigen. The 1C9 B cell line displayed both high and low affinity IL-2 binding sites, but the levels of both were far below those seen on activated T cells and the HUT 102B2 line (Table I). PMA-stimulated HSB$_2$ cells and a sample of B cells of hairy cell leukemia origin, although positive for anti-Tac binding, displayed few, if any, high-affinity
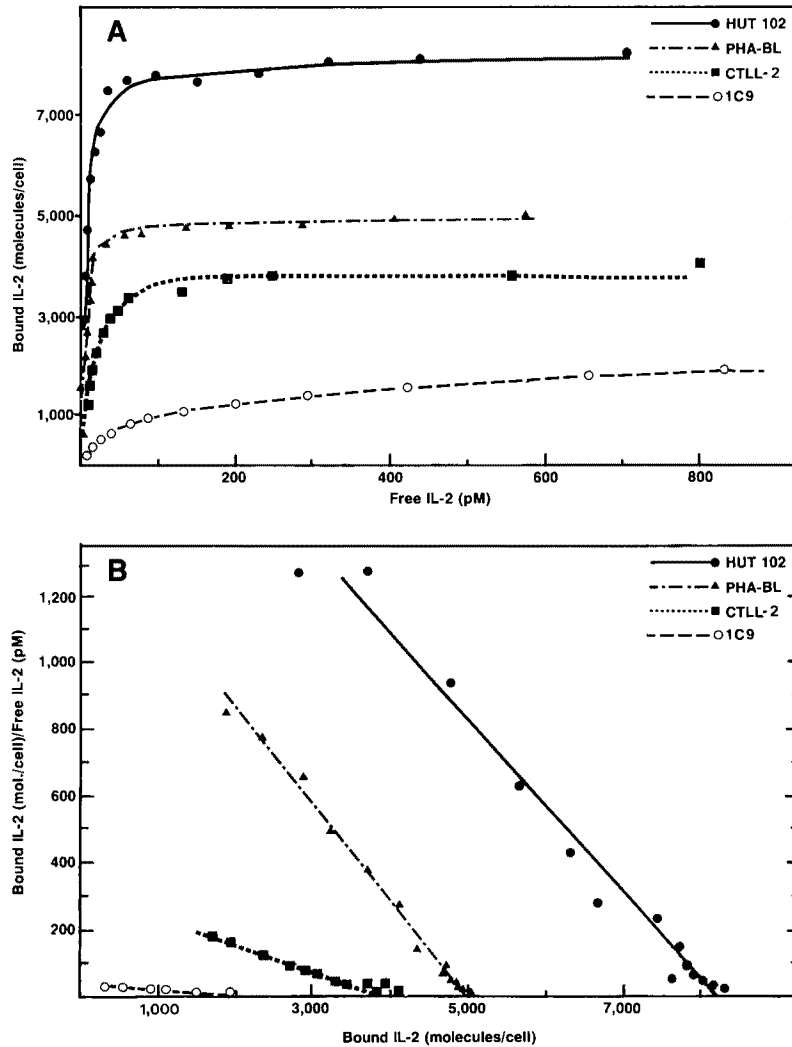
FIGURE 6. (A) Typical curves for the binding of [^{3}H]leu,lys IL-2 (3.82 × 10^5 dpm/pmol) at low IL-2 concentrations (i.e., high-affinity binding) to HUT 102B2, PHA-activated (72 h) human lymphoblasts, 1C9 and murine CTLL 2, subclone 15H cells. Nonsaturable binding was estimated by including 10 μM unlabeled IL-2 in selected tubes. The residual radiolabel associated with the cells was subtracted from the total bound fraction found in the absence of unlabeled factor to give specific binding. (B) A Scatchard plot of the data in A.

IL-2 binding sites (Table I). Almost all the sites on these cells were of the low-affinity variety. The ratios of total IL-2 binding sites for the three murine cell lines (Table I) were approximately the same as the ratios of mean fluorescence intensity determined with rat monoclonal antibody 7D4 (20), which is specific for the murine IL-2 receptor (data not shown). The demonstration of high levels of low-affinity IL-2 binding on these cell lines is consistent with the intensity of their fluorescence staining.

