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Cholera Toxin Mediated Agglutination of Ganglioside G_{m1} Containing Phospholipid Vesicles and G_{m1}-Coated Polystyrene Spheres†

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ABSTRACT: Quasi-elastic laser light scattering is used to examine the cholera toxin mediated agglutination of ganglioside G_{m1} containing phospholipid vesicles and G_{m1}-coated polystyrene spheres. We find that the ability of cholera toxin to agglutinate G_{m1}-containing phospholipid vesicles depends markedly on the lipid composition of the vesicle, with only those composed of short-chain lipids (C14, C16) being appreciably agglutinated. This is interpreted as due to poor mixing of these lipids with the longer chain gangliosides, resulting in lateral separation of the gangliosides in the membrane bilayer. A simple quantitative model, a modification of that developed by von Schulthess et al. [von Schulthess, G. K., Cohen, R. J., Sakato, N., & Benedek, G. B. (1976a) *Immunochemistry 13*, 955–962], is developed to describe these agglutination processes. Application of this model to the agglutination of G_{m1}-coated polystyrene spheres by cholera toxin allows an estimate of a minimum value of 4.5×10^4 M^{-1} for the association constant of cholera toxin for its initial G_{m1} receptor.

Cholera toxin is a multimeric protein consisting of two types of subunits: an A subunit, which contains the enzymatic activity of the toxin, and five identical B subunits, each capable of binding a membrane receptor. The receptor for cholera toxin has been shown to be the ganglioside G_{m1}[1] (van Heyningen et al., 1971; Cuatrecasas, 1973). The binding of the toxin to cells and to liposomes containing G_{m1} has been heavily studied and is well reviewed (Bennett & Cuatrecasas, 1976; Gill, 1977; Lai, 1980).

In addition to binding receptors on a single cell, Richards et al. (1979) have demonstrated that the pentavalent nature of the molecule confers a lectin-like quality on cholera toxin. They were able to show that cholera toxin was capable of agglutinating erythrocytes and phospholipid vesicles containing G_{m1}. This agglutination resembled antibody–antigen precipitation, showing a maximum at intermediate cholera toxin concentrations.

A simplified thermodynamic model of such agglutination reactions has been formulated by von Schulthess et al. (1976a) and is the basis of their laser light scattering immunoassay (von Schulthess et al., 1976a,b, 1980). In this technique, based on the earlier work of Singer (1961), polystyrene (PS) latex spheres coated with antibody are agglutinated with divalent antigen. The degree of agglutination is sensitively monitored by changes in either the translational diffusion coefficient obtained from quasi-elastic laser light scattering (QLS) (von Schulthess et al., 1976a,b) or angular anisotropy as measured by total intensity light scattering (von Schulthess et al., 1980). In addition to the sensitivity of immunoassay, it is possible from the analysis of these data to extract association constants for the initial attachment of antibody to antigen as well as those for the cross-linking of two PS spheres.

In this paper we utilize modifications of the above technique to study the cholera toxin mediated agglutination of G_{m1}-containing phospholipid vesicles as well as G_{m1}-coated PS spheres. The major modifications consist of substituting receptor-containing phospholipid vesicles for receptor-coated PS spheres and allowing the pentavalent cholera toxin to bind more than one receptor per vesicle (or PS sphere). We find that the ability of cholera toxin to agglutinate G_{m1}-containing vesicles depends markedly on the lipid composition of the vesicle, with only those composed of short-chain lipids (C14, C16) being appreciably agglutinated. This is interpreted as due to poor mixing of these lipids with the longer chain gangliosides, resulting in lateral separation of the gangliosides in the membrane bilayer. The agglutination of G_{m1}-coated PS spheres by cholera toxin was analyzed to obtain an estimate of at least 4.5×10^4 M^{-1} for the association constant of cholera

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[1] Abbreviations: QLS, quasi-elastic laser light scattering; G_{m1}, Galβ1→3GalNAcβ1→4Gal(3←2αAcNeu)β1→4Glcβ1→1′Cer; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; DOPL, dioleoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; PS, polystyrene; G_{m1}-OS, oligosaccharide portion of G_{m1}; T_m, liquid-crystal to gel transition temperature; Tris, tris(hydroxymethyl)aminomethane.

