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Modulation of Interleukin 2 High-Affinity Binding by Lymphocyte-Derived Tetrahydrobiopterin: Pterins as Potential Participants in the Control of Interleukin 2 Receptor Assembly

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In this report, we have examined whether (6R)-tetrahydrobiopterin (H_4biopterin) modulates the binding of interleukin 2 to high-affinity sites of the cloned mouse cytotoxic T-lymphocyte clone CTLL-2. Scatchard plot analysis of the equilibrium binding data reveals increased affinity when the cells are exposed simultaneously to interleukin 2 and to the pterin. The K_d values are statistically significantly reduced from 1.4×10^{-11}M to 0.78×10^{-11}M interleukin 2. The dissociation kinetics of the ligand were followed at 4°C after equilibrium binding under high-affinity conditions (1.2×10^{-10}M interleukin 2). In the presence of H_4 biopterin, the dissociation rate constant (k_{-1}) decreases from 6.2×10^{-3} min^{-1} to 3.0×10^{-3} min^{-1} and the half-time for dissociation increases from 106.8 min to 218.0 min. As a third approach interleukin 2 was bound to the surface of cells under high-affinity conditions by incubation in the cold and the internalization kinetics upon warming were determined. Sigmoidal-shaped kinetics of endocytosis in control cells indicate that the internalization rates increase only gradually. The presence of H_4 biopterin causes an apparent immediate transition from higher-order kinetics to a linear response so that maximum internalization rates are reached immediately upon warming. The data show that lymphocyte-derived H_4 biopterin in vitro at concentrations ranging from $2–8 \times 10^{-7}$M modulates interleukin 2 high-affinity binding and that H_4 biopterin potentially participates in the control of interleukin 2 receptor assembly.

Key words: interleukin 2 binding, high-affinity receptor

The clonal expansion of T cells is directed by interleukin 2 (IL-2), which mediates its effects by binding to specific sites on membrane receptor molecules. Receptors for IL-2 can be resolved into high-affinity ($K_d \sim 10^{-11}$M) and low-affinity ($K_d \sim 10^{-8}$M IL-2 [1]. Current evidence from receptor reconstitution [2–7] and from chemical crosslinking [2–5, 8–11] data suggests that the high-affinity form of the IL-2 receptor

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corresponds to a membrane receptor complex. This complex is composed of at least two glycoprotein chains, p55 and p75, each of which is independently capable of binding IL-2 with low (p55) and with intermediate (p75) affinity. They act in a synergistic manner whereby p55 assists in the formation of high-affinity binding sites and p75 is the moiety responsible for ligand endocytosis and for signal transduction [9].

In activated murine T cells and in the mouse cytotoxic T lymphocyte clone CTLL-2 additionally to p55 and p75 a putative γ-chain appears to be associated with the high-affinity receptor complex [12,13].

Immunoprecipitation studies with monoclonal antibodies directed against p55 [8,9,12] and p55 cDNA transfection studies [14] suggest that the receptor chains are not covalently linked, e.g., by disulfide bridges. Their interaction is likely to be dynamic. It is concluded that an IL-2-induced conformational change in the structure of either or both of the chains mediates their association [14,15] so that a high-affinity complex gradually emerges.

Pterins are synthesized and secreted by human peripheral blood mononuclear cells [16] and by T cells of human [17,18] or mouse [19] origin during defined periods of the immune response. Both the slowly progressing pterin synthesis after lectin induction of resting T cells [16,19] and the more rapidly occurring pterin formation after IL-2 stimulation of IL-2 receptor$^+$ T cells [17] culminate before the cells enter DNA synthesis. IFN-γ not only controls the activity of GTP cyclohydrolase and the release of neopterin in macrophages [20] but appears to contribute to the control of H_4 biopterin synthesis and its release in activated T cells [16, for review see ref. 21].

