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Aggregation of IgE-Receptor Complexes on Rat Basophilic Leukemia Cells Does Not Change the Intrinsic Affinity but Can Alter the Kinetics of the Ligand-IgE Interaction†

Richard G. Posner,‡ Benjamin Lee,‡ Daniel H. Conrad,§ David Holowka,‡ Barbara Baird,‡ and Byron Goldstein*,‖
Department of Chemistry, Baker Laboratory, Cornell University, Ithaca, New York 14853, Department of Microbiology and Immunology, Medical College of Virginia, Box 678 MCV Station, Richmond, Virginia 23298, and Theoretical Biology and Biophysics Group, Theoretical Division, T-10, MS K710, Los Alamos National Laboratory, Los Alamos, New Mexico 87545
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ABSTRACT: The aggregation of IgE anchored to high-affinity Fc_ϵ receptors on rat basophilic leukemia (RBL) cells by multivalent antigens initiates transmembrane signaling and ultimately cellular degranulation. Previous studies have shown that the rate of dissociation of bivalent and multivalent DNP ligands from RBL cells sensitized with anti-DNP IgE decreases with increasing ligand incubation times. One mechanism proposed for this effect is that when IgE molecules are aggregated, a conformational change occurs that results in an increase in the intrinsic affinity of IgE for antigen. This possibility was tested by measuring the equilibrium constant for the binding of monovalent DNP-lysine to anti-DNP IgE under two conditions, where the cell-bound IgE is dispersed and where it has been aggregated into visible patches on the cell surface using anti-IgE and a secondary antibody. No difference in the equilibrium constant in these two cases was observed. We also measured the rate of dissociation of a monovalent ligand from cell surface IgE under these two conditions. Whereas the affinity for monovalent ligand is not altered by IgE aggregation, we observe that the rate of ligand dissociation from IgE in clusters is slower than the rate of ligand dissociation from unaggregated IgE. These results are discussed in terms of recent theoretical developments concerning effects of receptor density on ligand binding to cell surfaces.

The cross-linking of immunoglobulin E (IgE)[1] bound to the high-affinity Fc_ϵ receptor ($Fc_\epsilon RI$) on the surface of mast cells, basophils, or their tumor analogue, rat basophilic leukemia (RBL) cells, is the first step in a cascade of events that culminates in cellular degranulation. In addition to initiating a variety of biochemical events, aggregation of IgE on RBL cells can be rapidly followed by internalization of IgE aggregates (Furuichi et al., 1984), increased fluid-phase pinocytosis, changes in the surface topology of the cell (Pfeiffer et al., 1985), an increase in the immobile fraction of cell surface IgE (Menon et al., 1986a; Myers et al., 1992), and interactions between Fc_ϵ receptors and the membrane skeleton (Robertson et al., 1986; Apgar, 1990).

Recently, fluorescence techniques to monitor the rate and extent of ligand binding to cell surface-bound IgE have been developed (Erickson et al., 1986, 1987, 1991; Seagrave et al., 1987). Seagrave et al. (1987) used flow cytometry to study the binding of a phycobilli protein highly conjugated with DNP groups (DNP_{42}-phycoerythrin) to RBL cells sensitized with anti-DNP IgE. They observed that this multivalent ligand became increasingly resistant to DNP-lysine-induced dissociation as the incubation time of the ligand with the cells was increased. With fluorescence quenching methods, we observed that a symmetric bivalent ligand with two DNP groups ($(DCT)_2Cys$) became increasingly resistant to dissociation induced by high concentrations of anti-DNP IgE in solution, but not by high concentrations of a monovalent hapten (DCT) (Erickson et al., 1991).

Two hypotheses have been proposed for the observed resistance of DNP_{42}-phycoerythrin to dissociation. In the first hypothesis, the aggregation of IgE-$Fc_\epsilon RI$ complexes triggers events that induce a conformational change in the IgE binding site resulting in an increase in its affinity for antigen (Seagrave et al., 1987). This type of change appears to occur with neutrophils which have receptors with both high and low affinity for the peptide ligand f-Met-Leu-Phe (Sklar et al., 1987). In this case, ligand binding induces a time-dependent conversion of the receptor from a low-affinity state to a high-affinity state that appears to be modulated by receptor interaction with a GTP-binding protein (Sklar et al., 1987). The second hypothesis is that a multivalent ligand binds to IgE-$Fc_\epsilon RI$ complexes which then form long-lived clusters associated with other cellular components (Seagrave et al., 1987; Erickson et al., 1991). Ligand dissociation from the cell surface is

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‡ Cornell University.
§ Medical College of Virginia.
‖ Los Alamos National Laboratory.

[1] IgE, immunoglobulin E; $Fc_\epsilon R1$, high-affinity Fc_ϵ receptor; RBL, rat basophilic leukemia; DNP, dinitrophenyl; DCT, [ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosine; FITC, fluorescein 5-isothiocyanate; $(DCT)_2Cys$, *N,N'*-bis[[ε-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosyl]-cystine; [^{3}H]-5HT, [1,2-^{3}H(N)]-5-hydroxytryptamine binoxalate.

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slowed because there is a high probability of rebinding to the IgE–receptor complexes that are held in proximity for some time period before they diffuse apart.

