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Ligand–Receptor Interactions: Detailed Evaluation of Occupancy-Dependent Affinity

Guy B. Faguet

Departments of Medicine and Cell and Molecular Biology, Medical College of Georgia, and Medical and Medical Research Services, Veterans Administration Medical Center, Augusta, Georgia 30912

The exact nature of the curvilinearity of Scatchard plots derived from hormonal and nonhormonal binding systems has not been definitively resolved. Such plots are compatible with heterogeneous receptors with different but fixed affinities and with negatively interacting binding sites resulting in occupancy-dependent affinity. In the current study we examined in detail the effect of receptor occupancy by the ligand on receptor affinity under a variety of experimental conditions. We chose the human lymphocyte–leukoagglutinin (LPHA) system, which closely mimics the IM9-insulin model. Reliable estimates of total binding capacity (728 ng/10^6 cells) essential to our report were calculated from a wide database by the least-squares model. At occupancies $\geqslant 0.085$, receptors are associated with low and fixed affinity ($1.5 \times 10^6 M^{-1}$), whereas at occupancies $\leqslant 0.085$, affinity is high and fixed ($1.8 \times 10^8 M^{-1}$) or high but variable ($1 \times 10^7 M^{-1}$ to $1.5 \times 10^6 M^{-1}$) depending on whether the binding is assumed to be noncooperative or cooperative, respectively. Calculation of receptor–ligand complex dissociation velocity over a wide range of occupancies (0.01–0.40) suggested that occupancy exerts an inversely proportional effect on affinity that is rapid and sustained. Cell activation (DNA synthesis) is initiated at receptor occupancy of $\cong 0.004$ and is magnified as ligand binding to high affinity receptors increases up to $\cong 0.07$ occupancy (functional sites), beyond which point further binding (to low affinity sites) becomes increasingly ineffective and cytotoxic (redundant sites). These findings suggest that occupancy influences affinity as postulated by the hypothesis of negative cooperativity. Through this effect occupancy may play a significant role in regulating ligand-induced cell responses.

Key words: binding interactions, occupancy, affinity

Abbreviations used: LPHA, leukoagglutinin; PHA-P, phytohemagglutinin-P; NaCl/Pi, phosphate buffered saline; ME medium, minimum essential medium; albumin, bovine serum albumin; CLL, chronic lymphatic leukemia; B-cells, Bursa-derived lymphocytes; T-cells, Thymus-derived lymphocytes.

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In 1973, De Meyts et al proposed that the curvilinear Scatchard plot generated by the binding of insulin to its receptors reflected negative cooperativity, ie, varying receptor affinity dependent on occupancy by the ligand [1]. This hypothesis was based primarily on finding enhanced dissociation of bound tracer insulin in the presence of an excess of native insulin that was assumed to increase overall occupancy. Although initially questioned [2,3], the direct relationship between ligand concentration and occupancy was experimentally supported in the LPHA-lymphocyte model [4]. However, more recent data have shown that the experiment proposed by DeMeyts et al does not provide unambiguous proof of the existence of negatively interacting binding sites. Indeed, some systems exhibit accelerated dissociation but linear Scatchard plots [5,6]. While present methodology precludes distinguishing between interacting and noninteracting binding kinetics, demonstration of occupancy-dependent affinity would support the former hypothesis. In this report we examine the interactions of leukoagglutinin (LPHA) with its receptors on human lymphocytes, a model that closely mimics the insulin-IM9 system, and provide data that strongly suggests that, in this system, affinity is occupancy-dependent. Cell function studies assessed in terms of both affinity for and occupancy by the ligand suggest that this type of receptor–ligand binding interaction might play a regulatory role in cell activation.

MATERIALS AND METHODS

Cells

Blood samples were drawn from healthy volunteers who were on no medications. Lymphocytes were isolated by ficoll-hypaque gradient centrifugation as previously described yielding lymphocyte suspensions virtually free of phagocytes, red cells, and platelets [7].