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toxin with its initial G_{m1} receptor.

Materials and Methods

Cholera Toxin. Cholera toxin was obtained as a lyophilized powder from (at various times) Schwarz/Mann, Calbiochem, and Sigma. In all cases rehydration in buffer (0.1 M NaCl, 0.05 M Tris, pH 7.5) resulted in a highly aggregated toxin preparation. Monodisperse toxin was obtained by a dilution–reconcentration procedure as described in the accompanying paper (Dwyer & Bloomfield, 1982). We found that it was imperative in these experiments for the toxin to be monomeric as the aggregated toxin was a much more efficient agglutinating agent, agglutinating G_{m1}-containing species that the monomeric toxin would not.

Ganglioside G_{m1}. The isolation and purification of the G_{m1} were as described by Dwyer & Bloomfield (1982).

Preparation of Phospholipid G_{m1} Vesicles. Phospholipids (PC, DMPC, DPPC, DOPC, and DSPC) were obtained from Sigma and used without further purification. Monodisperse phospholipid–G_{m1} bilayer vesicles were prepared by the ethanolic injection method of Kremer et al. (1977). G_{m1} (80 μg) and phospholipid (2 mg), both in organic solvents, were mixed and the solvent was removed by evaporation. The residue was dissolved in 0.5 mL of absolute ethanol. Of this, 0.4 mL was injected with stirring into 8.5 mL of buffer at 45 °C. Prior to use, the vesicles were dialyzed overnight to remove the alcohol. This procedure resulted in a phospholipid to G_{m1} ratio of roughly 50:1. The number of G_{m1} receptors per vesicle was estimated from the vesicle radius, determined from D by Stoke's law, and the density (0.9 g/mL) and thickness of the hydrocarbon bilayer (35–50 Å, depending on lipid) (Shipley, 1973). Typically, a 50:1 PC:G_{m1} vesicle with a 200-Å radius (bilayer thickness of 50 Å) would have about 135 functional G_{m1} receptors.

The vesicle size was stable for 4–5 h below the transition temperature and for more than 24 h above it. The exception is DSPC, with a transition temperature of 58 °C, which aggregated noticeably in 1 h. However, during the time required to make these measurements (approximately 20 min in a water bath at 20 °C and another 20 min for the QLS measurements) the DSPC size was fairly stable.

Preparation of PS–G_{m1} Spheres. Monodisperse PS latex spheres (mean diameter of 910 Å; SD = ±1%, as determined by Dow) were obtained from Dow Chemical Co. G_{m1} was surface absorbed onto PS by overnight incubation of 5 μg of G_{m1} (whose molecular weight is 1500) with 1 mg of PS spheres in 1 mL of buffer. From the density of 1.05 g/mL and the diameter, the molecular weight of the PS spheres is calculated to be 2.49×10^8. Thus if all the G_{m1} is functionally incorporated, this procedure results in about 800 G_{m1} receptors per PS sphere, or one receptor every 3250 Å^2.

QLS Measurements. The instrumentation and data analysis methods used in the QLS measurements have been recently described (Pletcher et al., 1980). Sample volumes of 0.75 mL were used, and dust was removed from the phospholipid vesicle samples by centrifuging at 8000 rpm for 40 min in a Beckman J-21 centrifuge. Measurements were performed at 20 °C.

Theory

In this section we present a modification of the theory of von Schulthess et al. (1976a) to allow for multivalent agglutinating agents. In the following, whenever "vesicle" appears, "PS sphere" may also be appropriate. We begin by defining the following: n = number of receptors per vesicle, n_{ct} = average number of toxin molecules bound per vesicle, m = cholera toxin valency of 5, and $\bar{m}$ = average number of receptors bound per bound toxin molecule.

