Structural Features of the Acyl Chain Determine Self-phospholipid Antigen Recognition by a CD1d-restricted Invariant NKT (iNKT) Cell*

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Little is known about the antigen specificity of CD1d-restricted T cells, except that they frequently recognize CD1d-expressing antigen-presenting cells in the absence of exogenous antigen. We previously demonstrated that the 24.8.A iNKT cell hybridoma was broadly reactive with CD1d-transfected cell lines and recognized the polar lipid fraction of a tumor cell extract. In the present study, the antigen recognized by the 24.8.A iNKT cell hybridoma was purified to homogeneity and identified as palmitoyl-oleoyl-sn-glycero-3-phosphoethanolamine (16:0-18:1 PE). The 24.8.A iNKT cell hybridoma recognized synthetic 16:0-18:1[cis] PE, confirming that this phospholipid is antigenic. Recognition correlated with the degree of unsaturation of the acyl chains. Using a panel of synthetic PEs, the 24.8.A iNKT cell hybridoma was shown to be activated by PEs that contained at least one unsaturated acyl chain. The configuration of the double bonds was important, as the 24.8.A iNKT cell hybridoma recognized unsaturated acyl chains in the cis, but not the trans, configuration. PEs with multiple double bonds were recognized better than those with a single double bond, and increasing acyl chain unsaturation correlated with increased binding of PE to CD1d. These data illustrate the potential importance of the acyl chain structure for phospholipid antigen binding to CD1d.

The CD1 proteins are β2-microglobulin-associated antigen-presenting molecules that present lipid antigens to T cells. CD1d-restricted T cells can be divided into two subsets based on their T cell receptors (TCR)1: one that uses a diverse TCR repertoire and a second that uses an invariant TCRα chain (Vα14-Jα281) paired with a limited number of TCRβ chains (Vβ2, -7, -8). Both subsets can coexpress cell surface markers associated with NK cells, such as the NK1.1 antigen, and hence are referred to as NKT cells. The second subset of NKT cells will be referred to as invariant NKT (iNKT) cells. It is this subset that has been implicated in a variety of pathological processes, and activation of iNKT cells promotes host rejection of tumors,ameliorates autoimmune disease, and enhances immunity to certain infectious diseases (1–9). In some cases, the beneficial effects of iNKT cells appear to be mediated by other cell types, and this finding has led to the concept that CD1d-restricted iNKT cells function as immunoregulatory T cells (10).

In many of these studies, iNKT cells were activated with the synthetic antigen α-galactosylceramide (αGalCer), an antigen originally isolated from marine sponges and rarely detected in mammalian cells or bacteria (11). Most human and mouse iNKT cells recognize αGalCer when presented by CD1d, and undergo activation characterized by rapid cytokine production and enhanced cytotoxic activity (11–13). Although αGalCer has been extremely useful in elucidating the biology of iNKT cells, little is known about the naturally occurring antigens that are recognized by iNKT cells. To understand how iNKT cells participate in immunity, the physiological antigens recognized by the iNKT cell TCR require identification. Many of the first CD1d-restricted NKT cells studied were self-reactive, and recognized both human and murine CD1d in the absence of exogenously added antigen (14–16). Several naturally occurring antigens potentially recognized by iNKT cells have since been identified. These include peptides, phosphatidylinositol (PI) and PI-containing compounds, and other phospholipids (17–19).

We have previously characterized a panel of CD1d-restricted T cells and T cell hybridomas (both invariant and diverse) that recognize CD1d-transfected cell lines in the absence of exogenously added antigen (14). However, these CD1d-restricted NKT cells are antigen-dependent and some have been shown to recognize endogenous cellular lipid antigens (17). Using an in vitro assay that was developed for the purpose of antigen discovery, the antigen recognized by the 24.8.A iNKT cell hybridoma was partially purified from RPMI-S tumor cells and char-

* This work was supported by operating grants from the Arthritis Society of Canada (to J. R.), the Canadian Institutes of Health Research (to J. R.), a Schering Traveling Fellowship from the Canadian Society for Clinical Investigation (to J. R.), the American College of Rheumatology Research and Education Foundation (to D. B. M.), NIAID, National Institutes of Health Grant R01-A149313, the Arthritis Foundation (to S. M. B.), and the National Center for Research Resources, National Institutes of Health (to C. E. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: TCR, T cell receptor; PBS, phosphate-buffered saline; iNKT, invariant NKT; PE, phosphatidylethanolamine; ER, endoplasmic reticulum; CID, collision-induced decomposition; MS, mass spectrometry.
acterized as a polar lipid (14, 17). We have now purified the antigen to homogeneity and identified the antigen as palmitoyl-oleoyl-sn-glycero-3-phosphoethanolamine (16:0–18:1 phosphatidylethanolamine (PE)). We show that unsaturation of acyl chains is important for recognition and suggest that this is likely because of its effect on lipid packing and the ability of the lipid tails to load into the CD1d binding groove.