Scatchard analysis of the assay results at low IL-2 concentrations (Fig. 6B) produced straight lines, indicating that the binding for each cell type over this
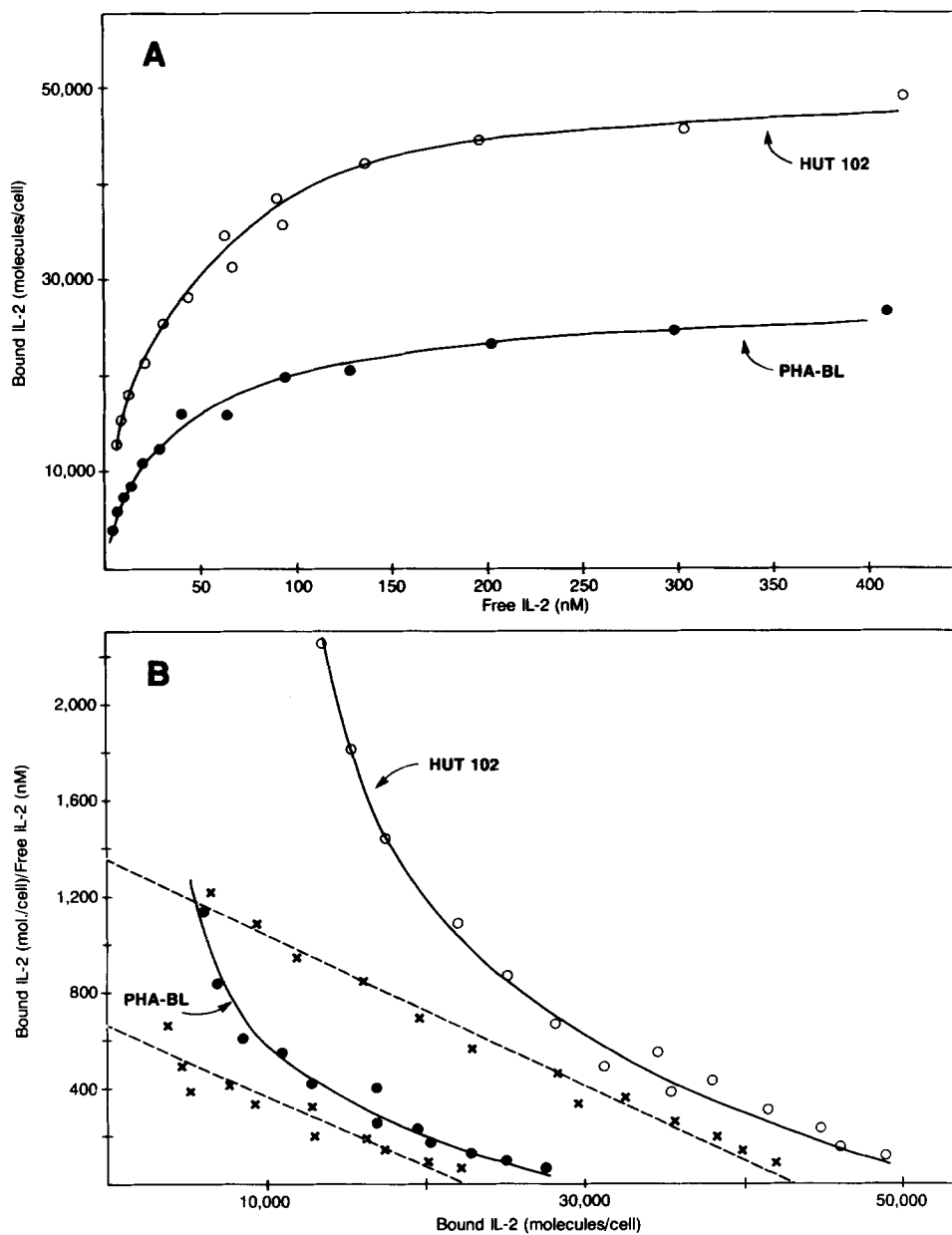
FIGURE 7.   (A) Typical curves for the binding of [³H]leu,lys IL-2 (3.09 × 10⁴ dpm/pmol) at high IL-2 concentrations (i.e., low-affinity binding) to HUT 102B2 and PHA-activated human lymphoblasts. The results were corrected for nonsaturable binding by measuring the residual binding in the presence of 10 μM unlabeled IL-2. (B) A Scatchard