In this paper, we directly test the first hypothesis with a relatively simple experimental system. We measure the equilibrium binding of monovalent ligand to cell surface IgE and the rate of ligand dissociation for two cases: when IgE is unaggregated and when it is aggregated into large patches on the cell surface. We show that the ligand–IgE equilibrium binding constant is unaffected by this aggregation, but that dissociation of the ligand from IgE is slowed when IgE is aggregated.

Materials and Methods

Reagents. Mouse monoclonal anti-DNP IgE from hybridoma H1 26.82 (Liu et al., 1980) was affinity purified and iodinated with ^{125}I as described previously (Holowka & Metzger, 1982). Final steps in the purification included ion-exchange chromatography on Bio-Rad AG 1-x4 at 37 °C to remove bound DNP-glycine (Holowka & Metzger, 1982), followed by concentration with a Millipore immersible CX-30 filter unit, and then gel filtration on a 2 $cm^2 \times 100$ cm column of Sephacryl S-300 in 135 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7.4, with 0.01% NaN_3 to separate monomeric IgE from any aggregates. Unlabeled, affinity-purified goat antibody against rat IgG (γ chain specific) was obtained from Southern Biotechnology. Monoclonal rat anti-mouse IgE antibody, B1E3, was prepared and purified as described (Keegan et al., 1991).

Fluorescein 5-isothiocyanate (FITC) was obtained from Molecular Probes, Inc., and sodium [^{125}I]iodide was from Amersham Corp. The preparation of FITC–IgE was previously described (Erickson et al., 1986). The monovalent ligand [ϵ-[(2,4-dinitrophenyl)amino]caproyl]-L-tyrosine (DCT) was obtained from Biosearch Inc. (San Rafael, CA) as the dicyclohexamine salt. The monovalent ligand DNP-lysine was obtained from Sigma Chemical Co. [1,2-^3H(N)]-5-hydroxytryptamine binoxalate ([^3H]-5HT) was obtained from NEN Research Products. Bovine serum albumin, conjugated with an average of 27 mol of DNP/mol of protein (DNP-BSA), was prepared as described previously (Eisen et al., 1959).

Degranulation Assays. RBL-2H3 cells (Barsumian et al., 1981) were grown adherent in 75 cm^2 flasks and used five days after passage. For the degranulation assay (Baird et al., 1983), a flask containing approximately 2.5×10^7 cells was incubated overnight with 10 μCi of [^3H]-5HT and 10 μg of anti-DNP IgE at 37 °C. Cells were harvested and washed in supplemented medium (minimum essential medium, 10% newborn calf serum, 20 mM Hepes, pH 7.4) and placed on a shaker inside a 37 °C water bath for 60 min. Cells were then washed twice at room temperature in buffered salt solution (BSS: 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5.6 mM glucose, 0.1% gelatin, 20 mM Hepes, pH 7.4) and resuspended to a final concentration of (1×10^6)/mL. Triggering reagents in BSS were serially diluted in the wells of a 96-well microtiter plate before the addition of cells. In experiments where B1E3 and polyclonal anti-IgG were employed together, the cells were incubated with B1E3 for 20 min at 4 °C prior to the addition of anti-IgG. The plate containing triggering reagents and cells was incubated for 90 min at 37 °C, and release of [^3H]-5HT was assayed by counting the supernatants of each well in a Beckman LS250 scintillation counter. The spontaneous release ($\leq$10%) was subtracted from all data points, and the degranulation data are shown as the fraction of the maximal amount of release obtained with an optimal concentration of DNP-BSA ($\approx$65% of the total [^3H]-5HT content).

Sensitization of RBL Cells with FITC-Labeled IgE and Fluorescence Measurement of Bound Ligand. Approximately 2.5×10^7 RBL cells within a flask were incubated overnight with 10 μg of ^{125}I and FITC-labeled anti-DNP IgE at 37 °C. Cells were harvested and washed as described for the degranulation assays. The cell concentration was determined by counting with a microscope and a hemocytometer, and the radioactivity of a known number of cells was measured. The specific activity of the ^{125}I label, determined as previously described (Erickson et al., 1986), allowed us to calculate the number of IgE molecules per cell.

The proportionate decrease in FITC–IgE fluorescence that accompanies Fab site occupation by DNP-ligands has been described in detail previously (Erickson et al., 1986). All fluorescence recordings were made with a spectrofluorometer (SLM Model 8000) operated in ratio mode with FITC excitation and emission wavelengths of 490 nm and 526 nm, respectively. The spectrofluorometer was interfaced with an AST Premium 286 computer for direct data acquisition. Cells sensitized with ^{125}I-FITC–anti-DNP IgE and suspended at $(2–4) \times 10^6$ cells/mL ($\approx$2–3 nM Fab binding sites) in BSS, were stirred continuously in acrylic cuvettes (W. Sarstedt) and placed in a cuvette holder thermostatically controlled at 15 °C or 30 °C. Cross-linking reagents (B1E3 and goat anti-rat IgG) were added sequentially, with B1E3 allowed to bind for 20 min prior to the addition of anti-IgG. The equilibrium titration began 20 min after the addition of anti-IgG. Known volumes of DNP-lysine solution were added in microliter amounts to the sample cuvette using microcapillary tubes. The resulting constant fluorescence signal was averaged over a 60-s interval. For all fluorescence measurements performed at 30 °C, the samples were prepared in BSS without glucose and containing 10 mM NaN_3 and 10 mM deoxyglucose to prevent internalization of the FITC-labeled IgE (Menon et al., 1986b).