EXPERIMENTAL PROCEDURES

Materials—Unless stated otherwise, all chemicals were obtained commercially and used without further purification. Dicaproyl-sn-glycero-3-phosphoethanolamine (di-6:0 PE), dicapryl-sn-glycero-3-phosphoethanolamine (di-8:0 PE), dicapryl-sn-glycerol-3-phosphoethanolamine (di-10:0 PE), dilauroyl-sn-glycero-3-phosphoethanolamine (di-12:0 PE), dimyristoyl-sn-glycero-3-phosphoethanolamine (di-14:0 PE), dipalmitoyl-sn-glycero-3-phosphoethanolamine (di-16:0 PE), distearoyl-sn-glycero-3-phosphoethanolamine (di-18:0 PE), dipalmityloleyl-sn-glycero-3-phosphoethanolamine (di-16:1(14:0) PE), dioleoyl-sn-glycero-3-phosphoethanolamine (di-18:2(16:1) PE), dielaidoyl-sn-glycero-3-phosphoethanolamine (di-18:3(16:1) PE), dilinoleoyl-sn-glycero-3-phosphoethanolamine (di-18:2(12:1) PE), dilinoleoyl-sn-glycero-3-phosphoethanolamine (di-18:3(12:1) PE), and dilinoleoyl-sn-glycero-3-phosphoethanolamine (di-18:3(14:0) PE) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Phospholipid stock solutions were prepared in chloroform (CHCl3) and stored at −70 °C. Phospholipid suspensions were prepared in dimethyl sulfoxide (Me2SO) as follows. Phospholipid in CHCl3, or an equivalent amount of CHCl3 (CHCl3 control), was dried down in a round-bottomed glass tube under nitrogen gas. Me2SO was added to the lipid film, and the sample was then vortexed vigorously and redissolved at 30 °C for 20 min in a sonicator bath. Phospholipid concentrations were determined by the method of Bartlett (20).

Cells and Cell Lines—The CD1d-restricted 24.8 iNKT cell clone and the 24.8.A iNKT hybridoma, both of which express a TCR encoded by the invariant TCRα (Va14-Jα281) and Vβ8.2 genes, have been previously described (14, 17). The RMA-S cell line was derived from the Balb/c mouse: It is a derivative of the Balb/c thymoma RMA, with the exception that protein G-coated plates (Pierce) were used in place of protein A-coated plates. This assay will be referred to hereafter as the plate-bound CD1d assay. Briefly, phospholipid antigens diluted in PBS were added to the wells of a protein G-coated plate, followed by mCD1d fusion protein (0.5–1.0 μg/ml in PBS). Following incubation of the plates for 24 h at 37 °C, the plates were washed with PBS and medium. 24.8.A iNKT hybridoma cells were added to the coated wells at a concentration of 1×10^5 cells/well in a total volume of 150 μl/well. Assays were performed using 3 replicate wells and repeated a minimum of twice. Controls included wells coated with anti-CD3 (positive) or PBS (negative). In some experiments, anti-CD1d (19G11), anti-MHC I (M1/42.3.9.8.HLK), or anti-MHC II (M5/114.15.2) blocking monoclonal antibody was included at a concentration of 20 μg/ml. The coefficient of variation between replicate wells was generally <10%.