plot of the data in A (solid lines). The contribution of high-affinity binding sites was subtracted from the values of total bound factor to reveal the approximate characteristics of the low-affinity binding phenomenon (dashed lines).
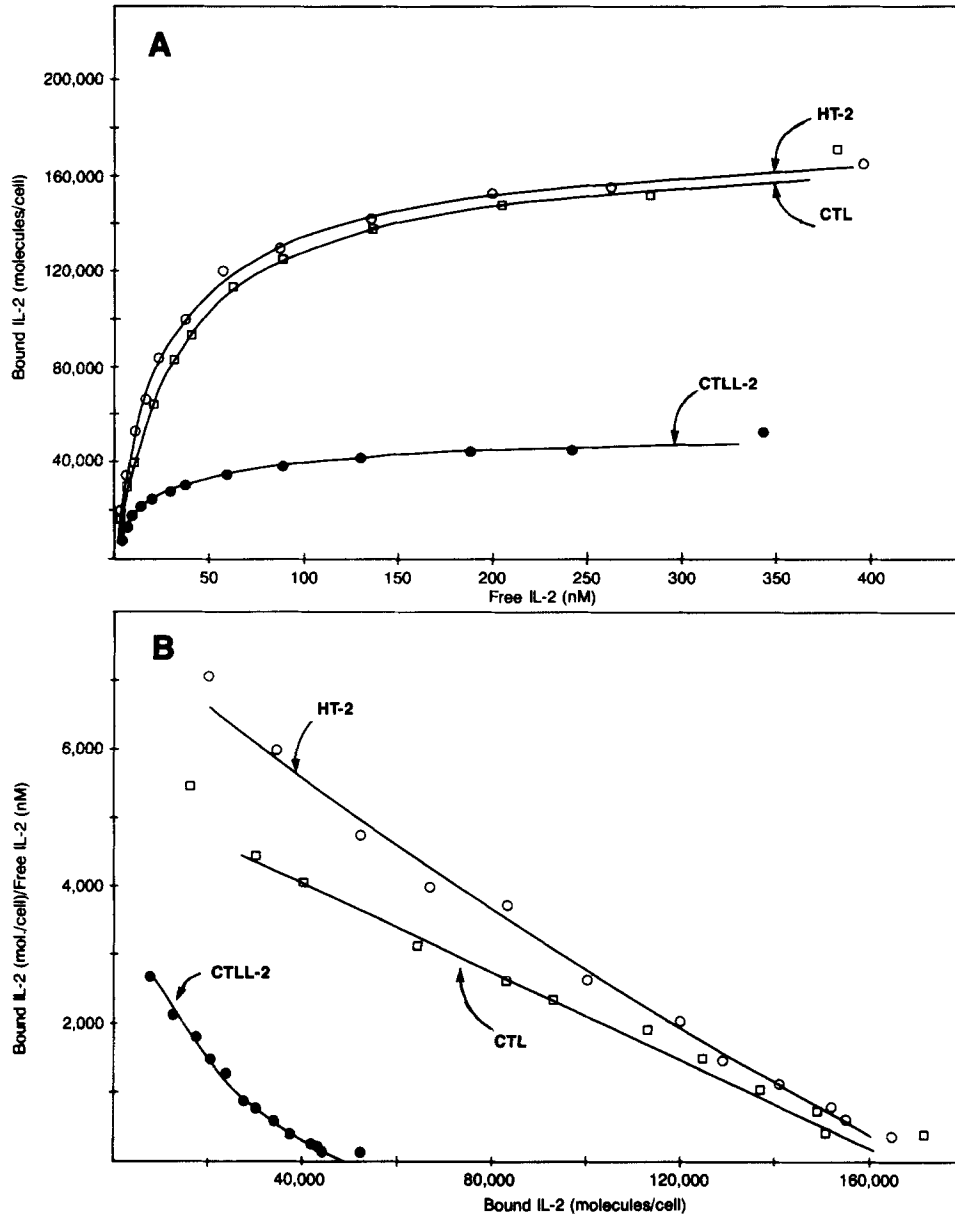
FIGURE 8. (A) Typical curves for the binding of [^{5}H]leu,lys IL-2 (3.09 × 10^4 dpm/pmol) at high IL-2 concentrations to murine CTLL-2 (subclone 15H), CTL, and HT-2 cells after correction for nonsaturable binding. (B) A Scatchard plot of the data in A.