In the dissociation experiments, sufficient DCT ($\approx$2 nM) was added to the labeled cell suspension to bind to a large fraction of Fab binding sites, thereby quenching the FITC fluorescence (maximally by $\sim$20% at saturation). After the fluorescence decrease was complete, a large excess of unlabeled anti-DNP IgE (115 nM IgE which is equal to 230 nM Fab binding sites) was added to the cell suspension. The rate of dissociation of the DCT from the Fab sites on the cell surface was monitored as the consequent increase of fluorescence with time. Data points were recorded at 2.4-s intervals. The temperature for these experiments was 15 °C.

Parameter Estimation for Equilibrium Binding Experiments. For a monovalent ligand binding to a receptor, we determined the equilibrium binding constant K as follows: The concentration of bound ligand $L^* = KR_TL/(1 + KL)$ where $L = L_T - L^*$ is the free ligand concentration, L_T is the total ligand concentration, and R_T is the total receptor site (Fab site) concentration. Substituting for L and solving for L^*, we have

$$L^* = [1 + KL_T + KR_T - [(1 + KL_T + KR_T)^2 - 4K^2R_TL_T]^{1/2}]/(2K) \quad (1)$$

L^* is related to the relative fluorescence F as binding leads to quenching of the fluorescence. In particular

$$L^*/R_T = 1 - (F - F_{min})/(F_{max} - F_{min}) \quad (2)$$

where F_{max} is the relative fluorescence when all the receptor sites are free and F_{min} is the relative fluorescence when all sites are filled. To determine K, we fit eqs 1 and 2 to a fluorescence titration curve taking as free parameters K, F_{max}, and F_{min}. To obtain parameter estimates, nonlinear least-squares data fitting was carried out using the International Mathematics

and Statistics Library (IMSL) routine ZXSSQ, which is based on a finite difference Levenberg–Marquardt algorithm.

Parameter Estimation for Dissociation Experiments. If k_f and k_r are the forward and reverse rate constants for the binding of a ligand to a receptor on the surface of a cell in the presence of other receptors, then for a cell with N free binding sites on its surface (Shoup & Szabo, 1982)

$$k_f = k_{on}/(1 + Nk_{on}/k_+) \quad (3)$$

and

$$k_r = k_{off}/(1 + Nk_{on}/k_+) \quad (4)$$

where k_{on} and k_{off} are the reaction-limited forward and reverse rate constants for the binding of a ligand to an isolated receptor, i.e., a cell surface receptor when the separation between receptors is large. The rate constant k_+ characterizes the transport of ligands from solution to the vicinity of the cell surface; i.e., it is the diffusion-limited forward rate constant to the cell surface. If the cell is modeled as a sphere of radius a, then k_+ is the Smoluchowski diffusion-controlled rate constant, i.e.

$$k_+ = 4\pi Da \quad (5)$$

where D is the diffusion coefficient of the ligand. Equation 3 has been directly tested for small DNP haptens (DCT, DNP-lysine, and DNP-glycine) binding to surface IgE concentrations in the range $N = (0 - 1.2) \times 10^6$ sites/cell and found to be in excellent agreement with the experiment (Erickson et al., 1987).

If receptors that can bind the ligand are also present in solution, then the rate at which ligands in solution reach the cell surface will be altered. In particular, if the soluble receptors are present at a high concentration, S, then eq 5 becomes

$$k_+ = 4\pi Da[1 + (k_{on}Sa^2/D)^{1/2}] \quad (6)$$

and this form of k_+ must be used in eqs 3 and 4 (Goldstein et al., 1989).

In our experimental system the fluorescence of FITC–IgE bound to the cell surface is quenched by a DNP ligand. Upon addition of excess unlabeled IgE to the solution, the fluorescence begins to recover. If at time t the relative fluorescence has a value $F(t)$, then $x(t)$, the fraction of ligand that has dissociated by time t, is given by

$$x(t) = (F(t) - F_{min})/(F_{max} - F_{min}) \quad (7)$$

and therefore

$$F(t) = F_{min} + (F_{max} - F_{min})x(t) \quad (8)$$

We have shown that $x(t)$ obeys the following equation (Goldstein et al., 1989):

$$(1 - \delta)x(t) + \ln(1 - x(t)) = -k_m(\infty)t \quad (9)$$

The parameter δ is the fractional decrease in the rate of dissociation from the start of the experiment ($t = 0$) until dissociation is completed ($t = \infty$). This decrease occurs because as ligand dissociates binding sites become free, which tends to increase rebinding and slow dissociation. $k_m(\infty)$ is related to k_r and k_f evaluated at $t = \infty$, i.e., when essentially all ligand has dissociated and N equals the total number of IgE binding sites on the cell surface. In particular

$$k_m(\infty) = k_r + k_f L_{eq} = k_r(1 + KL_{eq}) \quad (10)$$

L_{eq} is the final value of the free ligand concentration when dissociation has gone to completion. In our experiments $KL_{eq} \approx L_T/S_T \approx 0.01$, where L_T and S_T are the total ligand and unlabeled IgE concentrations, respectively, and therefore $k_m(\infty) \approx k_r$ (Goldstein et al., 1989).

