Availability of Dinitrophenylated Lipid Haptens for Specific Antibody Binding Depends on the Physical Properties of Host Bilayer Membranes*

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We have measured the binding of two radioliodinated monoclonal anti-dinitrophenyl antibodies (IgE and IgG2a) to two dinitrophenylated lipid haptens in lipid bilayer membranes having various compositions and physical properties. These antibodies bind strongly to the lipophilic dinitrophenyl group in some membranes. Dimyristoylphosphatidylcholine and dipentadecanoylphosphatidylcholine containing 2 mol % dinitrophenyl lipid hapten bind anti-dinitrophenyl antibodies below the chain-melting transition temperatures of these lipids (22 and 35 °C, respectively) but not above these temperatures. Evidently, the lipophilic dinitrophenyl group is partially or completely buried in the hydrophobic region of these bilayers at temperatures above the chain-melting transition temperatures. The inclusion of increasing concentrations of cholesterol in such membranes (e.g. in dimyristoylphosphatidylcholine at 37 °C) results in a marked enhancement of antibody binding. It was found that a third lipid hapten containing the dinitrophenyl group does not show this strong dependence of antibody binding on the physical state of the lipid membrane.

The weak immunologic degranulation of rat basophil leukemia cells by dimyristoylphosphatidylcholine target membranes at 37 °C can be attributed to a weak binding of anti-dinitrophenyl IgE to these membranes (Balakrishnan, K., Hsu, F. J., Cooper, A. D., and McConnell, H. M. (1982) J. Biol. Chem. 257, 6427-6433). However, if the antibody is first allowed to bind to this membrane below the lipid chain-melting transition temperature, these IgE-coated membrane targets are very effective in releasing serotonin from the rat basophil leukemia cells when the temperature is raised to 37 °C.

The dinitrophenyl group is a common hapten used in many immunological studies. When attached to the head group of lipid molecules, it has been used extensively in immunological studies of lipid membrane targets (1-5). Whether these model membranes are used as stimulators of afferent immunological response or as targets in efferent immunological attack, the exposure or availability of the hapten is crucial for binding and triggering. As described in the accompanying paper (6), dimyristoylphosphatidylcholine liposomes containing 2 mol % Dnp lipid hapten evoked only a weak immunologic degranulation response from rat basophil leukemia cells. We suspected that this weak response was due to the weak binding of anti-Dnp IgE to the Dnp lipid hapten in the DMPC membranes.

In other studies Kinsky and co-workers have noted a correlation between liposomal immunogenicity in mice and the transition temperature of the phospholipid (2, 3). They have suggested that these differences may be due partly to differential lipid hapten exposure. The nonavailability of the Dnp group in the Dnp lipid hapten may be the primary reason for the low immunogenicity of certain liposomes containing Dnp-caproylphosphatidylethanolamine seen by Yasuda et al. (2) and Dancey et al. (3). Antibody binding to lipid haptens in bilayer membranes can be affected by the length and nature of the molecular "linker" chain connecting the hapten to the head group of the lipid (see Ref. 7 and "Discussion"), the length of the fatty acid chains (8) and, as shown here, by interactions between the hapten and the bilayer itself. Because specific antigen-antibody binding is an early step in all these immunological responses, an examination of anti-Dnp antibody binding to Dnp lipid haptens in various lipid membranes was undertaken.

MATERIALS AND METHODS

anti-dinitrophenyl IgE—Monoclonal mouse IgE against the Dnp hapten was purchased from Miles-Yeda (Elkhart, IN).

Iodination—The antibodies were radioiodinated by a slight modification of the chloramine-T method (9). To 8 μg of antibody in 100 μl of 50 mM sodium phosphate buffer (pH 7.4) was added 1 μCi of Na125I. Five microliters of a freshly made solution of chloramine-T (10) was then added, followed by four subsequent additions at 2-min intervals. (In total, 20 μl of chloramine-T solution were added.) After a 10-min exposure to chloramine-T, 25 μl of a 0.15 mg/ml solution of sodium metabisulfite in the same buffered solution were added. Subsequent to the addition of 100 μl of 1% potassium iodide solution, the free 125I was removed by gel filtration through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden). Specific radiodactivities of approximately 30 μCi/μg of protein were obtained.