range of IL-2 levels was characterized by a single affinity. The enormous difference in the dissociation constants for the high and low affinity phenomena, which was emphasized by the binding curves depicted in Fig. 2, explains why the low-affinity binding has no detectable effect on the association curves at these IL-2 concentrations. The apparent dissociation constant, averaged over several experiments, was ~4 pM for the human PHA-lymphoblasts and HUT 102B2

TABLE I
Cellular Binding of Radiolabeled IL-2 and Anti-Tac*

Cell type	IL-2 binding				Average ratio of low-affinity to high-affinity binding sites	Average total IL-2 binding sites per cell	Anti-Tac binding	
	High-affinity		Low-affinity‡					
	Sites/cell	K_d (×10^-12 M)	Sites/cell	K_d (×10^-12 M)			Sites/cell	K_d (×10^-12 M)
Human								
T cells								
PBL	85 (1)	8.5 (1)	1,080 (1)	36,000 (1)	12.7	1,160	1,080 (1)	250 (1)
PHA-blasts (72 h)	3,560 ± 1,040 (7)	3.6 ± 0.7 (4)	25,200 ± 3,500 (6)	28,300 ± 11,200 (3)	7.1	28,800	59,100 ± 10,800 (5)	140 ± 40 (4)
PHA-blasts (10 d)	700 ± 150 (2)	—	1,900 ± 450 (2)	—	2.7	2,600	2,500 ± 600 (2)	—
Restimulated PHA-blasts (10 d)	1,270 ± 130 (2)	—	19,800 ± 3,280 (2)	33,200 (1)	15.6	21,100	27,600 ± 4,100 (2)	120 (1)
Molt 3	<10 (2)	—	<200 (2)	—	—	<200	<100 (2)	—
JURKAT	<10 (2)	—	<200 (2)	—	—	<200	<100 (2)	—
HUT 102B2	7,150 ± 1,700 (8)	4.6 ± 1.3 (5)	63,400 ± 15,800 (18)	32,100 ± 13,100 (6)	8.9	70,600	181,000 ± 61,000 (11)	160 ± 86 (6)
HSB₂	<10 (2)	—	<400 (2)	—	—	<400	<100 (2)	—
PMA-HSB₂	≤15 (2)	<50 (2)	3,950 ± 1,100 (2)	29,600 ± 840 (2)	≥260	4,000	3,100 ± 450 (2)	143 ± 11 (2)
B cells								
IC9 (early)§	1,640 ± 560 (3)	24.5 ± 14.2 (3)	8,200 (1)	25,100 (1)	5.0	9,800	7,500 (1)	130 (1)
(late)	805 ± 170 (4)	70.0 ± 4.3 (4)	3,990 ± 1,350 (3)	26,200 ± 9,500 (3)	5.0	4,800	2,880 ± 220 (3)	120 ± 50 (3)
HCL	≤20 (1)	<50 (1)	2,980 (1)	41,000 (1)	≥150	3,000	2,890 (1)	—
Raji	<20 (1)	—	<400 (1)	—	—	<400	<200 (1)	—
Daudi	<20 (1)	—	<400 (1)	—	—	<400	<200 (1)	—
Murine								
T cells								
CTLL-2(15H)	3,600 ± 900 (4)	13.2 ± 2.3 (4)	35,000 ± 4,200 (4)	24,300 ± 8,100 (3)	9.7	38,600	—	—
CTL	7,380 ± 1,250 (4)	39.0 ± 4.1 (4)	167,300 ± 9,100 (2)	32,200 ± 7,300 (2)	22.7	175,000	—	—
HT-2	9,380 ± 2,000 (4)	36.3 ± 3.7 (4)	187,400 ± 47,000 (2)	24,800 ± 7,000 (2)	20.0	197,000	—	—

* The results represent the mean ± standard deviation of multiple binding experiments (number in parentheses) after correction of the data for nonsaturable binding. Two independent preparations of radiolabeled probes were used in each case. These preparations had specific radioactivities of 5.21×10^5 dpm/pmol and 3.82×10^5 dpm/pmol for high-affinity IL-2 binding, 1.53×10^4 dpm/pmol and 3.09×10^4 dpm/pmol for low-affinity IL-2 binding, and 3.59×10^5 dpm/pmol and 8.33×10^4 dpm/pmol for anti-Tac antibody binding.

‡ The number and approximate K_d of the low-affinity IL-2 binding sites was calculated by subtracting the contribution of the high-affinity sites from the total bound ligand as was done in Fig. 7B.

§ Samples of IC9 cells were tested following a few weeks ("early") and a few months ("late") of in vitro culture.

cells (Table I). This value is in good agreement with that previously measured (3), particularly when corrections (16) are made in the specific radioactivity of the probe used in the former study. The 1C9 B cell line initially demonstrated an affinity for IL-2 at low factor concentrations that was fivefold lower than that of the human T cells (Table I). After 2 mo in culture, however, its level of receptors and the *apparent* affinity both declined. Freshly thawed cultures underwent the same apparent decline in affinity. The high-affinity factor binding sites on the murine CTLL-2 line had a dissociation constant comparable to that previously measured (3). In contrast, the CTL and HT-2 lines appeared to have threefold lower affinities for IL-2 for this range of factor concentrations (Table I).