Purification of the mCD1d Fusion Protein—A competitive phospholipid binding assay was developed to evaluate the ability of phospholipids to compete with biotinylated di-18:0 PE bound to streptavidin-coated plates for binding to mCD1d. The concentrations of biotinylated di-18:0 PE and mCD1d used in this assay were selected to fall on the linear portion of their binding curve. Immulon-2 (Dynatech, Chantilly, VA) plates were coated for ~16 h at 4 °C with 5 μg/ml streptavadin (ICN, Irvine, CA) in 0.015 μm carbonate buffer, pH 9.6. The coated plates were washed 3 times with PBS and then blocked with PBS containing 10% heat-inactivated fetal bovine serum (Invitrogen, Burlington, ON) and 0.05% Tween 20 for 1 h at 25 °C. Following three washes with PBS, 50 μl of biotinylated di-18:0 PE (Northern Biosynthesis Technologies, Chantilly, VA) was added to the coated streptavidin-coated wells and incubated for 1 h at 25 °C. Competitive binding samples for the assay were prepared by incubating 30 μl of phospholipid competitors, diluted from the Me2SO stock solution into PBS containing 0.1% ovalbumin (assay buffer), with 20 μl of purified mCD1d (approximate final concentration of 4 μg/ml). The samples were incubated in hirudin-cationized glass tubes for 16 h at 37 °C. 50 μl of the competitor/CD1d solution were added in duplicate to the streptavidin-coated plates, and incubated for 4 h at 25 °C. The plates were washed four times with PBS. Bound mCD1d was detected by the addition of alkaline phosphatase-conjugated human- or mouse-goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL), diluted at 1:1000 in PBS containing 0.4% bovine serum albumin, and incubation for 1 h at 4 °C. A colored reaction was developed by adding p-nitrophenyl phosphate substrate (Sigma) and developing for ~20 min at 37 °C. The OD405 was read in an ELISA reader (TiterTek Multiskan MCC/340, Flow Systems, Huntsville, AL). The coefficient of variation between replicate wells was generally ~10%.

RESULTS

Purification and Identification of Palmitoyl-oleoyl PE as the RMA-S Antigen Recognized by the 24.8.A iNKT Cell Hybridoma—Previous studies had shown that the iNKT cell hybridoma 24.8.A was most potently stimulated by the phospholipid-enriched methanol fraction of RMA-S cellular lipids (17). To determine the identity of the natural antigens, the methanol fraction was further fractionated by two-dimensional TLC, which yielded 20 distinct lipid species (data not shown). Ten spots were visualized based on the exclusion of a fine water mist (spots 1–10), and a further ten spots were visualized using iodine vapor (spots 11–20). The silica corresponding to each spot was scraped and the lipids eluted with CHCl3/methanol (spots 1–10) and CHCl3/methanol (spots 11–20). The silica corresponding to each spot was scraped and the lipids eluted with CHCl3/methanol. The ability of each of these fractions to stimulate the 24.8.A iNKT cell hybridoma was tested using the plate-bound CD1d assay. One of the fractions (fraction 7) strongly stimulated the 24.8.A iNKT cell hybridoma to produce IL-2 in the plate-bound CD1d assay, similar to the original methanol fraction (Fig. 1). Plate-bound CD1d alone, in the presence of PBS, showed little or no reactivity.

To determine the identity of the reactive lipid, fraction 7 was analyzed by negative ion mass spectrometry. It contained a single isotopic cluster with the nominal mass per unit charge of m/z 716. The accurate mass m/z 716.5233 was determined (calc. m/z 716.5236 for the C55H37NO3P− anion). Deuterium exchange experiments indicated that two exchangeable protons were present (data not shown). Both of these findings are consistent with the structure of palmitoyl-oleoyl PE. Further
structural analysis of this anion by collision-induced decomposition (CID) MS/MS revealed fragments at $m/z$ 255 and $m/z$ 281, consistent with the presence of 16:0 and 18:1 acyl chains (Fig. 2a). At slightly higher energy, fragmentation of the glycerol backbone or the glycerophosphate bond resulted in fragments at $m/z$ 196 \{C$_9$H$_{16}$O$_2$N$_2$\}, and $m/z$ 140 \{HPO$_4$CH$_2$CH$_2$NH$_2$\}, which are characteristic of PEs (Fig. 2c). Comparison of the MS/MS data to the literature for the two possible isomers, where carboxylate anion were preferentially generated from the sn-2 position during low energy CID of PEs, indicates that the 18:1 acyl group is located at the sn-2 position, although a mixture with lower levels of the other isomer cannot be ruled out (22). 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (16:0–18:1) is postulated as the most probable structure (Fig. 2c).

To confirm that 16:0–18:1 PE was an antigen recognized by the 24.8.A iNKT cell hybridoma, synthetic 16:0–18:1 PE was obtained and evaluated in the plate-bound CD1d assay. Synthetic 16:0–18:1 PE was recognized by the 24.8.A iNKT cell hybridoma and its recognition was completely blocked by antibodies specific for CD1d (Fig. 3). Antibodies specific for MHC I or MHC II did not block antigen recognition. These data indicate that PE is an endogenous self-lipid antigen that can be presented by CD1d to the self-reactive 24.8.A iNKT cell hybridoma.