Haptens—The synthesis of Dnp lipid hapten 1 is described in detail in the accompanying paper (6). Dnp-caproyl-PE was purchased from Avanti Biochemicals, Inc. (Birmingham, AL). The synthesis of the

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nitroxide lipid hapten II will be described elsewhere. The structures of Dnp lipid hapten I and nitroxide lipid hapten II can be found in the accompanying paper.

**Liposomes**—Unless otherwise stated for individual experiments, liposomes containing 2 mol % Dnp lipid hapten I were prepared as described elsewhere (6, 7) in a phosphate-buffered saline solution containing 0.2% w/v gelatin, 0.02% w/v sodium azide, and 5% w/v Ficoll-70 (Pharmacia). Large liposomes were preselected for the antibody binding assay by spinning for 5 min at 8000 × g through a cushion of 2.5% Ficoll 400 (Pharmacia) solution in the same incubation buffer (phosphate-buffered saline containing 0.2% gelatin and 0.02% sodium azide).

**Binding Assay**—One hundred microliters of liposomes were incubated with an equal volume of incubation buffer containing approximately 300 pg of iodinated antibody (20,000 cpm) for 1–3 h with gentle shaking in an equilibrated water bath at the appropriate temperature. The incubation mixture was layered over a 900-μl cushion of 2.5% w/v solution of Ficoll 400 and centrifuged for 5 min at 8000 × g in a 1.0-ml Fisher centrifuge tube. The tubes were then frozen in dry ice, and the tips containing the liposomal pellet were excised. The remainder of the tube contained the unbound antibody. Both samples were counted in a Beckman (Gamma 8000) γ counter (Beckman Instruments). In some experiments, the binding assay was carried out differently and is described in detail (see Table I).

**Immunologic Degranulation from RBL Cells**—As discussed in detail earlier (7), the extent of immunologic degranulation was measured using the release of [3H]5-hydroxytryptamine from labeled RBL cells.

**RESULTS**

**Binding of Iodinated Antibodies to Solid and Fluid Membrane Targets**—The binding of [125I]anti-Dnp IgE to DMPC, DPPC, DSPC, and DBPC membrane targets containing 2 mol % Dnp lipid hapten I was measured at 37°C. Antibody binding to the lipid hapten in the DMPC liposomes was weak and almost unmeasurable. The binding to hapten in the other membranes was strong and specific. When an excess of cold antibody was added, the radiiodinated antibody could be displaced quantitatively. On a Scatchard plot, all the three solid liposomes yielded a straight line with a slope or an apparent affinity constant of 2 ± 0.2 × 10^6 M^{-1} (Fig. 1). (All these binding constants are "apparent" binding constants because we have no information concerning the fraction of these antibodies that have zero, one, or two hapten-combining sites).

In separate experiments, the binding of [125I]anti-Dnp IgE to DMPC liposomes at 0°C (solid) was studied and yielded an apparent affinity constant of 7 ± 1 × 10^6 M^{-1}, which was similar to that obtained with DPPC liposomes at 0°C, viz. 8 ± 1 × 10^6 M^{-1}.