Scatchard analysis of the assay results at high IL-2 concentrations using PHA-lymphoblasts and HUT 102B2 cells (Fig. 7) demonstrated that the data formed curvilinear plots indicative either of a mixture of binding sites with different affinities or of negative cooperativity. It should be noted that the results were first corrected for nonspecific, nonsaturable binding before they were plotted. If the contribution of the high-affinity sites of each cell type was first subtracted, the plots usually approximated straight lines (Fig. 7B). The K_d for the various types of human cells was roughly 30 nM, indicating that this class of receptors had an affinity over 5,000 times lower than that of the high-affinity IL-2 binding sites. A similar dissociation constant was found for the three IL-2–dependent murine T cell lines (Fig. 8, Table I). The CTL and HT-2 lines expressed a much higher density of low-affinity binding sites on their surface than the CTLL-2 cells. For both these lines, the high-affinity sites represented a minor fraction of the total binding. As a consequence, the Scatchard plots at high IL-2 concentrations were nearly linear (Fig. 8B), even without correction for the high-affinity binding component.

Using [^{3}H]anti-Tac, the number and affinity of antibody-binding sites were estimated for each of the human cell types. As shown in Fig. 9 for a representative experiment, saturable binding of the antibody occurred on both PHA-lymphoblasts and HUT 102B2 cells and the Scatchard analysis demonstrated a single class of binding sites. Preliminary results with Fab fragments of anti-Tac suggested that the binding of the intact antibody, as measured under these conditions, was monovalent (J. M. Depper and W. C. Greene, unpublished observation). Thus, assuming only a one-to-one ratio of bound antibody to Tac protein, the average number of Tac proteins on the various human cell types was found to be reasonably close (usually within a factor of 2) to the average number of total IL-2 binding sites (Table I). This result suggests that each 55,000 mol wt Tac molecule is capable of binding one molecule of IL-2. The total number of factor binding sites was somewhat lower than the number of anti-Tac binding sites in the case of the PHA-lymphoblasts and HUT 102B2 cells. For the stimulated HSB$_2$ cells and the two Tac-positive B cell samples, the number of IL-2 binding sites equaled or slightly exceeded (1C9 cells) the number of sites detected with radiolabeled antibody (Table I).

The apparent affinity of anti-Tac binding was ~40-fold lower than that of high-affinity IL-2 binding to the HUT 102B2 cells but ~200-fold higher than that of the low-affinity IL-2 phenomenon (Table I). These values are quite similar
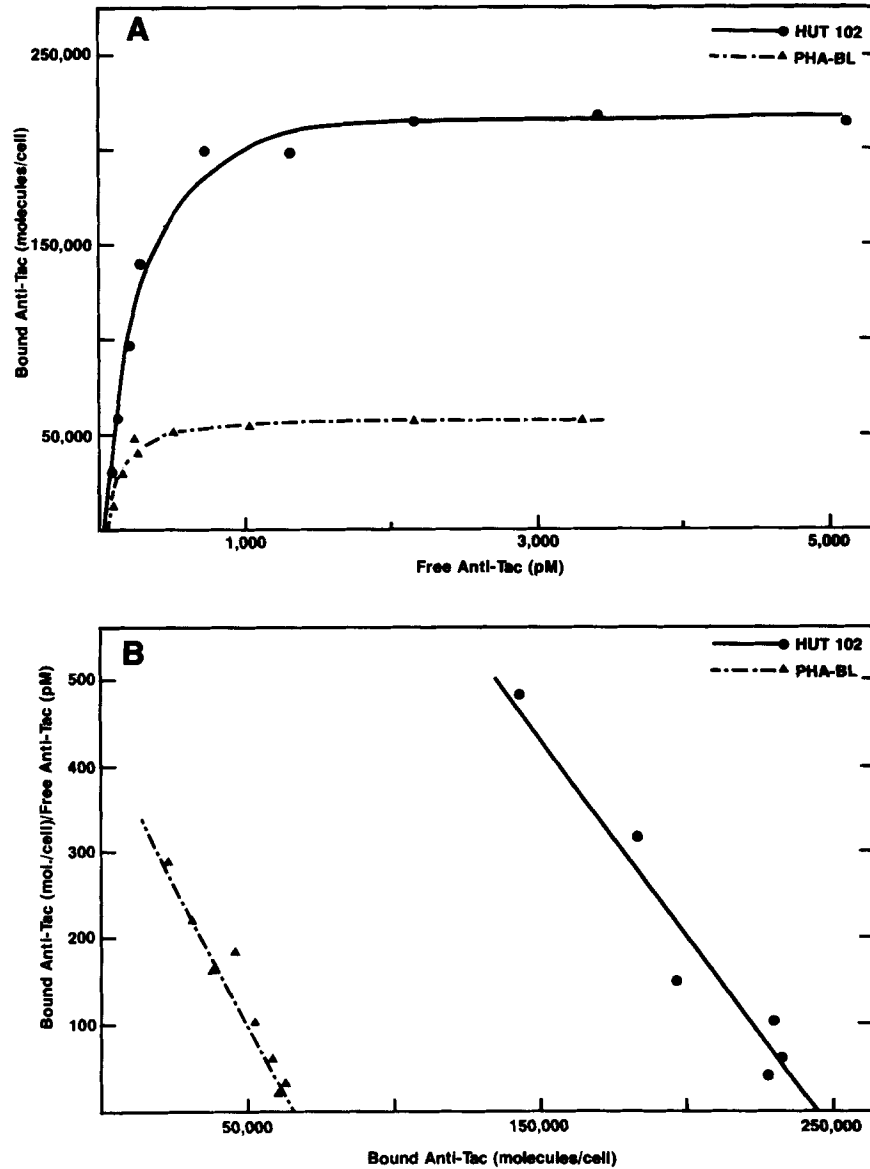
FIGURE 9. (A) Typical curves for the binding of [³H]anti-Tac antibody (8.33 × 10⁴ dpm/pmol) to HUT 102B2 cells and PHA-activated human lymphoblasts. Nonsaturable binding was estimated by including 1 μM unlabeled anti-Tac antibody in selected tubes. The residual radiolabel associated with the cells was subtracted from the total bound fraction found in the absence of unlabeled antibody to give specific binding. (B) A Scatchard plot of the data in A.