PHA Purification and Iodination

LPHA was prepared from a single batch of PHA-P (Difco Laboratories, Detroit, MI) according to a modification of the column chromatography method of Webber et al [8], as previously described [9]. This homogeneous mitogenic glycoprotein was utilized for both metabolic and receptor studies at concentrations appropriate for the purpose intended and based upon known receptor occupancy and metabolic response profiles previously reported [9,10]. For receptor studies, LPHA was iodinated (reductant-free ^{125}I, specific activity 17 Ci/mM, ICN, Life Sciences Group, Irvine, CA) by the chloramine-T method of Hunter [11] and adjusted to a final specific activity of 150,000 CPM/μg LPHA-protein in 0.5% albumin (bovine serum albumin, Grand Island Biological Co., Grand Island, NY). Details of the LPHA purification and iodination procedures have been published elsewhere [9]. Identity of ^{125}I-LPHA with native LPHA was ascertained by column chromatography, ultracentrifugation analysis in various density gradients, competitive binding studies, and by their biological activity judged by DNA synthesis responses of normal human lymphocytes [8–10].

Metabolic Studies

The LPHA-induced metabolic responses of isolated peripheral blood lymphocytes were ascertained by DNA synthesis measurements, as previously described [10]. Briefly, 10^6 lymphocytes were cultured in 2 ml volume of ME medium supplemented with fetal calf serum, antibiotics, and LPHA in concentrations ranging from

4×10^{-10} to 8×10^{-7}M. As previously reported [10], this concentration range encompasses the entire spectrum of metabolic responses to LPHA stimulation. After 3-day culture at 37°C, DNA was pulse-labeled (4 hr) with 0.5 μCi of tritiated thymidine (New England Nuclear, Boston, MA; specific activity 6.7 Ci/mM), and precursor incorporation into cell DNA was measured in a toluene-based scintillation fluid by an LS-150 Beckman Spectrometer (Beckman Instruments, Inc., Irvine, CA) with counting efficiency of 45% (SEM 0.9%). Metabolic responses to each lectin concentration measured in triplicate showed a mean standard deviation of less than 10%. Data are presented as mean of 26 experiments.

Characterization of LPHA Binding to Lymphocyte Receptors

The methodology used to evaluate the binding of LPHA to specific human lymphocyte membrane receptors has been described in detail elsewhere [4,9,10]. In brief, aliquots of 10^6 lymphocytes suspended in 0.1 ml of ME medium containing 0.1% albumin (ME medium/albumin) were incubated in duplicate plastic culture tubes presoaked with 0.5% albumin in phosphate buffered saline (NaCl-Pi, pH 7.2) with LPHA concentrations ranging from 1×10^{-9} to 2×10^{-5}M (using ^{125}I-LPHA as a tracer) in 0.4 ml of NaCl-Pi with 0.1% albumin (NaCl-Pi/albumin). After reaching equilibrium (60-min incubation at 22°C), the reaction was abruptly terminated by the addition of 10 ml of NaCl-Pi/albumin at 4°C. Unbound ligand was removed by washing thrice with ME medium/albumin at 4°C; cells were collected on 0.5% albumin-presoaked 0.45 μm filters (Millipore Corp., Bedford, MA), and cell bound radioactivity was measured in a Beckman Gamma 4000 counter (Beckman Instruments Inc., Irvine, CA). For dissociation kinetics studies, lymphocyte aliquots of 10^6 cells in 0.1 ml of ME medium/albumin were incubated with LPHA, ^{125}I-LPHA, and in some experiments ^{131}I-LPHA in NaCl-Pi/albumin over a wide range of concentrations designed to induce <0.01 to >0.50 receptor occupancy. After reaching equilibrium, unbound native and tracer ligand were removed by twice washing the cells with ME medium/albumin at 4°C and replacing the supernatants with 100 volumes of ligand-free NaCl-Pi/albumin to preclude reassociation of ligand released during the dissociation reaction [4,12]. Receptor–ligand complex dissociation was monitored from the radioactivity remaining on cells collected on 0.5% albumin-presoaked 0.45 μm millipore filters after discarding the supernatants from two centrifugations at 400g for 5 min at 4°C. In some experiments, this undisturbed, basal dissociation reaction was compared to receptor–ligand dissociation in the presence of an excess of ligand, according to De Meyts et al [1], as previously described for this system [4,9,12]. The nonspecific binding component assessed from identical concomitant incubations containing a 100-fold excess of LPHA and from blanks containing no cells were subtracted from the total to determine receptor-specific ligand binding.

Data Analysis

Data presented represent averages derived from replicate experiments as indicated in the figure legends. Within each experiment each data-point is assessed in duplicate for radio-receptor assays and in triplicate for DNA synthesis measurements with standard deviations generally less than 5 and 10%, respectively. Binding parameters were calculated from equilibrium binding experiments performed using varying concentration of hot ligand of constant specific activity. Equilibrium binding data

were analyzed by curve-fitting using the least-squares "Ligand" program [13] adapted to Applesoft Basic for the Apple II+ microcomputer and confirmed by the "Ligand" program on a DEC-10 computer (courtesy of J.E. Fletcher, NIH). Fitted parameters are displayed in Scatchard and semilogarithmic coordinates [14,15].