The 24.8.A iNKT Cell Hybridoma Recognizes Microbial, Mammalian, and Plant PEs—Once it was confirmed that PE was an endogenous antigen recognized by the 24.8.A iNKT cell hybridoma, synthetic 16:0–18:1 PE was evaluated in the plate-bound CD1d assay. Synthetic 16:0–18:1 PE was recognized by the 24.8.A iNKT cell hybridoma (Fig. 4). PE from E. coli was recognized by the 24.8.A iNKT cell hybridoma, but not better than PEs from plant or animal sources. The degree of recognition of E. coli PE was similar to that of liver PE. In general, we found that most sources of natural PEs were well recognized, and the degree of recognition correlated with the percentage of 18:1 + 18:2 + 18:3 acyl chains for the PEs for which this information is known (Fig. 4). For example, plant PE was the most potent activator of the 24.8.A iNKT cell hybridoma and has 73.2% of its acyl chains (18:1, 18:2, and 18:3) unsaturated. In contrast, liver PE was the least potent activator and contained only 14.7% unsaturated 18-carbon acyl chains. This observation led us to more carefully examine the role of the acyl chains in recognition of antigen by the 24.8.A iNKT hybridoma using a panel of synthetic PE.

Fatty Acyl Chain Unsaturation Is Critical for Recognition of PE—The paradigm for binding of lipid antigens to CD1 is that the acyl chains are buried deep in the hydrophobic cleft formed by the CD1 α1 and α3 domains, leaving the polar head group exposed on the surface of the molecule, a model confirmed by the crystal structure of murine CD1d, human CD1a, and human CD1b (25–29). Furthermore, alteration of the polar head group of the lipid antigen or the polar amino acid residues in the TCRβ CDR3 region both have a dramatic effect on TCR-dependent recognition of the antigen (24, 30). However, changes in the structure of acyl chains can also affect T cell recognition and lipid antigen processing (31, 32). The plate-bound CD1d assay detects the recognition of PE by the 24.8.A T iNKT cell hybridoma independently of antigen processing. As the lipid antigen is not processed and recognition depends only on the direct binding of the phospholipid to the CD1d fusion protein, we were able to determine the effect of different acyl chains on recognition of PE by the 24.8.A iNKT cell hybridoma.

To determine the effect of length and saturation of the fatty acyl chains on recognition of PE by the 24.8.A iNKT cell hybridoma, we first examined a series of synthetic saturated 1,2-diacyl-sn-glycero-3-phosphoethanolamines (Fig. 5b). Although the chain length varied between 6 and 18 carbons, none of the saturated diacyl PEs was recognized, with the exception of di-12:0 PE. As the PE purified from RMA-S cells (i.e., 16:0–18:1 PE) contained one monounsaturated acyl chain, we next examined whether PEs containing unsaturated acyl chains were better recognized than those containing saturated acyl chains. We first evaluated PEs containing acyl chains with a single double bond. Both di-16:1[cis] PE and di-18:1[cis] PE were recognized by the 24.8.A iNKT cell hybridoma, while PEs with saturated acyl chains of the same length (di-16:0 and di-18:0, respectively) were not (Fig. 5c). Furthermore, the introduction of a second double bond conferred greater recognition upon the PE, as shown by di-18:2[cis] PE (Fig. 5d). The introduction of a third double bond in each acyl chain, however, as in di-18:3[cis] PE, did not improve upon the recognition observed with di-18:2[cis] PE. Since the RMA-S cell-derived antigen, 16:0–18:1 PE,
contained mixed fatty acyl chains, we next investigated the reactivity of synthetic PEs with two different fatty acyl chains. Although di-16:0 PE and di-18:0 PE were not recognized, 16:0–18:1[cis] PE and 18:0–18:1[cis] PE were both well recognized, demonstrating that the presence of a single acyl chain containing one double bond can confer recognition (Fig. 5e).

Interestingly, the stereochemistry of the double bond had a great impact upon recognition by the 24.8.A iNKT cell hybridoma. We evaluated two different isomers of di-18:1 PE, di-18:1[cis] PE (cis isomer) and di-18:1[trans] PE (trans isomer). Chemically, these two forms differ only in the stereochemistry of the single double bond in the acyl chains, either cis or trans. However, structurally, these isomers take on very different forms in aqueous suspension at 37°C: di-18:1[cis] PE is in the hexagonal HII phase, while di-18:1[trans] PE is in the lamellar phase (33). We found that di-18:1[cis] PE was well recognized, while di-18:1[trans] PE was not recognized (Fig. 5f). The structures for di-18:0 PE, di-18:1[trans] PE, di-18:1[cis] PE, and di-18:2[cis] PE are shown in Fig. 5g. The 24.8.A iNKT cell hybridoma recognized structures with one or more double bonds in one or both acyl chains, but only if the double bonds were in the cis configuration.