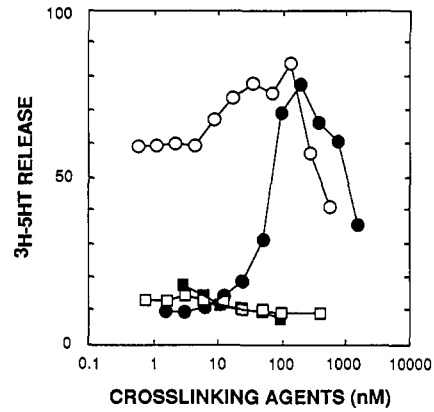

FIGURE 1: Degranulation from RBL cells that were sensitized with IgE and incubated with B1E3 (□), anti-IgG (■), varying concentrations of B1E3 together with 100 nM polyclonal anti-IgG (○), and varying concentrations of polyclonal anti-IgG with 100 nM monoclonal B1E3 (●). Results are normalized with respect to release caused by multivalent antigen as described in Materials and Methods.

To determine $k_m(\infty)$, we fit eq 8 to the data, supplying $x(t)$ by numerically solving eq 9 for $x(t)$. In our nonlinear least-squares fitting routine, which uses the IMSL routine ZXSSQ, we take the following as free parameters: $k_m(\infty)$, δ, F_{max}, and F_{min}.

RESULTS

Aggregation of IgE–Receptor Complexes. In order to test whether the aggregation of IgE and subsequent cellular activation results in an increase in the affinity of IgE for ligand, a set of reagents was required that could aggregate IgE and trigger degranulation yet not interfere with the anti-DNP combining sites. For these purposes, we employed a monoclonal rat anti-mouse IgE antibody (B1E3) which binds to the $C_\epsilon 4$ domain of IgE (Keegan et al., 1991) and cross-links cell receptor-bound IgE efficiently into dimers (Grassberger, 1989). Although B1E3 by itself does not effectively stimulate cellular degranulation on RBL cells (Figure 1), effective stimulation is accomplished by the addition of a secondary antibody (polyclonal goat anti-rat IgG) which serves to increase the size of the IgE–receptor aggregates. The combination of these two antibodies initiates a strong response, with maximal degranulation occurring at 100 nM B1E3 and 100 nM goat anti-rat IgG (Figure 1).

With fluorescence microscopy, we observed that RBL cells sensitized with FITC–IgE in the absence of cross-linking reagents have a characteristic smooth ring stain around the equator and essentially no intracellular fluorescence (not shown). Addition to these cells of 100 nM B1E3 and 100 nM anti-IgG antibodies causes visible patching of IgE–receptor complexes. These patches are similar in appearance to those caused by a highly multivalent antigen DNP-BSA (not shown) or oligomeric IgE (Menon et al., 1984, 1986a) or other types of anti-IgE (Menon, 1985; Menon et al., 1986b), although they are somewhat smaller and less well defined.

Equilibrium Binding Studies. Figure 2 shows a series of equilibrium titrations of receptor-bound FITC–IgE with DNP-lysine. In these experiments, the IgE–receptor complexes were either monomeric or preaggregated with B1E3 and anti-IgG. As can be seen in Figure 2, the data for the experiments where the IgE was aggregated are not discernibly different from the unaggregated case. These data were fit to eqs 1 and 2, and affinity constants were derived. Table I summarizes the results of a series of titrations performed on RBL cells sensitized with FITC–IgE either where the FITC–IgE has been preaggregated by the addition of B1E3 and

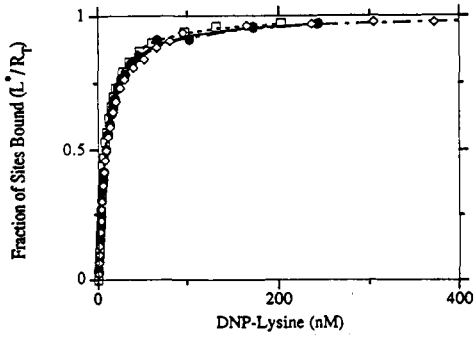

FIGURE 2: Equilibrium binding of DNP-lysine to FITC–IgE on RBL cells after different treatments at 30 °C in the presence of sodium azide and deoxyglucose. Key: no treatment, the IgE is disperse (● and solid line); incubation with 100 nM B1E3 together with 100 nM anti-IgG to aggregate the FITC–IgE into patches (□ and dashed line); incubation with 233 nM B1E3 and 233 nM anti-IgG (◇ and short dash and long dash line). The points represent the experimental data, and the lines are a nonlinear least-squares fit of the data according to eqs 1 and 2. The equilibrium constants (K) derived from these data are included in Table I.

Table I: Equilibrium Constants for the Binding of DNP-Lysine to an Fab Site for FITC–IgE Bound to $Fc_\epsilon R1$ on RBL Cells[a]

temp (°C)	B1E3 (nM)	anti-IgG (nM)	K^b ($M^{-1} \times 10^{-8}$)
15	0	0	3.4
	33	33	2.9
	100	100	2.8
	233	233	2.6
30	0	0	1.3
	100	100	1.5
	233	233	1.2

[a] Cells were saturated with FITC–IgE and then washed and incubated with the indicated concentration of B1E3 and anti-IgE before the addition of DNP-lysine, as described in Material and Methods. [b] Data such as that shown in Figure 2 were fit with eqs 1 and 2 to obtain values for K. For experiments done at 30 °C, 10 mM NaN_3 and 10 mM deoxyglucose were present in the sample buffer (BSS). Each determination of K is for a single experiment.

anti-IgG at various concentrations or where agents that aggregate IgE were absent. The experiments were performed at both 15 °C and 30 °C. No change in the equilibrium constant is observed at either temperature. The data demonstrate that aggregation of the cell-bound IgE does not significantly alter its affinity for ligand, and these results were obtained over a wide range of antibody concentrations.