to the ratios obtained from competition binding analysis. It took a 55-fold molar excess of anti-Tac to reduce high-affinity IL-2 binding on HUT 102B2 cells by half, but only a 1:130 ratio of antibody to IL-2 to block low-affinity factor binding by an equal amount (Fig. 4). Similarly, in the reverse experiment, it took a 100-fold molar excess of IL-2 to block the binding of [³H]anti-Tac to the

predominant low-affinity receptors by 50%, but only a 1:60 ratio of IL-2 to antibody to block the binding of anti-Tac to the small fraction of high-affinity IL-2 binding sites (HUT 102B2 cells, Fig. 5).

Discussion

The original radiolabeled IL-2 binding studies of Robb et al. (3) demonstrated that the growth factor interacted with activated T cells with a particularly high affinity. This and subsequent investigations (4, 19) provided substantial evidence that the high-affinity interaction directly correlated with the physiological response of these cells to IL-2. In separate studies, the cellular site for growth factor binding was shown to be associated with the protein recognized by the anti-Tac monoclonal antibody (6–8). Nevertheless, discrepancies between the IL-2 and anti-Tac binding assays raised questions as to whether the factor and antibody-reactive sites were due to coincident, or merely overlapping, sets of molecules (10). For the particular cell types studied here, three lines of evidence argue that the sets are, in fact, coincident. First, inclusion of the low-affinity phenomenon in the total for IL-2 binding greatly reduced the numerical discrepancy between the IL-2 and anti-Tac binding assays (Table I). The relatively minor quantitative difference remaining could certainly be due to inherent errors in estimation of the specific radioactivity of one or both probes. The radiolabeled IL-2 preparation, for example, required an indirect approach for estimation of its protein content (see Materials and Methods). In addition, potential endogenous production of IL-2 or other growth-promoting substances (21) may have competed for some of the radiolabeled factor binding in the case of the PHA-lymphoblasts and HUT 102B2 cells. The level of low-affinity IL-2 binding may also have been underestimated due to overcompensation for nonspecific interaction of IL-2 with the cells. Second, inclusion of unlabeled anti-Tac antibody substantially inhibited binding of radiolabeled IL-2 over the entire range of IL-2 concentrations (Fig. 4), indicating that the low-affinity, as well as the high-affinity, binding was probably attributable to the Tac protein. Thus, in the case of the HUT 102B2 cells, the low-affinity interaction, in addition to being largely saturable (Fig. 3), was not due to cross-reaction with an unrelated surface receptor. Finally, excess IL-2 totally blocked radiolabeled anti-Tac binding (Fig. 5), implying that all Tac proteins were capable of recognizing IL-2 with some affinity. The detection of a large pool of low-affinity factor binding sites on the murine cell lines suggests that a multiplicity of binding affinities explains a similar quantitative discrepancy with the anti-receptor antibody studies in that species.