The Phase Behavior of the Phospholipid Correlates with Its Ability to Stimulate the 24.8.A iNKT Cell Hybridoma—As the temperature is gradually raised, the macromolecular structures that phospholipids form in aqueous buffer become increasingly disordered. Each phospholipid has a characteristic temperature for the transition from the gel to the liquid crystalline state ($T_m$). In the liquid crystalline state, diacyl phospholipids like PE usually adopt two structures: the lamellar (bilayer) phase or the inverted hexagonal (HII) (nonbilayer) phase (34). For each phospholipid, there is also a characteristic temperature for the transition from the lamellar to HII phase ($T_h$). We noted an association between recognition of a given PE species and its transition temperature ($T_m$), likely as a result of the state or phase that the phospholipid assumes at a given temperature. Only species of PE with $T_m$ less than 37°C, which are likely to be in a fluid state (either lamellar or hexagonal phase depending on $T_h$), were able to activate the 24.8.A iNKT cell hybridoma in the plate-bound CD1d assay (Table I). In contrast, synthetic PEs with a $T_m$ greater than 37°C, such as di-14:0 PE, di-16:0 PE, and di-18:0 PE, did not activate the 24.8.A iNKT cell hybridoma. The clearest example of an association between antigenicity and transition temperature was observed with di-18:1[cis] PE and di-18:1[trans] PE. In physiological buffer, the $T_m$ and $T_h$ of di-18:1[cis] PE are ~16°C and
7–12 °C, respectively, while the $T_m$ and $T_b$ of di-18:1[trans] PE are 38 °C and 60–63 °C (33). Consequently, at 37 °C, the cis isomer of di-18:1 PE will be in the hexagonal phase, while the trans isomer will likely be in the gel phase.

In contrast with unsaturated PEs, PEs containing long saturated acyl chains, such as di-14:0 PE, di-16:0 PE, di-18:0 PE, and di-20:0 PE, have relatively high transition temperatures ($T_m = 50–80 °C$ and $T_b \approx 100 °C$, in water), making it probable that they will all be in the gel phase in our assay buffer at 37 °C. However, di-12:0 PE, with shorter saturated acyl chains, has a $T_m$ of 17 °C and $T_b$ of 43–44 °C in aqueous solution. Therefore, it would certainly be in the liquid crystalline phase and may even adopt the hexagonal (HII) phase in our system, as the presence of salt or Me$_2$SO can under certain circumstances decrease transition temperatures. This may explain why di-12:0 PE was reactive, while the other saturated PEs (with acyl chains >12 carbons long) were not recognized by the 24.8.A iNKT cell hybridoma. The lack of recognition of PEs with saturated acyl chains <12 carbons long (e.g. di-6:0, di-8:0, and di-10:0) (Fig. 5), which would be expected to be in the liquid crystalline phase at 37 °C, suggests that acyl chains of >10 carbons are required for recognition of saturated diacyl PEs.

**Fatty Acyl Chain Unsaturation Is Critical for Binding of the PE to mCD1d**—To address whether phospholipid recognition by the 24.8.A iNKT cell hybridoma reflected differences in the binding of lipids to mCD1d, di-18:n PEs of increasing unsaturation were evaluated in a competitive binding assay. We assessed the ability of each phospholipid to competitively inhibit the binding of mCD1d to biotinylated di-18:0 PE bound to streptavidin-coated wells. As shown in Fig. 6, both di-18:2[cis] and di-18:3[cis] PE inhibited the binding of mCD1d to biotinylated di-18:0 PE. However, di-18:3[cis] PE showed much greater competition of binding of biotinylated PE than di-18:2[cis] PE, suggesting a higher affinity of binding for this lipid to mCD1d. In contrast, neither di-18:1[cis] nor di-18:1[trans] were able to inhibit this binding. This was also the case for di-18:0 PE: and the CHCl$_3$ control. These data demonstrate that PEs with a higher degree of unsaturation (i.e. ≥ 2 double bonds) competitively inhibited the binding of biotinylated di-18:0 PE to mCD1d, while monounsaturated di-18:1[cis] PE or saturated di-18:0 did not inhibit binding in this assay.