Dipentadecanoylphosphatidylcholine has a chain-melting temperature of 35°C (intermediate between DMPC (22°C) and DPPC (42°C)). The binding of anti-Dnp IgE to liposomes

**TABLE I**

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Transition temperature</th>
<th>Anti-Dnp (IgE) antibody binding (bound/free × 100) at °C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0–4</td>
</tr>
<tr>
<td>Egg PC</td>
<td>&lt;0</td>
<td>2.9</td>
</tr>
<tr>
<td>DOPC</td>
<td>&lt;0</td>
<td>1.8</td>
</tr>
<tr>
<td>DMPC</td>
<td>22</td>
<td>18.0</td>
</tr>
<tr>
<td>DPPC</td>
<td>42</td>
<td>16.2</td>
</tr>
<tr>
<td>DSPC</td>
<td>55</td>
<td>19.5</td>
</tr>
<tr>
<td>DMPC + 30 mol % cholesterol</td>
<td>None</td>
<td>25.1</td>
</tr>
</tbody>
</table>

**Fig. 1.** Scatchard plot for the binding of anti-Dnp IgE to DPPC, DSPC, and DBPC membranes containing 2 mol % Dnp lipid hapten I. Bound hapten concentration was estimated to be twice the bound [125I]anti-Dnp IgE concentration. The free exposed hapten concentration (estimated as before (23) to be 5% of the total lipid hapten in the sample) was kept at least two to three orders of magnitude higher than the concentration of added [125I]antibody. Thus, almost all the hapten in solution was free. Hence a good approximation can be obtained for the value of (bound/free) hapten ratio. In the case of DMPC, the binding was weak or indetectable.

T. Some Lipid Haptens in Fluid Membranes Are Cryptic 6435

**TABLE II**

<table>
<thead>
<tr>
<th>Solid membranes</th>
<th>Fluid membranes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC at 0°C</td>
<td>DMPC at 37°C</td>
</tr>
<tr>
<td>0.14 ± 0.2 × 10^6 M^{-1}</td>
<td>1.5 ± 0.2 × 10^6 M^{-1}</td>
</tr>
<tr>
<td>0.15 ± 0.2 × 10^6 M^{-1}</td>
<td>1.5 ± 0.2 × 10^6 M^{-1}</td>
</tr>
</tbody>
</table>
Some Lipid Haptens in Fluid Membranes Are Cryptic

I 1 I I I
20 40
MOLE % CHOLESTEROL

FIG. 2. Binding of [125I]IgE to haptenated DMPC liposomes containing varying amounts of cholesterol. Notice the dramatic increase in the amount of antibody bound to the liposomes for cholesterol concentrations above 10–20 mol %.

composed of this phospholipid and 2 mol % Dnp lipid hapten I was studied at 30 and 40 °C. At 40 °C, the binding was weak and unmeasurable while at 30 °C there was a strong binding with an affinity of 1.5 ± 0.2 × 10^9 M⁻¹.

Table I shows essentially similar results for the binding of [125I]anti-Dnp IgG₂ to different lipid membrane targets composed of 1 mol % Dnp-cap-PE at various temperatures.

We have isolated a hybridoma cell line producing monoclonal mouse IgG₁ with a high specificity for the nitroxide hapten. [125I]Anti-nitroxide IgG₁ binds with a high affinity to lipid membranes containing 2 mol % nitroxide lipid hapten II (Table II) and does not cross-react with either DNP lipid hapten I or DNP-cap-PE. As shown in Table II, the anti-nitroxide IgG₁ binds to the nitroxide lipid hapten II in all phosphatidylcholine membranes with similar affinities, irrespective of whether the membrane is above or below the chain-melting transition temperatures.

Inclusion of Cholesterol in Phosphatidylcholine Membranes Above Their Chain-melting Transition Temperature Renders the Dnp Lipid Hapten Available for Antibody Binding—Experiments were carried out in which the binding of [125I]anti-Dnp IgE to DMPC membranes containing varying amounts of cholesterol was studied at 37 °C (Fig. 2). Above 10–20 mol % cholesterol, there is a marked increase in the binding of α-Dnp IgE to the Dnp lipid hapten I. The same effect is seen in the binding of α-Dnp IgG₂ to Dnp-cap-PE in liposomes composed of DMPC and cholesterol (Table I).