RESULTS

Equilibrium Kinetics

Curvilinear Scatchard plot resulting from noninteracting heterogeneous receptor sites. Once the curvilinear Scatchard plot (Fig. 1a) is assumed to reflect heterogeneous receptors each with different and fixed affinity, it can be mathematically resolved by weighted nonlinear least-squares curve fitting to estimate the R and K values of two or more binding sites. The "Ligand" computer program developed for that purpose [17,18] identifies a two-binding-sites model as "best fit" (two sites versus one site $p < 0.01$, three versus two sites $p > 0.5$) consisting of a small component (21 ng/10^6 cells) of high affinity receptors ($1.84 \times 10^8 M^{-1}$) and a second component corresponding to 707 ng/10^6 cells with low affinity ($0.86 \times 10^6 M^{-1}$) for the ligand. This model fit is graphically displayed in Figure 1b. However, because of questions raised recently regarding reliability of binding capacity measurements derived from Scatchard plots [15] and because our report is crucially dependent on binding capacity measurements, the fitted model was also displayed graphically in semilogarithmic coordinates (Fig. 1c). As shown, a symmetrical sigmoid curve with experimental

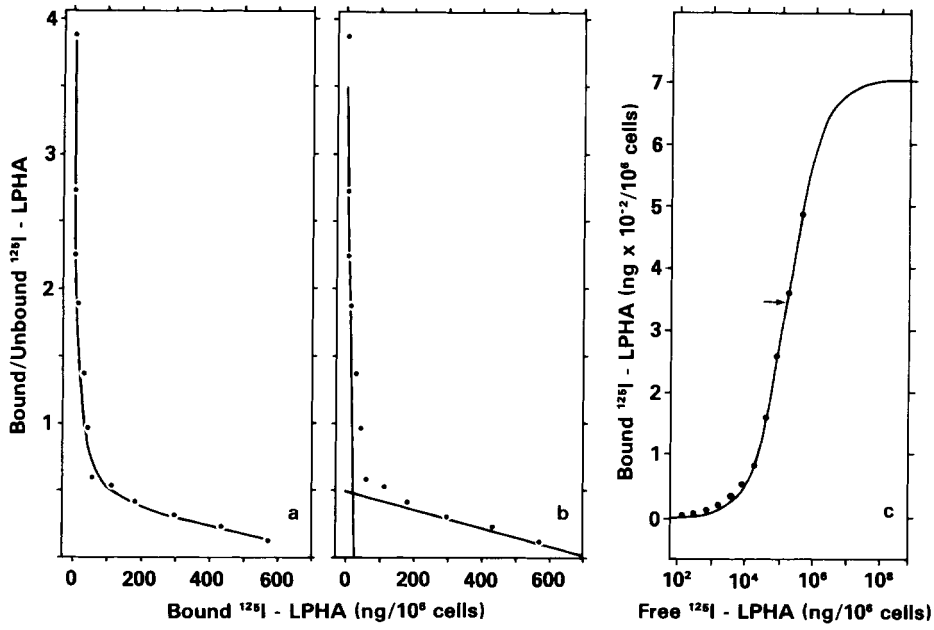

Fig. 1. Scatchard (bound/unbound versus bound) and semilogarithmic (bound versus log of free) plots of receptor-specific binding of ^{125}I-LPHA to human lymphocytes (n = 26). Panel a demonstrates the curvilinearity of the Scatchard plot. Panels b and c show graphic displays in Scatchard in semilogarithmic coordinates of the data analyzed according to the "Ligand" model.

data points distributed over more than half the model range [15] is generated. Good model fit as judged by statistical analysis [17,18] and by visual display of the fitted model in both Scatchard and semilogarithmic coordinate systems [15,18], support the adequacy of receptor capacity and fractional occupancy estimates.