H_4 biopterin was initially characterized as a co-mitogen during lectin stimulation of lymphocytes [22,23]. The modulation of IL-2-induced DNA synthesis in human lectin-induced blasts and in the murine T cell clone CSP 2.1 indicates that this pterin acts synergistically with the lymphokine [24]. The entry into DNA synthesis is markedly accelerated when human lectin-induced blasts are exposed to IL-2 and H_4 biopterin simultaneously and the kinetics of S-phase transition become linear [17]. Endocytosis and degradation of ^{125}I-IL-2 are accelerated by this pterin [25]. These findings suggest that H_4 biopterin cooperates with the lymphokine at an early stage and that the interaction may occur at the receptor level.

In this study we have begun to analyze the mechanisms by which H_4 biopterin modulates the perception of the IL-2 signal in CTLL-2 cells by means of equilibrium binding data, by kinetic studies of IL-2 dissociation, and by following the time course of ligand internalization.

MATERIALS AND METHODS

Reagents and Chemicals

The following compounds were used: nIL-2 (LYMPHOCULT-T-HP Biotest AG; Offenbach), rIL-2 (Boehringer; Mannheim), r^{125}I-IL-2 sp. act. 1.11-2.59 $\times$ 10^{13} Bq/mmol, according to the manufacturer (NEN; Boston), FCS (Gibco; Paisley), other medium components (Biochrom; Berlin), (6R)-H_4biopterin (Schircks; Jona), silicone oil AR (WackerChemie; München), and other analytical-grade chemicals (Sigma; St. Louis). Millititer GV plates (96 wells) with 0.22 μm Durapore membranes were mounted in the Millititer TM filtration system from Millipore (Bedford).

Cell Cultures and Preparation of the Cells

The IL-2-dependent cell line CTLL-2 was a gift from Dr. E. Rüde, Dept. of Immunology, University of Mainz. The cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2×10^{-3}M L-glutamine, and 1×10^{-4}M α-thioglycerol. For biopterin analysis the cells were sedimented, washed, and incubated with IL-2-free medium for 1 h at 37°C at a density of 0.6×10^6 cells/ml. At the periods indicated aliquots of $1–5 \times 10^6$ cells were harvested and quickly washed.

Determination of H_4biopterin by Reverse-Phase HPLC

The analysis of reduced biopterins was performed according to [26]. Experimental details and the instrumentation assembly are described in [19].

Equilibrium Binding of ^{125}I-IL-2

Cells were washed and incubated twice with IL-2-free medium for 1 h at 37°C with washings between and after the incubation to deplete the cells of endogenous IL-2 [1]. This treatment also reduces and standardizes the H_4biopterin levels in cells harvested from cultures of varying densities. After suspension in cold RPMI/bovine serum albumin (BSA) the cells were incubated at 4°C with serial dilutions of ^{125}I-IL-2 in RPMI/BSA (2:3 dilution steps) in 96-well Millititer GV plates for 30–40 min according to [27]. Each well contained 3×10^5 cells. Using this protocol triplicate sets of control cells and of cells which were additionally exposed to H_4biopterin were obtained simultaneously. After the binding period they were washed five times with ice-cold RPMI/BSA and the radioactivity on the individual filters was determined. Nonspecific binding was obtained by including a 200-fold excess of rIL-2 in the assay and was not altered by including H_4biopterin during the binding period.

Scatchard Analysis

The LIGAND computer program [28] was used for Scatchard analysis of the equilibrium binding data. It uses a statistically valid, appropriately weighted least-squares curve-fitting algorithm which evaluates the quality of fit. The program, moreover, allows nonspecific binding to be treated as a fitted parameter. In selected sets of experiments binding data were generated by subtraction of experimentally obtained nonspecific binding. They varied <15% from the results obtained by treating the nonspecific binding as a fitted parameter. Confidence intervals and SE were calculated according to the program operation guide. The correlation of affinity (K) and capacity (R) and the joint confidence ellipses were generated from the data in the covariance matrix of the LIGAND program as described [29]. The LIGAND computer program system (1983 version) together with the program operation guide (1987 version) was kindly provided by Dr. P.I. Munson (National Institute of Child Health and Human Development, Bethesda, MD 20205).