The binding of cholera toxin to $\bar{m}$ noninteracting receptors is governed by an association constant, K_0, such that

$$K_0 = (\bar{m}n_{ct}/[CT])(n - \bar{m}n_{ct})$$

where [CT] is the free cholera toxin concentration and $n - \bar{m}n_{ct}$ is the average number of free receptors per vesicle. By conservation of mass

$$[CT]_t = [CT] + n_{ct}[V]_t$$

where $[CT]_t$ and $[V]_t$ are the total cholera toxin and vesicle concentrations. Thus K_0 may be rewritten

$$K_0 = [\bar{m}n_{ct}/([CT]_t - n_{ct}[V]_t)](n - \bar{m}n_{ct}) \quad (1)$$

In considering agglutination, we will to a first approximation consider only dimer formation between two vesicles or PS spheres. von Schulthess et al. (1976a) have shown that this is sufficient to illuminate those factors that control agglutination. If dimerization is governed by an association constant, K_1, then

$$[V_2] = K_1(n - \bar{m}n_{ct})(m - \bar{m})n_{ct}[V_1]^2 \quad (2)$$

where $[V_2]$ is the concentration of vesicle dimers, $(m - \bar{m})n_{ct}$ is the average number of free protein binding sites per vesicle, and $[V_1]$ is the concentration of vesicle monomers. The product $(n - \bar{m}n_{ct})(m - \bar{m})n_{ct}$ is a statistical factor that gives the number of ways in which a free receptor on one vesicle may bind to a free protein binding site on another. Note that eq 2 predicts no agglutination when $n_{ct} = 0$, $\bar{m}n_{ct} = n$, or $m = \bar{m}$, and thus agglutination is abolished at both high and low cholera toxin concentrations, as is also typical of antigen–antibody precipitation curves.

By maximizing eq 2 with respect to n_{ct}, one finds that maximum dimerization (as well as n-merization) occurs when $n_{ct} = n/(2\bar{m})$. Inserting this into eq 1 and rearranging yields

$$[CT]_{t,max} = 1/K_0 + n[V]_t/(2\bar{m}) \quad (3)$$

where $[CT]_{t,max}$ is the total cholera toxin concentration at which maximum agglutination occurs. This point is determined experimentally as the minimum in the diffusion coefficient determined by QLS.

Equation 3 is analogous to eq 7 obtained by von Schulthess et al. (1976a). The differences are the presence of $\bar{m}$ in the denominator of the second term of the right of eq 3 and the fact that our K_0 represents an association constant for $\bar{m}$ receptors. In their experiments, $\bar{m} = 1$ as antigen was assumed to bind no more than one antibody per PS sphere.

Results

The ability of cholera toxin to agglutinate G_{m1}-containing vesicles of various lipid compositions was examined with QLS. The results are summarized in Figure 1. We have plotted the change in hydrodynamic radius of the vesicle as a function of added cholera toxin. The hydrodynamic radii of the vesicles and the cholera toxin–vesicle complexes were obtained from the corresponding translational diffusion coefficients by using Stokes' law:

$$R_h = kT/(6\pi\eta_0\bar{D})$$

where k is the Boltzmann constant, T is the temperature, η_0 is the viscosity of water, and $\bar{D}$ is the z-average diffusion coefficient.