Curvilinear Scatchard plots resulting from interacting binding sites. Interpretation of curvilinear Scatchard plots assumed to reflect interacting binding sites calls for an entirely different approach for estimating apparent equilibrium constants [19]. Figure 2a depicts a Scatchard plot under the assumption of a homogeneous class of interacting binding sites with affinity inversely proportional to fractional occupancy according to the postulate by DeMeyts et al [1]. According to this model, the highest affinity corresponds to the unoccupied receptor conformation. If all receptors were homogeneous with respect to affinity and if affinity remained fixed regardless of occupancy, the ratio of bound over free receptors (B/F) would be linear throughout the occupancy spectrum, joining the lowest (X intercept) to the highest (Y intercept) B/F. The negative of the slope of that line would reflect the affinity (K). However, in the presence of interacting binding sites dependent upon occupancy, increasing occupancy will result in decreasing affinity represented by decreasing slope of lines joining each experimental point of the curve to the X intercept. Plotting K at each point ($B/F/[R_o-B]$) as a function of log Y gives rise to the affinity profile (Fig. 2b) that depicts an uncertain (and unmeasurable) affinity for

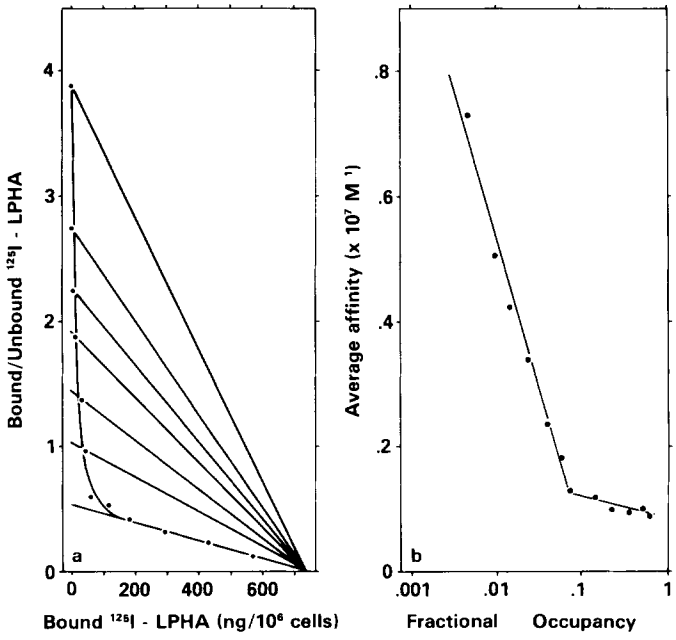

Fig. 2. Scatchard plot (panel a) of ligand binding to an assumed single class of interacting binding sites (Ro_t = 728 ng/10^6 cells). With increasing binding (thus, increasing occupancy), the average affinity decreases from the highest affinity (empty sites conformation) to a limiting low affinity conformation shown as straight lines connecting each data point to the abscissa intercept. The calculated affinity at each measured occupancy (K = [B/F]/[Ro - B]) is depicted in panel b, which shows the affinity profile expressed as a function of fractional occupancy.

the "empty sites" conformation, decreasing affinity ($0.7 \times 10^7 M^{-1}$ to $1.3 \times 10^6 M^{-1}$) between $\cong 0.005$ and $\cong 0.085$ occupancy, and a limiting low affinity (1.3 to $0.9 \times 10^6 M^{-1}$) beyond $\cong 0.085$ saturation not further affected by occupancy, corresponding to the "filled sites" conformation.

Curvilinear Scatchard plots resulting from distinct classes of interacting binding sites. Finally, distinct classes of interacting binding sites could account for the observed binding data. Such a model would be not only indistinguishable from a single class of interacting binding sites but also compatible with and encompassed by the hypothesis of negative cooperativity.

Dissociation Kinetics

As shown in Figure 3a, addition of native ligand in our system increases the dissociation of ^{125}I-LPHA-receptor complexes, as it does in many other systems [reviewed in 21]. While in this type of experiment receptor occupancy is assumed to