Kinetics of ^{125}I-IL-2 Dissociation

Cells were pre-incubated in IL-2 as was described for the equilibrium binding experiments. Equilibrium binding under high-affinity conditions was achieved by incubating the cells (1×10^7 cells/ml) with ^{125}I-IL-2 (1.2×10^{-10}M) in RPMI/BSA for

30–40 min at 4°C. Nonspecific binding was determined by including a 100-fold excess of rIL-2. After centrifugation, ^{125}I-IL-2 dissociation was initiated by resuspension in a fourfold volume of cold RPMI/BSA. The dissociation kinetics were determined at 4°C to prevent internalization of the ligand. Aliquots (5×10^5 cells in 200 μl) were pelleted through 150 μl silicone oil (density 1.026; 2 parts Wacker AR 200 + 1 part Wacker AR 20) into a cushion of 20 μl 15% perchloric acid by centrifugation for 30 s in a Beckman Microfuge B. The tips of the tubes were cut off and the cell-associated radioactivity was counted. Control samples and samples in which H_4biopterin was included during equilibrium binding and during subsequent dissociation were each processed fivefold. These data points varied <12% from the mean value. The time interval over which the dissociation was measured started with the dilution of the centrifuged cells. Four independent experiments were performed according to this protocol with comparable results. The $t_{1/2}$ for dissociation was calculated from the natural log plots of the data. The dissociation rate constants (k_{-1}) are the slopes of these plots.

Kinetics of ^{125}I-IL-2 Internalization

Cells were incubated in IL-2-free medium as described for the equilibrium binding experiments. They were resuspended at a density of 1×10^7 cells/ml in RPMI/BSA and incubated for 5 min at 37°C with 1×10^{-4} M chloroquine which was maintained throughout the following steps [30]. IL-2 equilibrium binding was achieved by incubation of 2.5×10^6 cells/ml with $1.1–1.4 \times 10^{-10}$M ^{125}I-IL-2 at 4°C for 20–40 min. Internalization was started by quick warming to 37°C. To determine the levels of total cell-associated (surface bound + internalized) ligand, 200 μl aliquots were separated by silicone oil density gradient centrifugation as described for the dissociation experiments. Acid treatment of the cells prior to centrifugation through silicone oil was used to selectively remove IL-2 attached to the cell surface [30,31]. All samples were processed in triplicate. Six independent experiments were performed according to this protocol with comparable results. Nonspecific binding was determined by including a 200-fold excess of rIL-2 during the binding period.

Addition of H_4biopterin

A stock solution of H_4biopterin (1 μg/μl) was dissolved in 1×10^{-5}M 2-mercaptoethanol and was appropriately diluted with RPMI/BSA to yield the final concentrations used ($4–8 \times 10^{-7}$M). During the equilibrium binding assays these pterin concentrations were adjusted in the Millititer plate wells before the cells were added. In the dissociation and in the internalization experiments IL-2 and the pterin were simultaneously added to the cells. These pterin concentrations were maintained throughout the binding period at 4°C and during the dissociation and internalization periods. Care was taken to prevent exposing the highly photosensitive compound to daylight. A 2-mercaptoethanol control incubated with the cells had no effect on equilibrium binding data, on dissociation progress, or on internalization kinetics.

Curve Fitting

The data points of the dissociation and of the internalization kinetics were processed by the SAS procedure REG [32].

RESULTS

H_4biopterin Levels in CTLL-2

The H_4biopterin concentration in CTLL-2 cells harvested from cultures of different densities varies markedly, with values averaging 10.8 ± 6.7 pmol/10^6 cells (n = 6). Upon resuspension in medium the cellular levels decline within 10 min to about half of the original values and gradually level off to reach uniform concentrations of 3–5 pmol/10^6 cells after 40–60 min. Due to the limits of detection (0.5–0.8 pmol biopterin/10^6 cells), determination of SD values is no longer meaningful at these concentrations. We first obtained these preliminary data with CTLL-2 to define optimum experimental conditions. They suggested that the pre-treatment which is recommended for IL-2 depletion prior to IL-2 equilibrium binding also provides adequate conditions for studying the effect of exogenously added H_4biopterin.

Equilibrium Binding of ^{125}I-IL-2

To examine a modulator function of H_4biopterin in high-affinity receptor formation the binding data of the cells were examined at concentrations of 2×10^{-12}–2.4×10^{-10}M ^{125}I-IL-2. Equilibrium binding at 4°C was achieved after 20 min and increasing incubation times to 40 min did not change the results.