**DISCUSSION**

Little if anything is known about the natural antigens recognized by CD1d-restricted iNKT cells. We have previously characterized the 24.8.A iNKT cell hybridoma and shown that it recognizes a polar lipid of RMA-S cells (17). In the present study, we have purified the antigen to homogeneity and identified it to be palmitoyl-oleoyl PE (16:0–18:1 PE) by mass spectrometry. Synthetic 1-palmitoyl-2-oleoyl-phosphoethanolamine (16:0–18:1 PE) was recognized by the 24.8.A iNKT cell hybridoma in a plate-bound CD1d assay, and this recognition was specifically blocked by anti-CD1d monoclonal antibodies. The 24.8.A iNKT cell hybridoma also recognized natural PEs of other cellular origins, including E. coli, mammalian tissues, and plant (soybean). The strength of recognition correlated with the degree of unsaturation of the PEs, with plant PE being the most potent activator, but all of the natural PEs were recognized by the 24.8.A iNKT cell hybridoma.

To better understand how the structure of the acyl chains can influence recognition of PE, a panel of synthetic PEs was analyzed. We focused primarily on synthetic PEs that contain 16- and 18-carbon acyl chains, since these were the chains found in the native antigen and are more commonly represented in mammalian phospholipids. We determined that di-16:1[cis] PE and di-18:1[cis] PE were recognized but not di-16:0 PE and di-18:0 PE. These findings demonstrate, first, that the 18:1[cis], and not the 16:0, acyl chain is likely responsible for recognition of 16:0–18:1[cis] PE by the 24.8.A iNKT cell hybridoma. Second, these data suggest that monounsaturated, but not saturated, forms of the 16- and 18-carbon acyl chains are recognized by the 24.8.A iNKT cell hybridoma.

To demonstrate definitively that the 24.8.A iNKT cell hybridoma shows preferential recognition of unsaturated, but not saturated, acyl chains, we then evaluated two series of synthetic PEs: 1) PEs with saturated acyl chains of different lengths (from 6 to 18 carbons) and 2) PEs with increasing degrees of unsaturation in acyl chains of one length (18 carbons). The results were striking. Of the seven saturated PEs tested, only one (di-12:0 PE) was recognized by the 24.8.A iNKT cell hybridoma. On the other hand, increasing unsaturation in the di-18 PE series resulted in strong recognition by the 24.8.A iNKT cell hybridoma, with maximal recognition of di-18:2[cis]
Fine specificity of the 24.8.A iNKT cell hybridoma.

Phospholipids (PL), or the CHCl₃ control, were diluted serially in PBS and incubated with plate-bound CD1d. All phospholipids shown are synthetic forms of PE. 24.8.A iNKT cell hybridoma cells (10⁵/well) were incubated for 18–24 h in the CD1dpolypeptide-coated wells. The data represent the mean pg/ml IL-2 detected in 24.8.A iNKT cell hybridoma supernatants from three replicate wells and are representative of two independent experiments. a, structure of synthetic 1,2-diacyl-sn-glycerol-3-phosphoethanolamines: 1) di-18:0 PE; 2) di-18:1 [trans]-PE; 3) di-18:1[cis]-PE; and 4) di-18:2[cis]-PE. b, a series of synthetic 1,2-diacyl-sn-glycerol-3-phosphoethanolamines with saturated (di-n:0) acyl chains were examined for their recognition by the 24.8.A iNKT cell hybridoma. c, synthetic PEs containing acyl chains with a single double bond (monounsaturated) were recognized significantly better than PEs with saturated acyl chains of the same length. For example, neither di-18:0 PE nor di-16:0 PE were recognized; in contrast, both di-18:1[cis]-PE and di-16:1[cis]-PE activated the 24.8.A iNKT cell hybridoma. d, the introduction of double bonds into the acyl chains modulates antigen recognition by the 24.8.A iNKT cell hybridoma. For acyl chains containing 18 carbons, the sequential introduction of double bonds conferred recognition upon the PE. In this series, di-18:0 PE was recognized best. Introduction of a third double bond (di-18:3[cis]-PE) in each acyl chain did not improve activation of the 24.8.A iNKT cell hybridoma. e, a single double bond in one of the two acyl chains is sufficient to confer recognition. Synthetic PEs containing mixed acyl chains were recognized if one chain was monounsaturated. For example, (16:0-18:1[cis]) PE and (18:0-18:1[cis]) PE were recognized, but di-16:0 PE and di-18:0 PE were not. f, the stereochemistry of the double bond affects recognition. The cis, but not the trans, isomer of di-18:1 PE was recognized by the 24.8.A iNKT cell hybridoma.