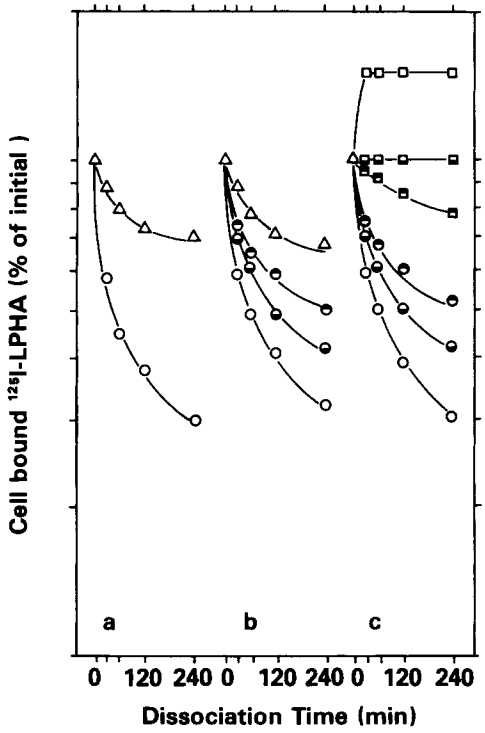

Fig. 3. Dissociation of ^{125}I-LPHA from receptor-ligand complexes. Replicate aliquots of 10^6 cells were incubated with tracer amounts (5.3×10^{-9}M) of ^{125}I-LPHA. After reaching equilibrium, supernatants were replaced with 100 volumes of fresh medium alone, or with medium containing one or more concentrations of LPHA or ^{131}I-LPHA. At the intervals shown, duplicate aliquots from each set were processed as described in the text to measure cell-bound ^{125}I-LPHA during tracer dissociation in medium alone (△) and in medium containing 2.7×10^{-8}M LPHA (○), panel a) or 5.3×10^{-10}M(◓), 5.3×10^{-9}M (◒), and 2.7×10^{-8}M (○) of LPHA, panel b) or equivalent concentrations of ^{131}I-LPHA, panel c) In the latter set, cell-bound ^{131}I-LPHA was monitored concomitantly to ascertain total receptor occupancy (□, ◪, and ◨, respectively). Data are expressed as percentage of initially bound and plotted as a function of dissociation time.

increase by the added native ligand, the correctness of this assumption and therefore the dependency of dissociation on occupancy is demonstrated in the experiments depicted in Figure 3b. As shown, spontaneous dissociation of ^{125}I-LPHA (top curve) is variously accelerated (lower three curves) by addition of native LPHA at concentrations chosen so that during the dissociation total occupancy would be expected to decrease, remain the same, and increase, respectively. These assumed occupancies are proven correct by substituting ^{131}I-LPHA for native LPHA to destabilize ^{125}I-LPHA-receptor complexes. As shown in Figure 3c, occupancies induced by ^{125}I-LPHA plus ^{131}I-LPHA (top three curves) are greater than occupancy by ^{125}I-LPHA alone at any time during the dissociation reaction, increasing in proportion to the concentrations of ^{131}I-LPHA added. This demonstrates that the enhanced tracer ligand–receptor complex dissociation induced by LPHA does reflect increased receptor occupancy by LPHA above and beyond occupancy by ^{125}I-LPHA. This is so at each time-point during the dissociation reaction.

Spontaneous occupancy-dependent receptor–ligand complex dissociation. The effect of occupancy on receptor–ligand complex dissociation was further evaluated as described above but concentrations of tracer ligand were selected to generate initial receptor occupancies ranging from 0.015–0.40. The time and occupancy dependence of spontaneous dissociation of receptor–lectin complexes is illustrated in Figure 4. While at an occupancy of 0.015 only 5 and 30% of tracer ligand initially bound dissociated in 15 and 240 min (Fig. 4a and b, data points W and X, respectively), at occupancy of 0.40 over 25 and 70% of ligand initially bound dissociated in comparable time (Fig. 4a and b, data points Y and Z, respectively). This occupancy and time dependence is quantitatively illustrated in Figure 4c which replots the data as cumulative ligand dissociation per unit of time expressed as a function of initial fractional occupancy. Over the occupancy spectrum studied (0.15–0.40), dissociation of receptor–lectin complexes is initially brisk but becomes less efficient with time as dissociation progresses. To illustrate, let us examine the dynamics of receptor–ligand complex dissocation occurring at 0.015 and 0.40 occupancy: As shown in Figure 4c, data points W and Y, the cumulative dissociation velocity averages 6.40 and 15.7 pg/ng bound/min respectively during the first 15 min. However, as dissociation continues over time (and occupancy diminishes concurrently) the velocity of subsequent dissociation progressively diminishes to a calculated 5.4 and 12.8, 3.2 and 9.7, 2.3 and 6.6, and 1.5 and 4.5 pg/ng bound/min (the latter two shown as data points X and Z, respectively) during the following 15, 30, 60, and 120 min time intervals, respectively, averaging 1.53 and 4.69 pg/ng bound/min during the entire 240-min dissociation time. Similar calculations of dissociation velocity over this wide range of occupancies demonstrated the continuous dynamic dependency of receptor–ligand complex dissociation on occupancy. The effect of occupancy on affinity is rapid and sustained suggesting energy requirement.