The binding data as obtained from five independent experiments, done in triplicates, are listed in Table I. In each of them the K_d values are reduced by approximately half when the cells are exposed to the ligand together with H_4biopterin. The increased affinity appears to correlate with a reduced number of high-affinity binding sites. Nonspecific binding, however, is not altered by the pterin.

To determine the confidence intervals and SE and to generate the confidence ellipses triplicate binding data from multiple binding experiments may be pooled by the LIGAND program. The Scatchard plot analysis (Fig. 1a) indicates that for 2,300 binding sites the K_d value is 1.40×10^{-11}M IL-2, a number which does not necessarily coincide with the arithmetic mean of the K_d values shown in Table I (Fig. 1a). This K_d value for control cells has a 95% confidence interval from 1.25–1.58×10^{-11}M IL-2. Inclusion of H_4biopterin during the binding period reduces the number of binding sites to 1,600 but increases the apparent affinity ($K_d = 0.78 \times 10^{-11}$M IL-2). The K_d values for these cells have a 95% confidence interval from 0.66–0.93×10^{-11}M IL-2.

TABLE I. Characteristics of ^{125}I-IL-2 Equilibrium Binding to CTLL-2 Cells*

	Experiment K_d ($\times 10^{-12}$M)		Binding sites/cell	
	Controls	With H_4 biopterin	Controls	With H_4 biopterin
I	16.3	7.3	2,300	1,600
II	9.1	5.6	1,655	1,415
III	11.3	6.4	1,577	1,026
IV	13.4	7.4	1,524	1,450
V	16.6	7.7	1,443	884

*CTLL-2 cells were incubated with ^{125}I-IL-2 at 4°C for 40 min in Millititer GV plates. H_4 biopterin was added during the incubation period at a concentration of 4×10^{-7}M. Triplicate determinations were processed by the LIGAND program [29].