The extent to which the vesicles were agglutinated by cholera toxin depended on the lipid composition of the vesicle. Those composed of short-chain lipids (DMPC and DPPC) were strongly agglutinated, while those composed of longer chain lipids (PC, DOPC, and DSPC) were not appreciably

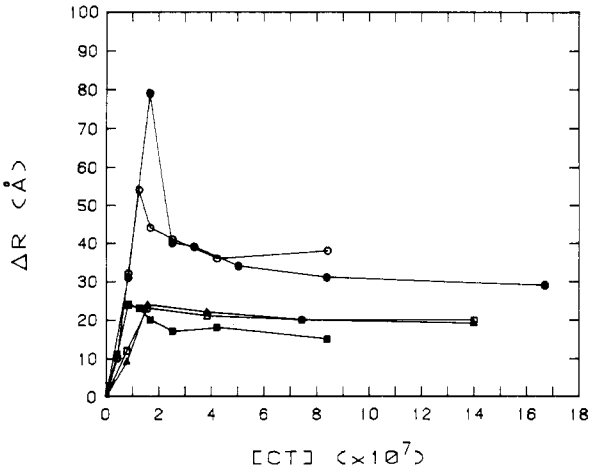

FIGURE 1: Cholera toxin mediated agglutination of G_{m1}-containing phospholipid vesicles of various compositions: DMPC (●); DPPC (○); PC (▲); DOPC (□); DSPC (■).

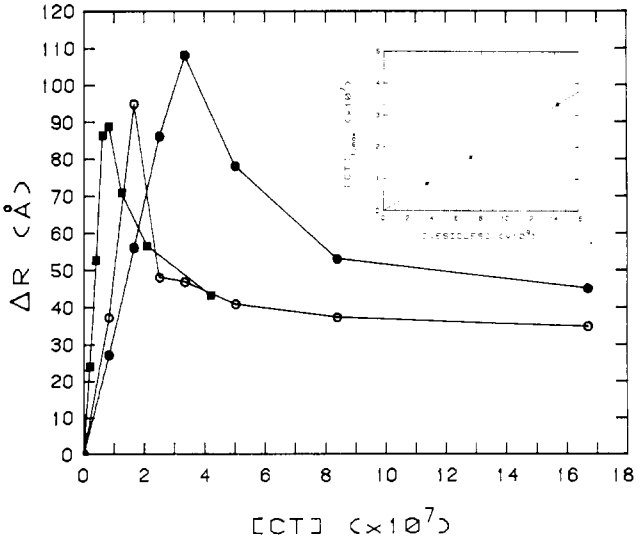

FIGURE 2: Effect of vesicle concentration of cholera toxin agglutination of DMPC–G_{m1} vesicles. [Vesicle] = 1.43×10^{-8} (●), 7.17×10^{-9} (○), and 3.58×10^{-9} M (■). (Inset) Fit of agglutination data to eq 3. From the intercept, $1/K_0 = -2 \times 10^{-10} \pm 1 \times 10^{-8}$ mM. From the slope, $\bar{m}$ = 2.4 based on an estimate of n = 90.

agglutinated. When these experiments were repeated at 37 °C, the agglutination of DMPC–G_{m1} and DPPC–G_{m1} vesicles was enhanced while the others remained unagglutinated. The additon of 10 mol % cholesterol to the vesicles did not alter these results.

So that the validity of eq 3 could be assessed, three solutions of DMPC–G_{m1} vesicles containing different vesicle concentrations were titrated with cholera toxin. The results are shown in Figure 2. As predicted by eq 3 there was a cholera toxin concentration that gave maximum agglutination and was a function of the vesicle concentration. Application of eq 3 was made to these data by plotting the total cholera toxin concentration that produced maximum agglutination, $[CT]_{t,max}$, against the corresponding total vesicle concentration. This is shown in the inset to Figure 2. The intercept gives $1/K_0 = -2 \times 10^{-10} \pm 1 \times 10^{-8}$ M. The uncertainty, much larger than the estimate of the intercept, is due mainly to the 5% uncertainty in the protein concentration. Of course, the intercept is required physically to be positive. From the slope and an estimate of the number of receptors per vesicle we calculate $\bar{m}$ = 2.4. Assuming the affinity constant for each bound G_{m1} to be the same, we obtain a lower limit of $K_0^{1/2.4} = 2.2 \times 10^3$ M^{-1} for the attachment of a single G_{m1} to cholera toxin.