Accelerated occupancy-dependent receptor–ligand complex dissociation. The next series of experiments were designed to evaluate the effect on affinity of a wide spectrum of initial occupancies maintained constant during dissociation. In these experiments, spontaneous ^{125}I-LPHA dissociation was measured at fractional occupancies ranging from 0.005–0.5, and compared to ^{125}I-LPHA dissociation in the presence of LPHA at concentrations sufficient to maintain during the dissociation period the equilibrium initially induced by ^{125}I-LPHA. As shown in Figure 5a, the fraction of ^{125}I-LPHA spontaneously dissociated is directly proportional to initial

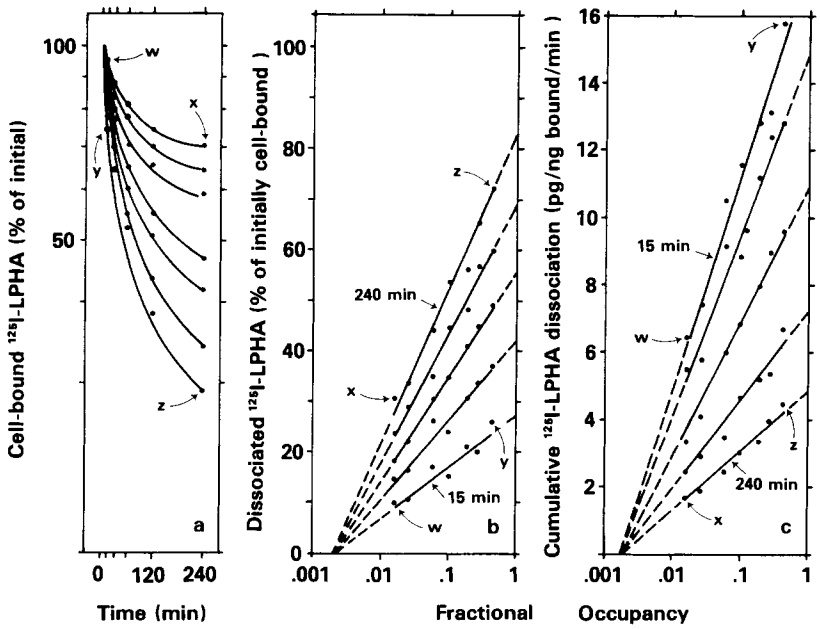

Fig. 4. Spontaneous dissociation of ^{125}I-LPHA from receptor–ligand complexes. Panel a, cell aliquots were incubated with ^{125}I-LPHA to induce fractional occupancies of 0.015, 0.024, 0.06, 0.09, 0.15, 0.24, and 0.40 (from upper to lower curve, respectively). After reaching equilibrium, spontaneous ^{125}I-LPHA dissociation was monitored for up to 240 min. Data are shown as percentage of initially bound ^{125}I-LPHA and expressed as a function of time. Panels b and c plot dissociated ^{125}I-LPHA as percentage of initially bound and as cumulative picograms dissociated/nanograms initially bound/minute, respectively, for each time interval both expressed as a function of initial receptor occupancy by the tracer. Ascending lines of panels b and c represent percentage and cumulative tracer dissociation, respectively, after 15, 30, 60, 120, and 240 min of dissociation in the sequence shown.

fractional occupancy by the ligand (represented by the hypothetical line A) at occupancies less than $\cong$0.03. However, at occupancies greater than 0.03 an occupancy-dependent geometric acceleration of the dissociation reaction takes place. Thus, while about 30% of bound ligand dissociates at occupancies $<$0.03, approximately 90% does so at 0.50 occupancy. As expected, addition of LPHA in concentrations sufficient to maintain the level of occupancy initially induced by the tracer further enhanced the dissociation of bound tracer ligand regardless of the level of initial receptor occupancy. However, the contribution of LPHA to the overall dissociation, ie the impact of sustained occupancy on average affinity, diminishes with increasing initial occupancies, particularly above $\cong$0.085 for such occupancies are associated with limiting low affinity conformation. The relative effect of initial and sustained occupancies on affinity is illustrated in Figure 5b, which plots spontaneous and LPHA-induced ^{125}I-LPHA dissociation expressed as a percentage of initially bound tracer. As shown, while spontaneous complex dissociation (Fig. 5b, curve S) is proportionately and increasingly dependent upon initial occupancy, LPHA-enhanced dissociation (Fig. 5b, curve E) represents a relatively constant percentage of the initially bound tracer at occupancies below $\cong$0.085 but rapidly decreasing percentages beyond this