Measurement of Antibody Off Times from Membrane Targets—Membranes composed of DSPC and 2 mol % Dnp lipid hapten I were preincubated with [125I]anti-Dnp IgE at 37 °C, and the free antibody was removed by centrifugation for 5 min at 8000 × g. When these [125I]antibody-coated liposomes were resuspended in fresh incubation buffer, the antibody was released with an apparent off time of about 10–15 min (Fig. 3). However, if a 10- to 1000-fold excess of cold anti-Dnp IgE was present in the incubation buffer used to resuspend the liposomes, the iodinated antibody came off much faster (with an off time of <5 min). The presence of cold IgE presumably inhibits the rebinding of the dissociated [125I]anti-Dnp IgE to the vesicles. We have not attempted a quantitative analysis of these kinetic data.

This observation, that anti-Dnp IgE bound to liposomes remains associated with them for about 10–15 min, suggested that we might observe stimulation of RBL cells by DMPC membrane targets which had been previously coated with anti-Dnp IgE below the transition temperature.

Serotonin Release from RBL Cells by DMPC Liposomes Precoated with IgE—In the companion paper (6) we suggested that the weak immunologic degranulation seen from RBL cells stimulated with DMPC membrane targets containing 2 mol % Dnp lipid hapten I could be due to the weak binding of the anti-Dnp IgE to Dnp hapten in these membranes.

In order to circumvent this problem, DMPC membrane targets containing 2 mol % Dnp lipid hapten I were incubated with an excess of anti-Dnp IgE at 0 °C for 1–3 h. In control

Fig. 3. Kinetics of antibody release from DSPC membranes coated with [125I]anti-Dnp IgE. DSPC liposomes containing 2 mol % Dnp lipid hapten I were coated with [125I]anti-Dnp IgE by incubation with the antibody followed by pelleting of the liposomes. The liposomes were resuspended in the incubation buffer with no added antibody, and the amount of radioactivity that remained associated with the liposomes was determined for various time points by pelleting the liposomes. A₀ and Aₜ represent the radioactivity associated with the liposomes at time 0 and t, respectively.
experiments, the DMPC liposomes were prepared with no Dnp lipid hapten I, or the hapten-containing DMPC liposomes were incubated with anti-Dnp IgE at 37 °C. These liposomes were then pelleted, resuspended, and incubated with [3H]serotonin-labeled RBL cells. As shown in Fig. 4, the DMPC liposomes that were coated with IgE at 0 °C stimulated the RBL cells to release serotonin, while the DMPC liposomes that were preincubated with IgE at 37 °C did not. Similarly, DMPC liposomes containing no Dnp hapten failed to stimulate the RBL cells to release serotonin. If IgE-coated DSPC liposomes were used, the level of degranulation seen was higher (Fig. 4), consistent with the results reported in the accompanying paper (6). Evidently, the IgE remains bound to the DMPC target membranes for a time long enough for an RBL cell target membrane conjugate to be formed. Once such a conjugate is formed, the Dnp hapten on the membrane evidently remains accessible and bound to the IgE, facilitating an immunologic degranulation response from the RBL cells.

In separate experiments, we studied the binding of DMPC liposomes containing 2 mol % Dnp lipid hapten I to RBL cells coated with anti-Dnp IgE at 0 and 37 °C (Fig. 5). (See companion paper (6) for experimental details.) RBL cells coated with anti-Dnp IgE do not bind to the DMPC membrane targets containing 2 mol % Dnp lipid hapten I at 37 °C and bind very well to the same DMPC membrane targets at 0 °C. The above experiments involving RBL cells were carried out in a buffer containing 135 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5.6 mM glucose, 0.5 mg/ml of gelatin, 10 mM 1-(2-hydroxyethyl)-1-piperazinethanesulfonic acid, at pH 7.2.