Dissociation Kinetics. The dissociation of bound DNP-ligand from its receptor (FITC–IgE) on the cell surface can be initiated by addition to the solution of an excess of free receptors (unlabeled IgE) (Goldstein et al., 1989). As the ligand dissociates from the cell-bound FITC–IgE, it is free to bind to the unlabeled IgE. Dissociation experiments were carried out with DCT because it dissociates more slowly than DNP-lysine and therefore is better suited for this study. Figure 3 shows how the relative fluorescence of FITC–IgE is quenched by the binding of DCT and then is restored after the addition of excess unlabeled IgE to initiate DCT dissociation. In this experiment the FITC–IgE had been aggregated by the addition of 100 nM B1E3 and 100 nM anti-IgG. The solid line starting at $t \approx 568$ s is the theoretical fit to the dissociation portion of the curve. Equations 8 and 9 were used to fit the dissociation curve and obtain $k_m(\infty)$.

Similar experiments and fits of the data were performed on samples in which the cell-bound FITC–IgE was not aggregated and dissociation for the two types of samples are compared in Figure 4. These plots clearly show that dissociation of DCT from aggregated FITC–IgE is significantly slower. Two separate experiments for each type of FITC–IgE distribution

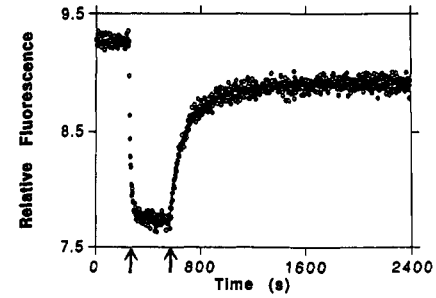

FIGURE 3: Fluorescence data from a typical dissociation experiment. RBL cells (2.6×10^6 cells/mL) with 2.65×10^5 FITC–IgE/cell (5.3×10^5 Fab sites/cell) were present in solution at 15 °C. At $t = 260$ s (first arrow), DCT was added to a final concentration of 2.0 nM. DCT became bound to a large fraction of the FITC–IgE binding sites and partially quenched the FITC fluorescence (~20% of total signal). At $t = 568$ s (second arrow), unlabeled IgE is added at a final concentration of 230 nM Fab sites, causing net dissociation of the DCT and accompanying recovery of fluorescence. The recovery is incomplete due to dilution and other effects (Goldstein et al., 1989). The data corresponding to the DCT are fit according to eq 8 and 9 (solid line beginning at $t = 568$ s). In the experiment shown, the cell bound IgE was preaggregated by the addition of 100 nM B1E3 for 20 min followed by the addition of 100 nM polyclonal anti-IgG. These data yielded the following parameters: $k_m(\infty) = 4.6 \times 10^{-3}$ s^{-1}, $F_{max} = 8.91$, and $F_{min} = 7.56$.

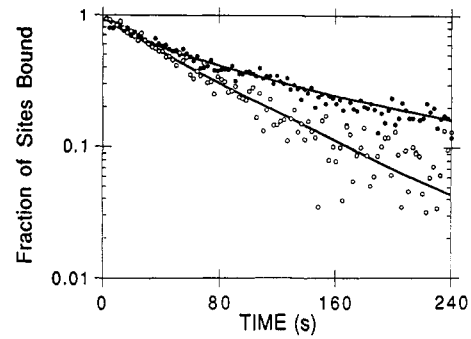

FIGURE 4: DCT dissociation from cell surface FITC–IgE that were disperse (○) or aggregated by a combination of 100 nM B1E3 and 100 nM anti-IgG (●). The points are calculated with eq 2 from fluorescence data: the data shown in Figure 3 were used for the aggregated receptor sample, and the sample containing unaggregated receptors were measured in parallel at 15 °C. The solid curve represents fits of the two data sets according to eqs 8 and 9; the values obtained for $k_m(\infty)$ are 4.6×10^{-3} s^{-1} for aggregated receptors (●) and 1.2×10^{-2} s^{-1} for dispersed receptors (○).

were carried out and the following average values and standard deviations were obtained: for unaggregated IgE, $k_m(\infty) = (1.0 \pm 0.2) \times 10^{-2}$ s^{-1}; for aggregated IgE, $k_m(\infty) = (5.4 \pm 1.2) \times 10^{-3}$ s^{-1}.

Because B1E3 and anti-IgG can interact with the unlabeled IgE in solution as well as the cell-bound FITC–IgE, it was necessary to test whether the presence of these two antibodies was reducing the effectiveness of the solution IgE in binding free ligand. To check this possibility, unlabeled IgE (230 nM Fab sites final concentration) was preincubated with B1E3 (100 nM final concentration) and anti-IgG (100 nM final concentration) prior to using it to initiate dissociation of bound DCT from unaggregated FITC–IgE. The average and standard deviation for two experiments using unlabeled IgE that had been preincubated with B1E3 and anti-IgG was $k_m(\infty) = (0.95 \pm 0.15) \times 10^{-2}$ s^{-1}. This is in good agreement with the value $k_m(\infty) = (1.0 \pm 0.2) \times 10^{-2}$ s^{-1} obtained when unlabeled IgE alone was used to induce dissociation.