The near equivalence of the binding site numbers obtained in the IL-2 and anti-Tac assays for PHA-lymphoblasts is consistent with the finding for this cell type that anti-Tac and IL-2-coupled affinity supports were each capable of removing all the cellular proteins reactive with the alternative support (7). Furthermore, the relationship between the affinities of the two types of IL-2 binding sites and that of the anti-Tac antibody provides a potential explanation for the seemingly contradictory finding that a large molar excess of each ligand was necessary to block the binding of the other ligand (Figs. 4 and 5; references 7 and 9). Based on the ratios of affinities, a molar excess of antibody would be necessary to block *high-affinity* IL-2 binding with its 40-fold lower dissociation

constant. On the other hand, a molar excess of IL-2 would be necessary to block binding of anti-Tac to the more prevalent *low-affinity* IL-2 sites, since for this class of receptors the antibody has the higher affinity. This simple model assumes that the antibody and IL-2 interact with the Tac protein at overlapping sites and that the inhibition of binding arises from steric hindrance. More complicated models would have to be invoked if the two ligands bind at distant sites on the Tac molecule and block each other's binding by inducing conformational changes.

To a first approximation, the IL-2 binding appeared to be composed of a single high-affinity and a single low-affinity component (Fig. 7 *B*). It was difficult, however, to distinguish between the existence of two distinct binding phenomena and a mixture of sites with a continuous range of affinities. Moreover, negative cooperativity between receptor molecules could account for some of the apparent differences in affinity and the curvature of the low-affinity Scatchard plots (22). The variability seen within a species in the dissociation constant for the high-affinity binding phenomenon (i.e., early and late 1C9 cultures and the three IL-2–dependent murine lines) was unexpected considering the consistency seen in a previous survey of receptor-positive cells (23). Since equilibrium binding can be affected by such things as ligand-receptor internalization and ligand degradation, however, conclusions about the variable nature of the IL-2 binding must await additional approaches, including kinetic analysis of ligand binding on isolated cell membranes and molecular analysis of the different states of the receptor molecule. The apparent dissociation constants listed in Table I result from the use of a certain methodology and set of conditions and arise from a simplistic mathematical derivation (Fig. 7 *B*). Although these values may thus be biased, any errors will have a negligible effect upon the enormous difference in K_d's measured for the high and low affinity classes of IL-2 receptors and thus will not alter the central argument that IL-2 can interact with certain cells with more than a single affinity.

The Tac protein was recently purified to homogeneity by immunoaffinity chromatography (24). Amino-terminal sequence analysis indicated the presence of a single polypeptide chain. Using a synthetic oligonucleotide probe based on the sequence information, cDNAs corresponding to the IL-2 receptor mRNA were isolated and sequenced. Expression of one of these cDNA clones in Cos-1 cells resulted in display of molecules on the cell surface that were capable of binding radiolabeled IL-2 and anti-Tac (24). Moreover, examination of several cell types using the cDNA clone suggested that a single structural gene coded for this component of the IL-2 receptor (24). Thus, molecular heterogeneity of the Tac protein (25) is probably generated by differences in posttranslational modifications, which include sulfation, disulfide bonding, phosphorylation, and extensive glycosylation.[2] Such differences could account for the disparity between low and high affinity IL-2 binding. Although each of the precursors in the maturation of the Tac protein was capable of binding to an IL-2–coupled affinity

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[2] Leonard, W. J., J. M. Depper, M. Krönke, R. J. Robb, T. A. Waldmann, and W. C. Greene. The human receptor for T-cell growth factor (TCGF): evidence for sulfation, phosphorylation, variable post-translational processing, and the ability of precursor forms of the receptor to bind TCGF. Submitted for publication.

support,[2] it was not possible from that analysis to determine whether their affinity for IL-2 varied.

The finding of IL-2 binding sites with multiple affinities is not unexpected, given the results obtained with other polypeptide growth factors. The receptor for nerve growth factor was shown to exist in two states which differed in affinity 17-fold (26, 27). These forms could be interconverted by interaction with wheat germ agglutinin, indicating that the affinity differences were due to conformational changes (26, 28). Similarly, the receptor for epidermal growth factor was found to exist in low and high affinity states (29), which could be interconverted by binding of a monoclonal anti-receptor antibody (30). As with IL-2 and T cell proliferation, the less numerous high-affinity sites appeared to be responsible for the growth-promoting properties of epidermal growth factor on one particular cell line (29). Finally, cellular receptors for platelet-derived growth factor were also shown to express two distinctly different affinities, depending upon conditions of temperature and ligand binding (31). Thus, in addition to potential differences in posttranslational modification, the variation in IL-2 receptor affinity could be due to conformational changes caused by interaction of the Tac protein with a different hypothetical receptor subunit (10) or by dimerization of the Tac molecule, as was postulated in the case of the nerve growth factor receptor (28). Alternatively, separate high and low affinity binding sites for IL-2 could be present on the same molecule.