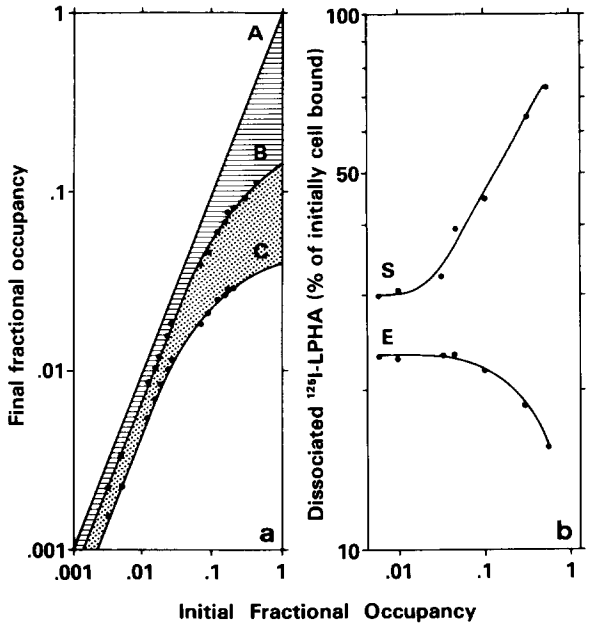

Fig. 5. Spontaneous and accelerated receptor–ligand complex dissociation. Panel a, cell aliquots were incubated with ^{125}I-LPHA to induce fractional occupancies ranging from 0.005–0.5. After reaching equilibrium, ^{125}I-LPHA dissociation occurring spontaneously (striped area) and in the presence of LPHA at concentrations sufficient to maintain initial occupancy through the dissociation reaction (stippled area) was monitored after 240 min. Lines B and C depict cell-bound ^{125}I-LPHA after spontaneous and LPHA-enhanced dissociation, respectively, compared to a hypothetical absence of dissociation (line A). All data points are expressed as a function of both initial and final fractional occupancy. Panel b replots selected data points of experiments shown on panel a. Spontaneous (S) and LPHA-enhanced ^{125}I-LPHA dissociation (E) are presented as percentage of initially cell-bound and expressed as a function of initial fractional occupancy.

point. These data further support the notion that the effect of occupancy on average affinity is dependent upon initial occupancy that need not be sustained to exert its effect on affinity.

Functional Relevancy of Cell-Bound LPHA

As shown in Figure 6, 2.8 ng/10^6 cells (occupancy of $\cong$0.004) is required to trigger significant lectin-induced DNA synthesis, the so-called activation threshold [20]. Once the number of bound receptors surpasses the activation threshold, further receptor recruitment enhances the cell response reaching a maximum at $\cong$52 ng of LPHA bound/10^6 cells (occupancy of $\cong$0.07) beyond which further binding is progressively ineffective. Assuming one LPHA molecule bound per receptor, an average of 1.3×10^4 and 2.4×10^5 LPHA molecules bound per cell are required to initiate and maximize cell activation, respectively. When receptor function (Fig. 6) is assessed in terms of both affinity for and occupancy by the ligand (Fig. 2b), it becomes apparent that a small number of high affinity sites are the most metabolically active, the remaining sites exhibiting low affinity and being metabolically redundant as judged by their eliciting a negligible response to the ligand. Increasing ligand

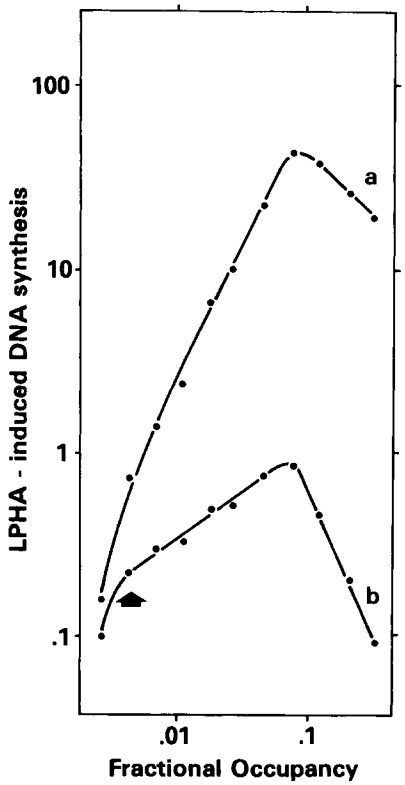

Fig. 6. Functional relevancy of cell-bound LPHA. DNA synthesis (dpm $\times 10^{-3}/10^6$ cells, line a) dpm $\times 10^{-3}$/ng of cell-bound LPHA, line b) is expressed as a function of fractional occupancy induced by the concentrations of LPHA triggering the metabolic responses.

binding to active sites is initially associated with both a progressively decreasing receptor affinity (toward low affinity conformation) but a more efficient metabolic response per unit of bound ligand. Further binding to low affinity, redundant sites is associated with progressively diminishing metabolic cell responses due to the increasing cytotoxic effects of excessive ligand binding [20].

