Immunity, Vol. 13, 255–264, August, 2000, Copyright ©2000 by Cell Press

The αβ T Cell Response to Self-Glycolipids Shows a Novel Mechanism of CD1b Loading and a Requirement for Complex Oligosaccharides

Abdijapar Shamshiev,* Alena Donda,* Theodore I. Prigozy,‡ Lucia Mori,* Vanna Chigorno,§ Chris A. Benedict,‡ Ludwig Kappos,† Sandro Sonnino,§ Mitchell Kronenberg,‡ and Gennaro De Libero*‖

*Experimental Immunology
Department of Research
University Hospital
Hebelstrasse 20
†Department of Neurology
University Hospital
Petersgraben 4
CH-4031 Basel
Switzerland
‡La Jolla Institute for Allergy and Immunology
10355 Science Center Drive
San Diego, California 92121
§Study Center for the Functional Biochemistry of Brain Lipids
Department of Medical Chemistry and Biochemistry–LITA–Segrate
Medical School, University of Milan
Via Fratelli Cervi 93
I-20090 Segrate
Italy

Summary

The structural basis for the T cell recognition of lipoglycans remains to be elucidated. We have described autoreactive T cells responsive to GM1 ganglioside presented by CD1b. We show that glycosphingolipids bind to CD1b on the cell surface at neutral pH and are recognized without internalization or processing. Furthermore, soluble GM1–CD1b complexes stimulate specific T cells. Oligosaccharide groups containing five or more sugars are required to build a minimal epitope for TCR recognition. This suggests a mechanism for T cell recognition of glycosphingolipids in which much of the CD1b-bound ligand is exposed. Binding to CD1b is a highly reversible process and other ceramide-containing glycosphingolipids displace GM1. These nonantigenic compounds act as blockers and may prevent harmful autoreactivity in vivo.

Introduction

It is now known that T lymphocytes can recognize a variety of antigens in addition to peptides, including lipids and glycolipids that are presented by nonpolymorphic CD1 molecules (Burdin and Kronenberg, 1999; Porcelli and Modlin, 1999). Humans have four CD1 proteins (CD1a–d), which are divided into two groups on the basis of their sequence homology (Calabi et al., 1989), while the mouse only has homologs of CD1d. The structure of mouse CD1 has been solved, and, despite its low sequence homology with classical MHC molecules, it strikingly resembles that of MHC class I glycoproteins (Zeng et al., 1997). As for the class I molecules, the α1 and α2 domains of mouse CD1 form two parallel α helixes and a floor containing antiparallel β strands, which together define a groove with the potential capacity to bind ligands. This antigen binding groove contains two pockets surrounded by neutral and hydrophobic amino acids (Zeng et al., 1997). These two pockets have been postulated to accommodate the lipid tails of the antigens that are presented by CD1 molecules to T cells. Molecular modeling suggests that similar characteristics are conserved in human CD1 molecules (Grant et al., 1999).

A number of lipids presented by group I and II CD1 molecules have now been characterized. Human CD1b presents bacterial lipids such as mycobacterial mycolic acids (Beckman et al., 1994), bacterial glycolipids such as lipoarabinomannan (LAM) (Sieling et al., 1995) and glucose monomycolate (GMM) (Moody et al., 1997), and self-glycosphingolipids (GSLs) such as GM1 ganglioside (Shamshiev et al., 1999). Also, the CD1a and CD1c molecules present bacterial phospholipids and lipids (Beckman et al., 1996; Rosat et al., 1999; Moody et al., 2000) and self-GSLs (our unpublished results) to T cells. The group II mouse and human CD1d molecules bind and present the sphingolipid α-galactosylceramide (α-GalCer), a compound isolated from marine sponges that stimulates NK (natural killer) T cells (Kawano et al., 1997; Brossay et al., 1998). However, α-GalCer is not present in normal mammalian cells (Yamaguchi et al., 1996), and the physiological ligand stimulating NK T cells is not identified yet.