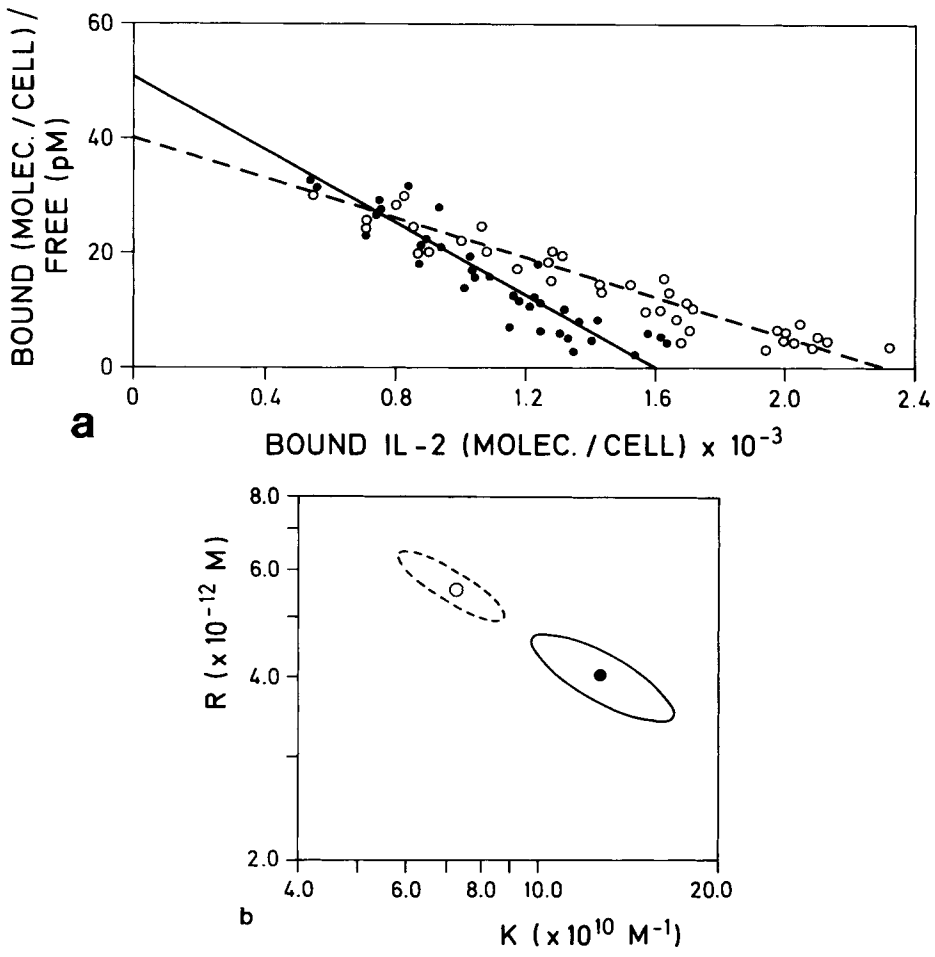

Fig. 1. **a:**Scatchard analysis of equilibrium binding of ^{125}I-IL-2 to CTLL-2 cells. Each data point represents triplicate determinations which were processed by the LIGAND program. Five independent experiments were pooled. o----o control cells, K_d = 1.4 × 10^{-11}M IL-2; ●—● with (6R)-tetrahydrobiopterin (4-8 × 10^{-7}M), K_d = 0.78 × 10^{-11}M IL-2. **b:** Joint confidence ellipses generated from the data in a. Nonoverlapping ellipses represent results which are statistically significantly different at the P = <0.05 level. o----, control cells; ●— with (6R)-tetrahydrobiopterin (4-8 × 10^{-7}M).

The affinity (K) and the capacity (R) are typically negatively correlated at a level of −0.90 to −0.95 in binding measurements [29]. The joint confidence ellipses (Fig. 1b) show the confidence regions for this pair of parameters at the probability level of P = <0.05. The two ellipses do not overlap or touch, proving that H_4biopterin induces a significant increase in apparent affinity of the cells for IL-2.

Kinetics of ^{125}I-IL-2 Dissociation

In order to obtain further information as to the modulator function of H_4biopterin in high-affinity binding the kinetics of dilution-induced IL-2 dissociation were examined after association of the ligand with 1.2 × 10^{-10}M ^{125}I-IL-2 (high-affinity conditions) (Fig. 2). The dissociation of IL-2 follows first-order kinetics. It is more rapid in controls

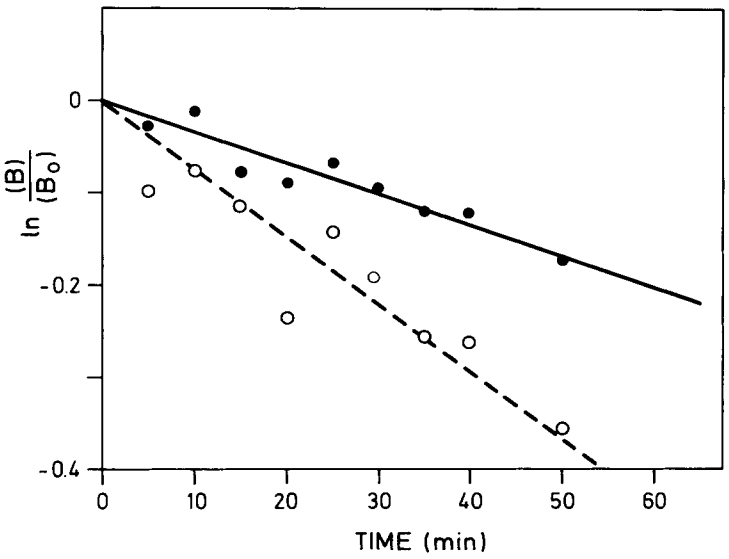

Fig. 2. Kinetics of IL-2 dissociation from CTLL-2 cells at 4°C. After equilibrium binding of ^{125}I-IL-2 (1.2×10^{-10}M) the centrifuged cells were resuspended in a fourfold volume of RPMI/BSA. Each data point represents the mean of five fold determinations. B_0 = specific binding at t_0; B = specific binding at the times indicated. O----O, control cells; ●—● with (6R)-tetrahydrobiopterin (8×10^{-7}M). The pterin was included both during the period of equilibrium binding and during the period of dissociation.

than in cells which have been supplied with H_4biopterin during the binding and the dissociation period. The pterin decreases the dissociation rate constant (k_{-1}) from 6.2×10^{-3} min^{-1} to 3.0×10^{-3} min^{-1} and increases the $t_{1/2}$ for dissociation at 4°C from 106.8 min to 218.0 min.