Discussion

In this study, we compared the equilibrium binding and rates of dissociation of monovalent DNP ligands with anti-DNP IgE

on the surface of RBL cells under two conditions, where the IgE–receptors were dispersed and where they were aggregated in patches by anti-IgE together with a secondary antibody. For equilibrium binding, no significant difference was detected for the two cases (Table I), both at 15 °C and at 30 °C in the presence of sodium azide and deoxyglucose. Under both of these conditions, internalization is prevented during the time course of these measurements (Menon et al., 1986b). Sodium azide and deoxyglucose also prevent cellular degranulation that would otherwise occur within minutes after receptor aggregation at 30 °C. Similarly, receptors aggregated at ≤15 °C do not stimulate degranulation, but they are fully competent to do so if the cells are subsequently warmed to 37 °C (Menon et al., 1986a). Changes in receptor dynamics due to receptor cross-linking, such as the loss of rotational (Myers et al., 1992) and lateral (Menon et al., 1986b; Posner, 1990) mobility, occur with the cross-linking reagents we have used. Thus, our results are directly relevant to the previous studies where highly multivalent DNP ligands were used to cross-link anti-DNP IgE bound to receptors on RBL cells and dissociation was initiated by the addition of monovalent DNP (Seagrave et al., 1987). These previous experiments showed that the rate and extent of dissociation of the multivalent ligand depended on the length of time before the addition of monovalent ligand. The longer the waiting period, the slower the rate of dissociation and the less multivalent ligand that dissociated (Seagrave et al., 1987). Our experiments clearly indicate that this phenomenon is not due to an aggregation-dependent change in the intrinsic binding affinity of the IgE for DNP.

When a multivalent ligand cross-links IgE on RBL cells, interactions of the IgE–Fc_ϵ R1 complex with the cytoskeleton occur (Robertson et al., 1986; Seagrave et al., 1987; Apgar, 1990) and rapid losses in mobility of the cross-linked IgE are observed (Menon et al., 1986b). Small oligomers of IgE (trimers or larger) also can cause the receptors to lose mobility and undergo large-scale patching (Menon et al., 1984; 1986a). Apparently, IgE–receptor complexes are clustered by external cross-linking agents as well as by interactions with other cellular components. If the cellular interactions are time dependent, so that more stable, and possibly larger clusters of receptors form with time, rebinding to these clusters could account for the observed slowing in dissociation of the multivalent ligand. Rebinding of a multivalent ligand to clustered receptors is a complicated process because of the multiple attachment sites of the ligand. Opening of one or even a few bonds will not lead to ligand detachment from the cell surface if other bonds remain intact. If the receptors are held in proximity, then the broken bonds can reform before the receptors diffuse away. Such means for enhanced rebinding are not possible for monovalent ligands, but if the cluster of IgE binding sites is large enough, monovalent ligands may rebind simply because the local concentration of receptors is high. In this latter type of rebinding, a ligand dissociates from a receptor and then diffuses in the solution near the cell surface to a new receptor held within the same cluster.

We monitored the kinetics of dissociation of a monovalent DNP ligand, DCT. We found that the dissociation of DCT initiated by the addition of unlabeled solution IgE was affected by clustering of cell-bound FITC–IgE. In the presence of clusters, dissociation of DCT was slowed. We propose that this slowing is due to rebinding within a cluster, i.e., a ligand that dissociates from one receptor within a cluster has a high probability of binding to a second receptor in a cluster before escaping into the bulk fluid. At 37 °C, aggregation of IgE–Fe_ϵR1 complexes induces changes in RBL cell surface topology, transforming the surface from a microvillous to a lamellar morphology (Pfeiffer et al., 1985). If similar changes also occur at 15 °C, they may enhance rebinding. Dissociation from a receptor in a valley with free receptors distributed over the surface of the valley will be slower than dissociation from a flat plane with the same receptor density. If a ligand dissociates from a receptor on a flat plane, half of all possible diffusive steps the ligand can take will carry it away from the plane and reduce its probability of rebinding. However, if the ligand dissociates from a receptor in a valley, fewer than half of the diffuse steps will be away from the surface and rebinding will be more likely.

In the appendix, we estimate the patch size and density of IgE required to induce significant rebinding for flat patches and show that the estimates are consistent with rebinding occurring within a patch made on an RBL cell surface. It is important to note that rebinding of monovalent ligand to clustered receptors can decrease both the forward and reverse rate constants in the same way such that their ratio, the equilibrium constant, is unchanged (Goldstein, 1989). We infer from our results that the forward rate of monovalent ligand binding to receptors in patches is reduced compared to dispersed receptors. We think that rebinding to clustered receptors does not explain the time-dependent loss of bivalent ligand dissociability from FITC–IgE bound to RBL cells that we previously observed with solution IgE to induce the dissociation (Erickson et al., 1991). The bivalent ligand employed in the previous study does not cause visible patching under the conditions of those experiments but rather appears to form cyclic dimers of IgE–receptor complexes that may interact with other membrane components.