**TABLE I**

| Phospholipid | Tm | Th | Recognition by 24.8.A |
|--------------|----|----|----------------------|
| Di-12:0 PE   | 17 | 44 | Yes                  |
| Di-14:0 PE   | 50 | 123d | No                  |
| Di-16:0 PE   | 65 | 118 | No                  |
| Di-18:0 PE   | 74 | 100 | No                  |
| Di-16:1[cis] PE | ~33 | 0 | Yes                  |
| Di-18:1[cis] PE | ~16 | 10 | Yes                  |
| Di-18:1[trans] PE | 38 | 64 | No                  |
| Di-18:2[cis] PE | ~40 | ~15 | Yes                  |
| Di-18:3[cis] PE | NA* | 30 | Yes                  |
| 16:0–18:1[cis] PE | 25 | 71 | Yes                  |

* Tm is the transition temperature from the gel to fluid liquid crystalline phase.  
+ Th is the transition temperature from the lamellar to hexagonal (Hhex) phase.  
~ Recognition by the 24.8.A iNKT cell hybridoma in the plate-bound CD1d assay (based on data from Fig. 5).  
° All transition temperatures are approximate and for lipid in aqueous solution (water or physiological buffer), with the exception of di-14:0 PE, which appears to undergo transition to the hexagonal phase in aqueous solution only at high salt concentrations (e.g., 2.4 M NaCl). Transition temperatures were obtained from LIPIDAT databases (www.lipidat.chemistry.ohio-state.edu).  
* NA, not available.

**Fig. 5.** Fine specificity of the 24.8.A iNKT cell hybridoma. Phospholipids (PL), or the CHCl₃ control, were diluted serially in PBS and incubated with plate-bound CD1d. All phospholipids shown are synthetic forms of PE. 24.8.A iNKT cell hybridoma cells (10⁵/well) were incubated for 18–24 h in the CD1dphospholipid-coated wells. The data represent the mean pg/ml IL-2 detected in 24.8.A iNKT cell hybridoma supernatants from three replicate wells and are representative of two independent experiments. a, structure of synthetic 1,2-diacyl-sn-glycerol-3-phosphoethanolamines: 1) di-18:0 PE; 2) di-18:1[trans]-PE; 3) di-18:1[cis]-PE; and 4) di-18:2[cis]-PE. b, a series of synthetic 1,2-diacyl-sn-glycerol-3-phosphoethanolamines with saturated (di-n:0) acyl chains were examined for their recognition by the 24.8.A iNKT cell hybridoma. c, synthetic PEs containing acyl chains with a single double bond (monounsaturated) were recognized significantly better than PEs with saturated acyl chains of the same length. For example, neither di-18:0 PE nor di-16:0 PE were recognized; in contrast, both di-18:1[cis]-PE and di-16:1[cis]-PE activated the 24.8.A iNKT cell hybridoma. d, the introduction of double bonds into the acyl chains modulates antigen recognition by the 24.8.A iNKT cell hybridoma. For acyl chains containing 18 carbons, the sequential introduction of double bonds conferred recognition upon the PE. In this series, di-18:0 PE was recognized best. Introduction of a third double bond (di-18:3[cis]-PE) in each acyl chain did not improve activation of the 24.8.A iNKT cell hybridoma. e, a single double bond in one of the two acyl chains is sufficient to confer recognition. Synthetic PEs containing mixed acyl chains were recognized if one chain was monounsaturated. For example, (16:0-18:1[cis]) PE and (18:0-18:1[cis]) PE were recognized, but di-16:0 PE and di-18:0 PE were not. f, the stereochemistry of the double bond affects recognition. The cis, but not the trans, isomer of di-18:1 PE was recognized by the 24.8.A iNKT cell hybridoma.

**Fig. 6.** Binding specificity of mCD1d for unsaturated PE. Phospholipids (PL), or the CHCl₃ control, in Me₂SO were diluted serially in assay buffer and incubated with mCD1d. The samples were then added to biotinylated di-18:0 PE bound to streptavidin-coated plates and incubated with mCD1d. Goats were added to the plates and incubated with mCD1d. The data represent the mean OD₄₅₀ values of duplicates and are representative of two independent experiments.