Kinetics of ^{125}I-IL-2 Internalization

Pre-binding of IL-2 to the cells under conditions which permit specific binding only to high-affinity sites (10^{-11}M IL-2) requires the formation of a complex with properly paired receptor chains (p55 + p75) for its subsequent internalization [9]. This event was therefore used as an indicator to indirectly follow the rates of such high-affinity receptor complex formation.

After equilibrium binding of 1.2×10^{-10}M ^{125}IL-2 (high-affinity conditions), 2,250 ± 120 IL-2 molecules are associated with each cell, 273 molecules being unspecifically adsorbed. Treatment of these cells with pH 3.6 buffered medium results in a rapid dissociation of 96–98% of bound IL-2 and indicates that nearly all ligand molecules remained at the extracellular receptor domain. When the cells are transferred to 37°C without washing and lysosomal degradation is inhibited by including of chloroquine, the total cell-associated IL-2 linearly increases up to 2,830 ± 147 molecules/cell in both control and H_4biopterin-exposed samples during the following 50 min (data not shown). This 30% increase in total cell-associated IL-2 may reflect the reassociation of free ^{125}I-IL-2 to recycled or newly expressed receptors [33]. Nonspecific binding remains at the same relative level during this period and comprises <15% of the total cell-associated ligand. It was not subtracted from the values presented in Figure 3. Consideration of the nonspecific binding does not appreciably change the relative shapes or proportions of the

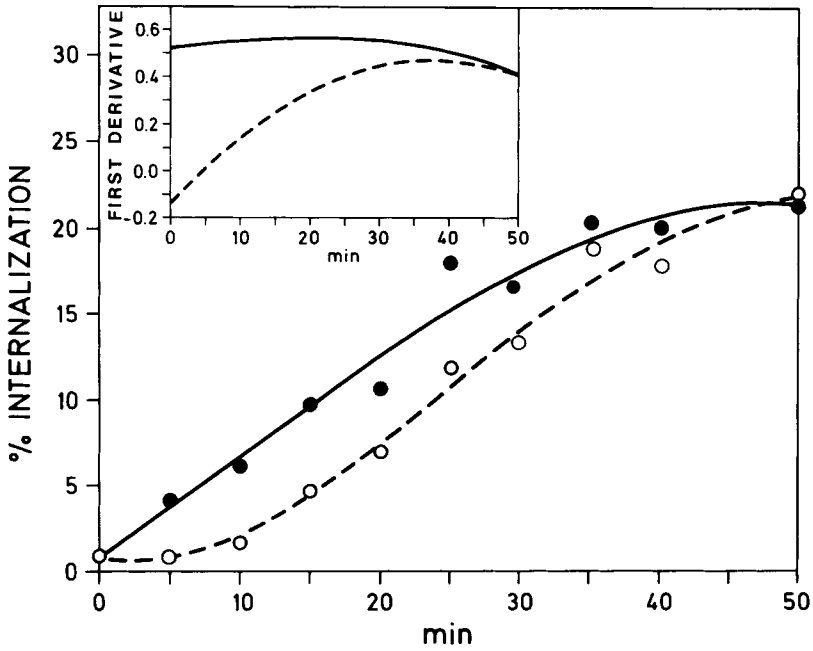

Fig. 3. Internalization time course of IL-2 in CTLL-2. Cells were incubated for 20 min at 4°C with ^{125}I-IL-2 (1.2×10^{-10}M) plus chloroquine (1×10^{-4}M) and subsequently warmed. The acid-resistant portion of total cell-associated ligand was determined. Each data point represents the mean of triplicate determinations. Insert: First derivatives. The pterin was added together with ^{125}I-IL-2 and was thus present during equilibrium binding and during subsequent internalization. O----O, control cells; ●—●, with (6R)-tetrahydrobiopterin (4×10^{-7}M).

curves or the interpretation of the data but could introduce an additional value subject to experimental variation.