Although we have not directly demonstrated that rebinding of a monovalent ligand occurs preferentially within a patch of receptors, our kinetic experiments clearly point to the possibility that large-scale clustering of cell surface receptors can slow dissociation of monovalent as well as multivalent ligands from the cell. In our experimental system, receptor clustering was accomplished by antibodies; receptors clustered by other means are found in native biological system. For example, some receptors, including the receptors for low-density lipoprotein (Anderson et al., 1975), asialoglycoprotein (Wall & Hubbard, 1981), and transferrin (Watts, 1985), cluster in coated pits in the absence of ligand. For these receptors, at low ligand concentration when most of the clustered receptor sites are free, it is possible that rebinding slows the dissociation of a bound ligand from a coated pit. In this manner the cell could make use of rebinding to keep ligands in coated pits until the pits invaginate and internalize the ligand. Another example is provided by the neuromuscular junction where acetylcholine receptors are concentrated and clustered. Receptors within these clusters are in large excess over the number necessary to bind the neurotransmitter and mediate the cellular depolarization. Part of the reason for this may be that the extra receptors facilitate acetylcholine molecules rebinding in this critical region and thereby reduce their escape and degradation by acetylcholinesterase (Salpeter, 1987). Our results suggest that rebinding to retain a ligand in proximity of its clustered cell surface receptors may be a general phenomenon occurring in a broad range of circumstances. The actual importance of this rebinding phenemenon in physiological signaling remains to be determined.

Appendix

Fluorescence micrographs show that the clusters of IgE–receptor complexes formed on RBL cells by the addition of anti-IgE and a secondary antibody are considerably smaller

than the radius of the cell. Here we estimate how large a cluster of IgE–receptor complexes must be in order for there to be significant rebinding within a cluster. If our estimates show that the cluster size must be larger than, or equal to, a significant fraction of an RBL cell radius, this would indicate that clustering cannot account for the slowed kinetics of dissociation seen in our experiments. However, if our estimates show that the cluster size is considerably smaller than an RBL cell radius, then this would suggest that clustering could influence the dissociation kinetics.

When ligands bind to receptors on cell surfaces, the forward and reverse rate constants, k_f and k_r, become functions of the density of free receptor binding sites. For example, if receptors are uniformly distributed over the cell surface, k_f and k_r are given by eqs 3 and 4, where N is the number of free receptor sites per cell and k_{off} and k_{on} are the rate constants for binding to a single isolated receptor. If receptors are in clusters on the cell surface, these equations are modified. For example, if N^{cl} clusters are uniformly distributed over the cell surface, then

$$k_f = k_{on}/(1 + (N/N^{cl})k_{on}/k_+^{cl} + Nk_{on}/k_+) \quad (11)$$

$$k_r = k_{off}/(1 + (N/N^{cl})k_{on}/k_+^{cl} + Nk_{on}/k_+) \quad (12)$$

where k_+^{cl} is the diffusion-limited forward rate constant for binding to a cluster (Goldstein, 1989). If the cluster is modeled as a disc of radius R then (Hill, 1975)

$$k_+^{cl} = 4DR \quad (13)$$

When receptors are in clusters on the cell surface, the rate constants for ligand–receptor binding (eqs 11 and 12) are smaller than when receptors are uniformly distributed (eqs 3 and 4). A dissociating ligand can either rebind to other receptors in the same cluster or rebind to a receptor in a different cluster. However, comparison of eqs 3 and 11 shows that rebinding within clusters will only be significant when

$$nk_{on}/k_+^{cl} \geq 1 \quad (14)$$

where $n = N/N^{cl}$ is the number of receptors per cluster. If the receptor density within a cluster is $d = n/(\pi R^2)$, then significant rebinding within a cluster will occur when

$$R \geq 4D/(\pi k_{on} d) \quad (15)$$

As is to be expected, the bigger the cluster the more likely there is to be rebinding within a cluster. For DCT, the monovalent ligand used in our kinetic studies, $k_{on} = 1.9 \times 10^{-13}$ cm^3/s and $D \approx 10^{-5}$ cm^2/s (Erickson et al., 1987). In our experiments, we have not determined the receptor density within a cluster, but we can make a crude estimate of its value. After sensitization, the RBL cells in this study had 2.7×10^5 IgE/cell or 5.4×10^5 sites/cell. For a smooth spherical cell of radius 4 μm with 2.7×10^5 IgE/cell randomly distributed over its surface, $d = 2.7 \times 10^{11}$ sites/cm^2.[2] It has been estimated that approximately 50% of the RBL cell surface is covered by protein and that on the basis of surface iodination studies $Fc_\epsilon R1$ make up approximately 1% of the total surface protein on an RBL cell (Ryan et al., 1988) and therefore cover approximately 0.5% of the cell surface. If these $Fc_\epsilon R1$ were close packed into 0.5% of the surface, then $d = 5.4 \times 10^{13}$ sites/cm^2. The effective density of sites within a cluster of IgEs in our experiments must be between these limits, i.e.

$$5.4 \times 10^{13} \text{ sites/cm}^2 > d > 2.7 \times 10^{11} \text{ sites/cm}^2 \quad (16)$$

Ryan et al. (1986) determined that the average IgE density within a patch induced by the addition of a polyclonal anti-IgE was 5 times higher than the diffuse IgE density (no anti-IgE present). If we assume that for our patches the patch density is 5 times higher than the diffuse IgE density, then $d = 1.3 \times 10^{12}$ sites/cm^2, well below the close packing limit. For $d = 1.3 \times 10^{12}$ sites/cm^2, we find from eq 15 that $R \geq 0.5$ μm. A patch of radius 0.5 μm at this density would contain 1.1×10^4 sites/patch or 5.3×10^3 IgE/patch. If all the IgE were in patches of equal sizes there would be approximately 50 patches per cell (N^{cl}).