Recently, the structures of two different complexes of a classical class I molecule with a glycopeptide have been solved (Glithero et al., 1999; Speir et al., 1999). In both cases, the peptide portion anchors the ligand to the MHC groove, with the carbohydrate available for TCR recognition. Based upon these structures, Speir et al. have proposed that the presence of no more than one or two sugars is consistent with a mode of antigen recognition similar to that carried out by αβ TCRs, in which a surface consisting of exposed portions of the ligand and α-helical amino acids on the antigen-presenting molecule are contacted by the TCR (Speir et al., 1999). A similar model may apply to the recognition of lipoglycan antigens, with the carbohydrate exposed but with lipid, rather than peptide, responsible for anchoring the ligand to the antigen-presenting molecule. This model is consistent with the small polar head groups or simple sugars present on many of the lipid antigens presented by CD1 (Porcelli and Modlin, 1999). It has been presumed that antigens such as LAM, with complex carbohydrate structures, are processed in order to be presented by CD1 molecules. Presentation of purified LAM by dendritic cells is dependent upon binding to the mannose receptor, followed by internalization and movement to a late endosomal compartment similar to MIICs, in which CD1b is also routed (Prigozy et al., 1997). Consistent with this, binding of glycolipids to CD1b was observed to occur only at acidic pH (Ernst et al., 1998),

‖To whom correspondence should be addressed (e-mail: Gennaro.DeLibero@unibas.ch).

Table 1. List of Antigens

	Antigen	Structure[a]
1	GM1	β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
2	Asialo–GM1	β-Gal-(1-3)-β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
3	GM2	β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
4	Asialo–GM2	β-GalNAc-(1-4)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
5	GM3	α-Neu5Ac-(2-3)-β-Gal-(1-4)-β-Glc-(1-1)-Cer
6	Lactosylceramide	β-Gal-(1-4)-β-Glc-(1-1)-Cer
7	Glucosylceramide	β-Glc-(1-1)-Cer
8	Deacylated GM1	β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc
9	GM1–acetyl	GM1 containing acetyl group as acyl moiety
10	Lyso–GM1	GM1 lacking side acyl chain
11	GM1–C24	Contains C24 instead of C18 acyl chain
12	GM1–sphinganine	Single bond C4–C5 instead of double bond of sphingosine
13	GM1–Neu5Gc	GM1 containing Neu5Gc
14	GM1–alcohol	Sialic acid contains CH_2OH instead of COOH
15	GalNAc–GD1a	β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
16	GD1a	α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
17	GD1b	β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
18	GT1b	α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer
19	GQ1b	α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)-β-Gal-(1-3)-β-GalNAc-(1-4)-[α-Neu5Ac-(2-8)-α-Neu5Ac-(2-3)]-β-Gal-(1-4)-β-Glc-(1-1)-Cer

[a] Ganglioside nomenclature is in accordance with IUPAC-IUB recommendations (IUPAC, 1977): Cer, ceramide; Neu5Ac, N-acetylneuraminic acid; Neu5Gc, N-glycolylneuraminic acid.

although direct evidence for processing of lipoglycans is lacking.

Several important questions regarding the presentation of lipid antigens by CD1 molecules remain unanswered. First, while the results from several studies suggest that most of the glycolipid–CD1b complexes expressed by APC are formed intracellularly, predominantly in late endosomes, it remains to be determined if antigen binding to CD1b can occur elsewhere. Second, the stability of lipid antigen-CD1 complexes on APC has not been assessed in any experimental system. Finally, the ability of the TCR to discriminate between different carbohydrate structures of a natural antigen has not been investigated extensively.

In this study, we have addressed these questions through the analysis of the CD1b-mediated presentation of gangliosides. Because of the autoreactivity of these cells and their increased frequency in multiple sclerosis patients (Shamshiev et al., 1999), this response may be relevant for the pathogenesis of multiple sclerosis. Furthermore, the great variety of gangliosides available permits a more detailed analysis of structure function relationships than has been carried out previously, particularly regarding the recognition of different carbohydrate structures. Here we show that the recognition of these self-antigens can occur without antigen processing and requires the presence of complex oligosaccharides. Additionally, the mechanism for association of antigen with CD1b is strikingly different from that found previously for microbial lipoglycans.

Results

Acyl Chains Influence Presentation of GM1 to T Cells

The antigenicity of a series of gangliosides with identical carbohydrate moieties, but with different acyl chains, was tested by using two T cell clones (Shamshiev et al., 1999), which provided similar results throughout these studies. The structures of these compounds are reported in Table 1. The lipid portion of the lipoglycan antigens is very likely to be buried in the CD1b groove, with the carbohydrate part exposed for TCR recognition. Therefore, differences in antigenicity between compounds most likely reflect also differences in the ability of a particular antigen to bind in a stable fashion and in the correct orientation to CD1b. The solubility and critical micelle concentration also may affect the immune response to different compounds, by altering the availability of the acyl chains for CD1b binding. This will be true for studies of any lipid antigens. There are two consequences of this fact. First, as a result of micelle formation, only a fraction of the GSLs are free as monomers in culture and therefore the concentrations required for T cell activation are probably overestimated. Second, caution must be exercised in concluding that a glycosphingolipid has less activity than the reference compound, GM1, especially if it has a more hydrophobic character.