The portion of cell-bound radioactivity which is resistant to acid treatment is defined as the internalized fraction. It is regarded in relationship to the total of cell-associated ligand which increases linearly with time. Determinations of the acid-resistant and the surface-bound radioactivity are each independently subject to experimental variations. Therefore, the acid-resistant internalized fraction varies between 20–61% of the total cell-associated ligand in six experiments. The progression of internalization, however, is uniform. The internalized portion increases only gradually in the control cells during the first 15 min and levels off after 30 min. The data points are best fitted by a polynominal curve of the third grade (Fig. 3). The first derivative of this curve represents its slopes at the given data points (Fig. 3, inset). It demonstrates that the internalization rate, starting from subzero levels, continuously increases and reaches maximum values only after 30 min, at which time the amount of internalized IL-2 already approaches maximum levels. When H_4biopterin is added together with the ligand the internalized portion grows linearly and the reaction levels off after 30 min. The first derivative of this curve demonstrates that immediately after warming maximum internalization rates are achieved. Time courses of control and H_4biopterin-treated cells were run strictly in parallel. Therefore, delayed warming of control cells which could have caused the convex upward slope can be ruled out.

DISCUSSION

In the present report three lines of evidence are presented which show a modulator function of H_4biopterin in IL-2 high-affinity binding and which support the hypothesis that pterins participate in the control of IL-2 receptor assembly.

The reported dissociation constants after equilibrium binding of IL-2 to CTL clones ($K_d = 1.3–3.9 \times 10^{-11}$M IL-2) [see ref. 1,34,35] agree with the data obtained from our control cells ($K_d = 1.25–1.58 \times 10^{-11}$M IL-2) and are reduced to half the original values ($K_d = 0.66–0.93 \times 10^{-11}$M IL-2) when the cells are exposed to the ligand and to H_4biopterin simultaneously. This relative difference in K_d values as measured by Scatchard analysis is also seen in the $t_{1/2}$ of IL-2 dissociation from control and H_4biopterin-exposed cells. Both approaches indicate that the pterin modulates the interaction between the receptor subunits and/or the ligand to double the efficiency of IL-2 binding. The reduction in the number of binding sites may possibly result from increasing clustering to high-affinity complexes. The pterin concentrations found to be effective in these in vitro assays are reached or even exceeded by the pterin production of activated T cells [16–19] or CTLL-2, as was shown above.

IL-2 internalization after its binding under high-affinity conditions was used as a criterion for the formation of the receptor complex in which the ligand as well as the receptor chains are properly associated to become endocytosed.

The period for maximum internalization varied in the different experiments from 38–47 min, which agrees with data obtained from CTL cells [33] when the experimental conditions allow for continuing ligand association during the internalization period. Contrary to others, our results present a sigmoidal-shaped instead of a linear internalization time course. This difference may possibly be explained by automated processing of data points which were gained at intervals as close as 5 min, even though the curves obtained earlier from data points at close intervals in HUT 102B2 cells [30] and in the human tumor T cell line IARC 301 [36] also indicate an initial lag in the internalization process. The sigmoidal-shaped time course suggests a slowly progressing transition to high-affinity complexes which are able to become endocytosed. H_4biopterin supplied simultaneously with the ligand causes an apparent immediate transition from higher-order kinetics to a linear response. This may indicate that the formation of the functional IL-2 receptor complex is assisted by the pterin during the pre-binding period in the cold and is ready for endocytosis upon warming.

The role of H_4pterins in aromatic amino acid hydroxylation is well established [37]. Our experimental conditions which used a 20–40 min incubation at 4°C can safely rule out such a cofactor function to explain the data. Replacing H_4biopterin by other reductants, e.g., 2-mercaptoethanol or dithiothreitol at 10^{-7} to 10^{-6}M concentrations, could not mimic the results described above, suggesting that the reducing potential of H_4biopterin [for review see ref. 38] is not involved. The mechanisms by which H_4biopterin modulates the efficiency of IL-2 high-affinity binding therefore await further clarification, as does the regulation of biopterin synthesis by the immune cells.

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