Partitioning of Dnp into Membrane Bilayers—We have obtained some evidence that the Dnp group binds to the DMPC liposomes at temperatures above the chain-melting transition temperature. E-Dnp-lysine (10⁻³ M) was incubated with preformed DMPC vesicles (10⁻² M) at 0 or 37 °C for 1-2 h, and the vesicles were removed by centrifugation at 9000 × g for 10 min. The concentration of E-Dnp-lysine in the supernatant, as measured by its optical density at 370 nm, decreased noticeably (to 60%) when the incubation was done at 37 °C and remained unchanged at 0 °C. When 50 mol % cholesterol was included in the DMPC vesicles at 37 °C the Dnp concentration in the aqueous solution did not change.

**DISCUSSION**

Lipid membrane targets containing lipid haptens have been used in numerous studies of afferent and efferent responses of the immune system (see Alving for recent reviews (8, 10)). Whether these liposomal targets are used for specific antibody-dependent afferent stimulation of the immune system or are used as targets for efferent immunological attack, it is clearly important to understand the physical properties of the lipid membrane and the lipid hapten that are significant for antibody-lipid hapten binding. In previous work it has been demonstrated that antibody binding to lipid haptens in bilayer membranes can be affected by the length and nature of the molecular linker chain connecting the hapten to the head group (7) and by the length of the fatty acid chains (8). The present work provides evidence that in the case of Dnp hapten one must also consider interactions between the Dnp group and the lipid bilayer itself.

The experimental results given in the present paper are most simply accounted for if, in the absence of antibody, there exists an equilibrium between two states, B and E

\[ B = E \quad (1) \]

\[ \frac{[B]}{[E]} = K_e \quad (2) \]
Here \( B \) represents a physical state of the Dnp group which is "buried" in the lipid bilayer and unavailable for antibody binding. \( E \) represents a physical state of the Dnp group which is "exposed" and available for antibody binding.

Let us assume that when IgE or IgG antibody is experimentally determined to be bound to the bilayer membrane it is bound with both combining sites.

\[
Ab + 2E \rightleftharpoons AbE_2
\]

\[
\frac{[AbE_2]}{[Ab][E]^2} = K_1
\]

Under the conditions of our experiments an adequate approximation to the bound-to-free ratio for antibody is

\[
\frac{[AbE_2]}{[Ab]} = K_0 ([H]//1 + K_0)^5
\]

where \([H]\) is the total hapten associated with the bilayer. In deriving Equation 5 it is assumed that \([AbE_2] \ll [H]\) because this is appropriate to our experimental conditions.

In the present work we have found that monoclonal anti-Dnp IgE does not bind significantly to I in DMPC or dipentadecanoylphosphatidylcholine membranes above their chain-melting transition temperatures. On the other hand, relatively strong binding is observed below these chain-melting transition temperatures. If \(K_0\) were of the order of one or less for the lower temperatures, and of the order of 10 or greater for the higher temperatures, our experimental results could be accounted for. According to this model, the introduction of cholesterol into a phosphatidylcholine membrane at temperatures above the chain-melting transition temperature decreases the value of \(K_0\) again resulting in enhanced antibody binding. All of our data on the binding of monoclonal anti-Dnp IgG\(_\alpha\) to Dnp-cap-PE (see Table I) are also consistent with this simple picture.

The differences seen in antibody-lipid hapten binding depend strongly on the physical properties of the lipid bilayer target that a quantitative analysis of the binding data is not essential. Therefore, we have not attempted to determine the fraction of the antibodies that had zero, one, or two active sites.

We have no direct physical evidence that the Dnp group is "buried" or intercalated within the hydrophobic region of pure phosphatidylcholine bilayers above the chain-melting transition temperatures. However, we have found that Dnp-lysine binds to DMPC at temperatures above the chain-melting temperature but not below. Dnp-lysine does not bind significantly to DMPC bilayers containing 50 mol % cholesterol. Dnp is a small planar lipophilic group that could quite plausibly intercalate between the hydrocarbon chains of a fluid lipid bilayer (e.g. DMPC at 37°C). Other lipophilic molecules such as diphenylhexatriene and fluorescent derivatives of pyrene and anthracene are believed to interact with lipid bilayers in this fashion (11). The above presumed behavior of the Dnp group is very similar to that of the spin label Tempo (2,2,6,6-tetramethylpiperidinyl-1-oxyl) that has been used in numerous studies of phase transitions in lipid bilayers (12-15).