For rebinding within a patch to significantly affect the kinetics of binding, a second condition must also be met. Not only must rebinding within a patch occur (eq 15), but its effect on the rate constants must be comparable to, or greater than, rebinding to receptors in different patches; i.e., the number of patches must not be too large. From the form of the denominator in eqs 11 and 12, it follows that

$$(N/N^{cl})k_{on}/k_+^{cl} \geq Nk_{on}/k_+ \quad (17)$$

or equivalently, from eqs 5 and 13

$$N^{cl} \leq \pi a/R \quad (18)$$

For example, for a cell of radius 4 μm and a patch of radius 0.5 μm, $N^{cl} \leq 25$. The value of $N^{cl} = 50$ we estimated above is of the same order of magnitude.

[2] Cell surfaces are generally not smooth but convoluted. Therefore, the actual surface area is larger than that of a smooth sphere, and the actual surface density of receptors is correspondingly less. However, our calculations approximate patches as flat discs such that the convolutions are compressed within the discs and the receptor density is correspondingly raised. Thus the density we use in eq 15 is the effective density when the surface area of the cell is approximated as a smooth sphere.

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Toxoplasma gondii Stimulates the Release of 13- and 9-Hydroxyoctadecadienoic Acids by Human Platelets†

William R. Henderson, Jr.,* Mohamed Rashed,‡ Elenita C. Yong,§ Thomas R. Fritsche, and Gertrude K. Chiang
Departments of Medicine, Medicinal Chemistry, Laboratory Medicine, and Microbiology, University of Washington, Seattle, Washington 98195

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ABSTRACT: We have recently demonstrated a novel cytotoxic effect of human platelets against *Toxoplasma gondii* and a role for thromboxane (TX) in this process (Yong et al., 1991). We now report on the spectrum of lipid mediators released by human platelets after interaction with *T. gondii*. In addition to TXB_2, human platelets after incubation with *T. gondii* for 90 min released 12-hydroxyheptadecatrienoic acid (12-HHT), 12-hydroxyeicosatetraenoic acid (12-HETE), and an unidentified peak (UV_{max} 234 nm) as determined by reverse-phase high-performance liquid chromatography. Thermospray-liquid chromatography/mass spectrometry analysis and straight-phase HPLC identified the unknown peak as a mixture of 13-hydroxyoctadecadienoic acid (HODE) and 9-HODE. Radiolabeling studies with [^{14}C]linoleic acid indicated that the platelets were the cellular source of the octadecanoids with 13-HODE (87.7%) > 9-HODE (12.3%). Inhibitor studies with indomethacin indicated that 13-HODE was a lipoxygenase product and 9-HODE was a cyclooxygenase product of linoleic acid. Thus, *Toxoplasma*-stimulated platelets release oxygenated products of both arachidonic acid and linoleic acid which may be important in the host response to *T. gondii* infection.

Toxoplasma gondii is an important pathogen in immunocompromised individuals, especially in patients with the acquired immunodeficiency syndrome (AIDS)[1] (Pfefferkorn, 1990; Yong et al., 1991). *T. gondii* can exert a variety of antiinflammatory effects including inhibition of mononuclear phagocyte respiratory burst activity (Wilson et al., 1980), phagolysosomal fusion (Jones & Hirsch, 1972), and 5-lipoxygenase activity (Locksley et al., 1985). For example, LTB_4, LTC_4, and LTD_4 formation in murine peritoneal macrophages is blocked by intracellular parasitism with *Toxoplasma* organisms (Locksley et al., 1985). Macrophages activated by cytokines such as γ-interferon (Murray et al., 1985; Suzuki et al., 1988), monocytes (Wilson & Remington, 1979; Murray et al., 1985), neutrophils (Wilson & Remington, 1979), and recently platelets (Ridel et al., 1988; Auriault et al., 1990; Yong et al., 1991) have been implicated in the host defense against *T. gondii* infection because of their toxoplasmacidal activities.

Platelets may interact with *T. gondii* during the bloodstream dissemination of the parasites throughout the body that occurs

† This research was supported by NIH Grant AI23713.
* To whom correspondence should be addressed.
‡ Present address: Metabolism Research, American Cyanamid, Pearl River, NY 10965.
§ Present address: Seattle Biomedical Research Institute, Seattle, WA 98119.

[1] Abbreviations: AIDS, acquired immunodeficiency syndrome; DEAE, diethylaminoethyl; DEAEC, diethylaminoethyl chloride; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; 12-HETE, 12-hydroxy-5(*Z*),8(*Z*),10(*E*),14(*Z*)-eicosatetraenoic acid; 12-HHT, 12-hydroxy-5(*Z*),8(*E*),10(*E*)-heptadecatrienoic acid; HODE, hydroxyoctadecadienoic acid; 9-HODE, 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid; 13-HODE, 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid; 12-HPETE, 12-hydroperoxy-5(*Z*),8(*Z*),10(*E*),14-(*Z*)-eicosatetraenoic acid; IFA, indirect fluorescence antibody; LTB_4, leukotriene B_4; LTC_4, leukotriene C_4; LTD_4, leukotriene D_4; PBS, phosphate-buffered saline; RIA, radioimmunoassay; TSP-LC/MS, thermospray-liquid chromatography/mass spectrometry; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2.

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