The low-affinity IL-2 binding sites were found on all Tac-positive cells tested but not on several Tac-negative lines (Table I). The presence of both high and low affinity IL-2 receptors on the 1C9 B cell line and the B cells obtained from the HCl sample was unusual but not without precedence (32). Selected EBV-transformed B cell lines, mitogen-activated normal B cells, and B cells from patients with hairy cell leukemia (HCL) were all shown to display the Tac protein (32, 33). In fact, one such B cell line was shown to respond to low concentrations of homogeneous IL-2 with increased immunoglobulin secretion (32). As expected, this response was blocked by the anti-Tac antibody. The excess of IL-2 binding sites over antibody binding sites on the 1C9 cell line, however, raises the possibility that a fraction of the low-affinity sites on this particular line might represent cross-reaction with a surface component which is unrelated to the Tac protein.

The proportion of high and low affinity binding sites for the different cell types varied (Table I). For example, most of the initial increase in Tac expression upon restimulation of 9-d-old PHA-lymphoblasts corresponded to low-affinity sites. The same was found for PMA-induced receptors on HSB$_2$ cells and the receptors displayed on the B cells in the HCL sample. In addition, the ratio of low to high affinity binding for the murine CTL and HT-2 lines was higher than that for the CTLL-2 line. Thus, display of the protein recognized by the anti-Tac or 7D4 antibodies was not always a quantitative measure of the capacity of the cells for high affinity interaction with IL-2. Analysis of IL-2 receptor expression should therefore include a determination of the relative prevalence of high and low affinity IL-2 binding sites.

The low-affinity binding sites for IL-2 are unlikely to directly participate in normal physiological responses to the factor, since at concentrations that might

occur in vivo (<200 pM)[3] only a minute fraction (<1%) of such sites would be occupied. In contrast, the same concentration would saturate the high-affinity sites. Nevertheless, a high enough local concentration of IL-2 might momentarily exist if the producing and responding cells are in contact or if the secreting cell also displays low-affinity receptors. Moreover, the low-affinity sites may act as a pool of components for generation of high-affinity receptors. If ways can be found to interconvert high and low affinity IL-2 binding sites, as was done for the nerve growth factor receptor (26), it may be possible to conclusively determine the contribution of each form of the molecule to any particular physiological response. In the meantime, there is ample reason to exercise caution in interpreting the results of experiments employing high concentrations of IL-2. In each case, the factor concentration necessary for the cellular response should be compared with the dissociation constant(s) and density of the IL-2 receptors. Both of the latter factors will be crucial in determining the extent of the response (4). If the analysis indicates that few, if any, receptors are present with an appropriate affinity, then alternative explanations, such as contaminants in the IL-2 preparation, should be explored. Similarly, putative responses to IL-2 by cells that are negative for the Tac protein may represent the effects of contaminants in the IL-2 preparation or an indirect effect upon residual T cells in the cell population (34). In addition, they might be due to cross-reaction of IL-2 at very high concentrations with receptors for distinct growth or differentiation factors. In fact, the latter possibility has been invoked to explain the IL-2–dependent induction of immunoglobulin secretion for a Tac-negative B cell line, SKW6.4 (35). Clearly, attribution of physiological effects to the kind of low-affinity IL-2 receptor described here requires the elimination of several competing explanations.

Summary

Interleukin 2 promotes proliferation of T cells by virtue of its interaction with a high-affinity cell surface receptor. This receptor is a 55,000 mol wt glycoprotein that is also recognized by the murine monoclonal antibody, anti-Tac. Quantitative binding studies with radiolabeled IL-2 and anti-Tac, however, initially indicated far more antibody binding sites per cell than IL-2 binding sites. Extension of the IL-2 binding analysis to concentrations several thousand-fold higher than that necessary for the T cell proliferative response demonstrated the existence of a class (or classes) of low-affinity IL-2 binding sites. Inclusion of the low-affinity IL-2 binding greatly reduced the quantitative discrepancy in the ligand binding assays. That the low-affinity binding, as well as the high-affinity interaction, was associated with the Tac molecule was indicated by the finding that the antibody could substantially or totally block the entire spectrum of IL-2 binding and by the finding that IL-2 could in turn block all radiolabeled anti-Tac binding. The low-affinity sites were found on activated T cells, several human and murine T cell lines and two examples of Tac-positive B cells. The physiological role of the low-affinity IL-2 binding sites and the molecular changes in the Tac protein that give rise to the affinity differences remain open to investigation.

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[3] Equivalent to the typical concentration of IL-2 released in vitro after 24 h by 1×10^6 PBL/ml following stimulation with PHA or by a mixed lymphocyte reaction.

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