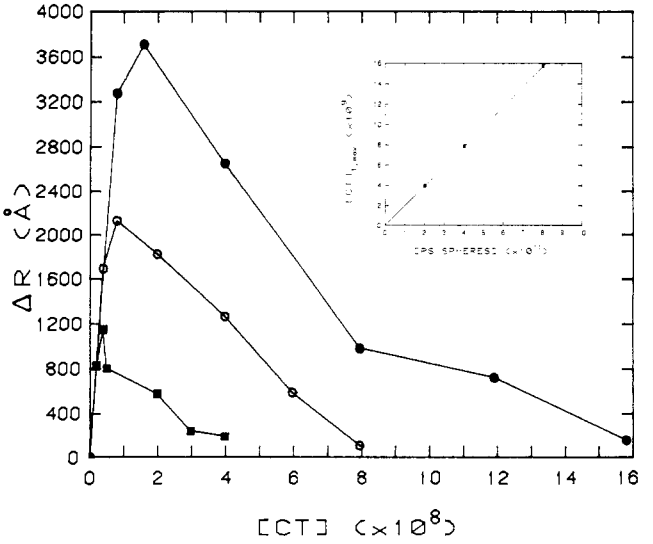

FIGURE 3: Effect of PS sphere concentration on cholera toxin agglutination of PS–G_{m1} spheres. [PS sphere] = 8.03×10^{-11} (●), 4.02×10^{-11} (○), and 2.01×10^{-11} M (■). (Inset) Fit of agglutination data to eq 3. From the intercept, $1/K_0 = 4 \times 10^{-11} \pm 5 \times 10^{-10}$ M. From the slope, $\bar{m}$ = 2.0 based on an estimate of n = 800.

In order to achieve greater sensitivity in the determination of K_0, we repeated the above experiment using G_{m1}-coated PS spheres in place of the DMPC–G_{m1} vesicles. PS spheres scatter light much more strongly than vesicles and may therefore be used at lower concentrations, thus necessitating lower cholera toxin concentrations to achieve agglutination. This results in reduced absolute uncertainty in the protein concentration and thus in K_0.

The results of this experiment are summarized in Figure 3. Three G_{m1}-coated PS samples of different concentrations were titrated with cholera toxin, and the toxin concentration that produced maximum agglutination was plotted against the corresponding PS sphere concentration (inset). Although the uncertainty in the least-squares calculated intercept of these data is again larger than the intercept itself ($K_0^{-1} = 4 \times 10^{-11} \pm 5 \times 10^{-10}$ M^{-1}), we can estimate $K_0 > 2 \times 10^9$ M^{-1}. From the slope and an estimate of n = 800 we calculate $\bar{m}$ = 2.0. Assuming the binding of these two receptors is governed by the same association constant, we estimate the affinity of cholera toxin for its initial G_{m1} receptor to be at least 4.5×10^4 M^{-1}.

Discussion

The ability of cholera toxin to agglutinate G_{m1}-containing phospholipid vesicles appears to be related to the lipid composition of the vesicle. This ability does not seem to be substantially related to the fluidity of the membrane as neither DOPC (T_m -22 °C) nor DSPC (T_m 58 °C) (Ladbrooke & Chapman, 1969) is agglutinated by cholera toxin. Bunow & Bunow (1979) have stated that short-chain lipids do not mix well with gangliosides, which tend to have longer acyl chains (Bunow & Levin, 1980). Strong head group interactions between the gangliosides also contribute to demixing. They find that this demixing results in laterally separated patches of gangliosides in the membrane bilayer. These patches of clustered gangliosides might be expected to behave toward cholera toxin in a manner similar to ganglioside micelles, which are readily agglutinated and precipitated by cholera toxin.