It is important to contrast briefly the above results and discussion with other studies of lipid hapten-antibody binding.

1) Our original motivation for the synthesis of I, having a long molecular linker between the glycerol backbone and the haptenic group, was because the monoclonal anti-Dnp IgE employed here bound very weakly to Dnp-cap-PE in liposomes, and consequently liposomes containing 2 mol % Dnp-cap-PE did not release serotonin from anti-Dnp IgE-coated RBL cells. Because the affinity of the IgE for Dnp-lysine alone is high, \(~10^6 \text{ M}^{-1}\) (16), the weak binding might be attributed to a property of the antibody molecules (e.g. depth of the combining site) and the shortness of the linker. Thus, a molecule with a longer linker was synthesized and proved effective.

2) Affinity-purified rabbit anti-Dnp antibodies do bind to Dnp-cap-PE in DMPC liposomes above the chain-melting transition temperature and are immunologically effective in phagocytosis (17). This can be attributed to a subpopulation of antibodies with appropriately oriented combining sites or to a subpopulation of antibodies with such a high hapten affinity that the equilibria (Reactions 1 and 3) are pulled in the direction of bound antibody. In other words, even with a larger \(K_0\), there could in principle be an antibody with high enough hapten affinity to overcome the tendency of the hapten to be buried.

3) Monoclonal anti-nitroxide antibody and lipid hapten II show none of the effects reported here for monoclonal anti-Dnp antibodies. For example, monoclonal anti-nitroxide IgG\(_\alpha\) binds equally well to II in DMPC bilayers, irrespective of whether the temperature is above or below the chain-melting transition temperature (see Table II). Steric as well as polar constraints doubtless inhibit the interaction of the headgroup of II with the bilayer. The binding of affinity-purified rabbit anti-nitroxide antibodies with a variety of nitroxide (spin label) lipid haptens shows some dependence on the length of the linker, on the temperature relative to chain-melting transition temperatures, and on cholesterol content (18, 19). However, none of these factors has such large effects as those described in the present paper.

In conclusion it will be seen that many factors, some not even mentioned here, must affect the binding of antibodies to lipid haptens in bilayer membranes. In many cases distinguishing between the various factors that affect antibody binding will be difficult. However, in the present work the very large effects of temperature and cholesterol content on the binding of monoclonal antibodies to the Dnp hapten I and Dnp-cap-PE appear to have a single straightforward interpretation. Depending on temperature and lipid composition, the Dnp hapten itself can bind to the bilayer and be unavailable for antibody binding.

The differences frequently noted in specific antibody-dependent activation of various components of the immune system (complement (19), macrophages (20), basophiles (6, 7)) by lipid hapten-containing liposomes or vesicles may arise partly from differences in antibody binding. This is particularly true for the enhancement of immune response by the inclusion of cholesterol in the target membranes. Spin label studies show directly that nitroxide hapten groups exhibit less restricted molecular motion when cholesterol is introduced into phosphatidylcholine bilayers.

The effects discussed in the present paper may very well be related to an earlier failure to elicit secondary cytotoxic T cells that were transplantation-antigen restricted and trinitrophenyl specific, when a trinitrophenyl lipid hapten was fused into purified lymphocyte plasma membranes and used for the secondary stimulation (21). In contrast, this elicitation is strong when the trinitrophenyl group was present on proteins associated with these plasma membranes.

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Availability of dinitrophenylated lipid haptens for specific antibody binding depends on the physical properties of host bilayer membranes.
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