The stereochemical configuration of the unsaturated acyl chain was important, however, as di-18:1[cis]-PE was recognized by the 24.8.A iNKT cell hybridoma, while di-18:1[trans]-PE was not. These two structures differ only in the configuration of the double bond at the C9 (nineth carbon) position in the acyl chains (Fig. 5a). The cis and trans configuration of double bonds and degree of unsaturation relate closely to the transition temperatures of PE (two double bonds in each acyl chain). Synthetic di-18 PEs with mixed fatty acyl chains were also recognized, as long as one acyl chain was unsaturated (e.g. 18:0–18:1[cis]). The struc-
the phospholipid. It is noteworthy that there is an association between transition temperature ($T_{m}$) and the ability of PEs to activate the 24.8.A iNKT cell hybridoma. Our findings indicate that PEs likely to be in the liquid crystalline phase at 37 °C are recognized by the 24.8.A iNKT cell hybridoma, while those in the gel phase at this temperature are not recognized. The adoption of the liquid crystalline phase (either lamellar or hexagonal [H$_{II}$]) and greater acyl chain fluidity may favor the interaction of the acyl chains with the CD1d protein, facilitating the loading of the lipid into the antigen-binding pocket. Similarly, unsaturation in the cis configuration results in looser packing of acyl chains, while the trans configuration exhibits tighter packing of acyl chains. Most eukaryotic membrane phospholipids bear unsaturations in the cis configuration, and the membranes are in the liquid crystalline (as opposed to the gel) phase at physiological temperatures. However, some bacteria produce trans isomers when subjected to certain environmental stress (e.g., heat); these isomers exhibit tighter packing of the acyl chains and higher transition temperatures (23).

To ascertain whether T cell recognition of unsaturated PE was due to better binding of unsaturated forms of PE to mCD1d, we determined the ability of unsaturated and saturated di-18:n PEs to competitively inhibit the binding of biotinylated di-18:0 PE to mCD1d. Fatty acid unsaturation was found to have an important effect on this interaction, and the ability of the PE to inhibit the binding increased with increasing fatty acyl chain unsaturation (di-18:3[cis] > di-18:2[cis] > di-18:1[cis]). These findings are consistent with our T cell recognition data. However, there are two minor discrepancies, both of which we believe can be explained. First, although both di-18:2[cis] and di-18:3[cis] PE were recognized by the 24.8.A T cell hybridoma, recognition by di-18:2[cis] PE was actually better than that of di-18:3[cis] PE. These data contrast with the finding that di-18:3[cis] PE bound to mCD1d better than di-18:2[cis] PE, and suggests that binding of the phospholipid to mCD1d may not be the only factor involved in its recognition by a T cell. Second, while di-18:1[cis] was recognized by the 24.8.A hybridoma, this phospholipid did not inhibit the binding of biotinylated di-18:0 PE to mCD1d. The latter can be explained by the differences between biotinylated and nonbiotinylated PEs. The biotinylated headgroup on the phospholipid is very reactive self-lipid antigens presented by CD1d may be responsible for the activated/memory phenotype observed for iNKT cells (41, 42). Although NKT cell activation is likely initiated by a high affinity interaction between the TCR and CD1d/lipid complex, preferential binding of certain acyl chains to CD1d may contribute to the selective displacement of endogenous self-lipids by antigenic lipids as they are loaded in the endosome (43).

Taken together, our data indicate that the effect of unsaturation on T cell recognition of PE is due to differences in the ability of PEs of differing degrees of unsaturation to bind to CD1d. Increasing fatty acid unsaturation appears to directly correlate with the ability of PE to bind to CD1d. It is not yet clear whether fatty acid unsaturation plays a similar role in the binding of the other phospholipids to mCD1d, but data from our laboratories suggest that this is likely a feature of other phospholipids. Our data are consistent with the possibility that PEs containing unsaturated acyl chains load more efficiently than PEs with saturated acyl chains, resulting in a higher affinity complex with CD1d. It is not yet clear whether this is related to a particular phase requirement, but it seems virtually certain that the antigenic lipids will be in the fluid phase.
in vivo. An alternative hypothesis is that unsaturated acyl chains may induce a conformational change in the CD1d protein that is recognized by the 24.8.A TCR. Finally, the third possibility is that phospholipids with unsaturated acyl chains bind in a different manner than saturated phospholipids, (e.g. a different portion of the phospholipid is presented to the TCR).

These considerations are important and lack of attention to acyl chain saturation may explain discrepant findings regarding CD1d-restricted T cell recognition of phospholipids in the current literature (17, 36, 44).

Acknowledgments—We thank Rebecca Subang for expert technical assistance (J. R.); Dr. Andrew Janoff for helpful discussions regarding in vivo binding in a different manner than saturated phospholipids, (e.g. the possibility is that phospholipids with unsaturated acyl chains may induce a conformational change in the CD1d protein that is recognized by the 24.8.A TCR. Finally, the third possibility is that phospholipids with unsaturated acyl chains bind in a different manner than saturated phospholipids, (e.g. a different portion of the phospholipid is presented to the TCR).

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