In the accompanying paper (Dwyer & Bloomfield, 1982) we showed that cholera toxin is well described by a structure in which the five B subunits are arranged radially about an

axially located A subunit, which extends well out one side of the molecule. This model makes it sterically unlikely for the cholera toxin to bind more than two receptors on a given PS sphere (or vesicle) and still participate in a cross-link to another. That is, three-point attachment would constrain the pentamer to lie on the surface of the vesicle or sphere. Therefore, although we calculated $\bar{m} \gtrsim 2$ at maximum agglutination, it is probable that only those toxin molecules bound to two or fewer receptors participate in cross-linking reactions.

In estimating association constants from these agglutination data, we found that up to 2 orders of magnitude greater sensitivity could be achieved by using G_{m1}-coated PS spheres in place of G_{m1}-containing phospholipid vesicles. As mentioned above, this was due to the greater scattering power of the PS spheres, which enabled the use of lower toxin concentrations. In the present experiments, agglutination produced by the addition of picomole quantities of cholera toxin was detectable. This confirms the high sensitivity of the light scattering agglutination assay, comparable to radioimmunoassay, claimed by von Schulthess et al. (1976a,b, 1980).

When multivalent agglutinating agents are allowed to bind more than one receptor on a given PS sphere (or vesicle), some assumptions are necessary in order to extract the association constant for the first bound receptor from K_0. The second term on the right of eq 3 contains two unknowns, n and $\bar{m}$. For calculation of one of these, an assumption or an independent measure of the other is required. von Schulthess et al. (1976a) assumed for their system that $\bar{m} = 1$ and thus calculated a value of n. This was reasonable given the divalent nature of the ligands they studied. They found that only 5% of the total added antibody receptors were functional when absorbed onto a PS sphere. We presume that this was due to the structural complexity of I_gA, leading to specific orientation requirements for binding and thus rendering the vast majority of absorbed receptors nonfunctional. In the present study we assumed that all of the added G_{m1} was incorporated functionally in the PS sphere, and thus n was given by the ratio of G_{m1} to PS sphere concentrations. With this estimate of n, we were then able to calculate of value of $\bar{m}$. Almost certainly the hydrocarbon side chains of G_{m1} are inserted into the bilayer or PS hydrophobic region, causing the oligosaccharide binding sites to project into solution. To the extent that it is incorrect, this assumption has the effect of overestimating $\bar{m}$, and thus our value of $\bar{m} = 2$ for PS spheres or 2.4 for vesicles may be viewed as an upper bound.

Another assumption necessary to extract the affinity of cholera toxin for its initial G_{m1} receptor is that the binding of receptors is neither cooperative nor anticooperative. We have asumed that all five receptors bind to cholera toxin with the same affinity, and thus the association constant for a single receptor is given by $K_0^{1/\bar{m}}$.

In support of these assumptions, Schafer & Thakur (1981) have recently developed a quantitative molecular model for the binding of the oligosaccharide portion of G_{m1} (G_{m1}-OS) to cholera toxin, which, when applied to equilibrium dialysis data, indicates that all five G_{m1}-OS bind to cholera toxin with roughly the same affinity. Furthermore, the values they obtain for these association constants, $(2.0–3.0) \times 10^6$ M^{-1}, are consistent with our minimum estimate.

These individual association constants for the binding of G_{m1} receptors to cholera toxin imply an overall affinity of cholera toxin for five receptors on the order of 10^{25}–10^{30} M^{-1}, i.e., essentially irreversible binding. Cuatrecasas (1973) has reported association constants on the order of 10^9 M^{-1} for the binding of cholera toxin to cell membranes containing G_{m1}. However, he also found that with time the toxin became irreversibly bound. Therefore, it is conceivable that the association constants he measured were for only partially bound cholera toxin and that with time the toxin was able to recruit and bind additional receptors and so become irreversibly bound. Our recent theoretical examination (Dwyer & Bloomfield, 1981) of the thermodynamics of binding multivalent ligands to mobile receptors in membranes indicates that ligands such as cholera toxin are thermodynamically favored to bind membrane receptors up to saturation.

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