DISCUSSION

A long and as yet unresolved controversy has arisen in the interpretation of ligand–receptor binding studies of binding isotherms suggesting cooperative binding interactions of the negative type. Conceptual and mathematical resolution of such data remain difficult and controversial particularly in view of limitations in graphical and mathematical analyses of binding data and of uncertainties relating to molecular models postulated as the basis to explain observed data. In addition, methodological and experimental shortcomings as possible explanation of results mimicking cooperative binding interactions must be recognized and avoided [4,16,17,21]. The Scatchard plot is currently the favored method for graphical display of ligand–receptor binding data. While binding of a homogeneous ligand to a single class of binding sites

with fixed affinity results in a straight-line between the Y and X intercepts, curvilinear plots of upward concavity are compatible with pre-existing asymmetry (PEA) of receptors, that is, more than one class of receptors with different but fixed affinities for the ligand or with one single class of negatively interacting binding sites. As initially defined [1], negative cooperativity refers to site–site interactions triggered by ligand binding and resulting in decreased average receptor affinity inversely proportional to occupancy by the ligand.

Although such a definition describes a phenomenon without inferences to the underlying molecular changes, Koshland, Nemethy, and Filmer (KNF) have proposed a molecular model [22] counterpart to the phenomenological model proposed by DeMeyts et al [1]. Inherent to its definition and because equilibrium binding data cannot distinguish the underlying binding model, support for the cooperative model has rested on demonstrating enhanced dissociation of bound, labeled ligand as receptor occupancy is increased by addition of an excess of unlabeled ligand in the dissociation reaction. However, there is mounting evidence that the dissociation experiment in itself is not an unambiguous proof of negatively interacting binding sites. Indeed, enhancement of the dissociation of ^{3}H-dehydroalprenolol from frog β-receptors by native alprenolol is seen in spite of a noncooperative binding Hill coefficient of 1.0 [6]. Similarly, enhanced ^{125}I-TSH dissociation results from increasing concentrations of TSH noncooperatively bound (linear Scatchard plots) to cultured thyroid cells [5]. In addition, an enhanced rate of dissociation of ^{125}I-Abrin and ^{125}I-Insulin from inert substances, such as Sephadex particles and talc, respectively, by their native ligand counterparts has been described [2,23]. These observations appear to eliminate the enhanced dissociation method as the definitive tool to prove negative cooperativity.

Our approach has been to evaluate the relationship between occupancy and affinity at equilibrium, and during dissociation under conditions conducive to explore their relationship and thus further characterize the binding isotherm. The present study supports the occupancy-dependent affinity of lymphocyte receptors for LPHA as evaluated under a variety of experimental conditions. Taken together, results of our equilibrium and kinetics studies are not compatible with the hypotheses of flexible or polyvalent ligand, mobile receptor or concerted substitution, or with the PEA model [4] but rather provide further experimental evidence in support of the concept of negative cooperativity. A more definitive interpretation of the underlying sequential conformational changes as proposed by the KNF model [22] has been proposed on the basis that changes in negative cooperativity occur when the primary ligand binds in the presence of a noncooperative competing ligand [24,25]. Using this approach, we have additional data in support of the KNF model in the binding of insulin to IM9 receptors.

We propose that in systems such as ours, high surface receptor density [9,10], ligand recycling through serial association–dissociation events [9], and the occupancy-dependent affinity shown here and elsewhere [4], result in a type of binding interaction that might exert a modulating role in cell responses. Such a model would (a) facilitate sufficient ligand binding to high affinity receptors to exceed the occupancy threshold necessary to trigger cell activation [20] and induce optimal cell responses; (b) amplify the response through receptor recruitment on the same or different cells facilitated by a series of ligand association–dissociation events; and (c) minimize the cytocidal effect of excessive ligand concentrations [1] through down-regulation.

Further studies correlating ligand binding to cell function will be necessary to validate this hypothesis.

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