As expected, deacylated GM1 (compound #8 in Table 1), which is devoid of the ceramide, was not stimulatory (Figure 1A). Lyso–GM1 (#10 in Table 1), a compound with only the sphingosine tail, and GM1–acetyl (#9), with an acetyl group substituting for the long fatty acid typical of ceramide, also were unable to stimulate GM1-reactive T cell clones (Figure 1A). Therefore, both the sphingosine and the acyl chain are important for antigenicity of the gangliosides, likely because they are required for stable anchoring of GM1 to CD1b. The length of the fatty acid present in the ceramide also may be important. Indeed, GM1 with stearic fatty acid (containing 18 C atoms) was more active than a ganglioside containing lignoceric acid (with 24 carbon atoms, #11) (Figure 1B), although as noted above, we cannot exclude the possibility that differences in solubility or availability are responsible for this difference. GM1 with sphinganine, which contains only saturated C–C bonds (#12), was less active than sphingosine-containing GM1, which has a double bond between the fourth and fifth carbons (Figure 1B). Sphinganine is less rigid than sphingosine, suggesting that the rigidity of one of the lipid tails also may influence the presentation efficiency, perhaps by facilitating the

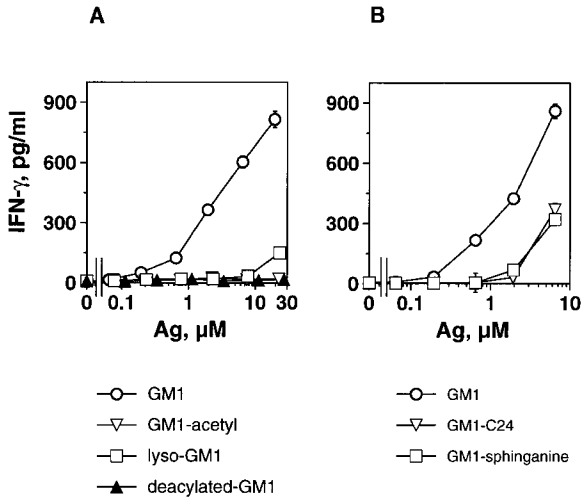

Figure 1. The Structure of GM1 Acyl Chains Influences Presentation to T Cells

(A) GM1 containing only one hydrophobic acyl chain and deacylated GM1 are not antigenic.

(B) Intact GM1 is optimal for activation of GM1-specific T cells. Sphinganine- and C24 acyl-containing GM1 are less active than natural GM1.

Dendritic cells were incubated with the indicated doses of different GSLs before addition of T cells. Released IFNγ is shown ± SD. Data are representative of the response of two T cell clones each tested three times with all compounds. Each time release of TNFα was also assessed and showed similar results (data not shown).

proper accommodation of antigen in the CD1b binding pocket.

In conclusion, stringent constraints in the ceramide structure control the generation of immunogenic GM1–CD1b complexes, the most important being the presence of two lipid tails, which presumably anchor GM1 to the antigen-presenting molecule.

GM1 Associates with CD1b on the Cell Surface without Being Internalized

The kinetics of the generation of GM1–CD1b complexes was studied in T cell activation experiments, with APC pulsed for different times with GM1. These kinetics are very fast, as APC pulsed for only 2 min are capable of some T cell activation, while 1 hr of pulsing led to near maximal T cell stimulation (data not shown). This fast binding and presentation suggested the possibility that GM1 is not internalized into APC before binding to CD1b. To test this hypothesis, we investigated the presentation capacity of cells expressing mutated CD1b molecules that are not targeted efficiently to endosomes. Previous studies have demonstrated that mutation of the tyrosine in the YXXZ motifs of the cytoplasmic tails of CD1b and CD1d results in defective endosomal targeting and accumulation of molecules on the plasma membrane (Sugita et al., 1996; Brossay et al., 1997; Jackman et al., 1998; Tangri et al., 1998). We therefore generated stable cell lines expressing either wild-type CD1b (CD1b wt), or a mutated CD1b having YQ-AA substitutions at positions 311 and 312 in the CD1b cytoplasmic tail (CD1b YQ-AA). Confocal microscopy was used to colocalize CD1b wt and CD1b YQ-AA with the lysosome-associated membrane protein 1 (LAMP1), a marker for late endosomes/lysosomes. In HeLa cells, it is clearly evident that the YQ-AA mutation leads to increased levels of CD1b molecules on the plasma membrane (Figure 2B) relative to the wild type (Figure 2A). Surprisingly, in the majority of the cells analyzed, a significant number of intracellular vesicles were labeled by the anti-CD1b antibodies (Figure 2B), although many of these do not colocalize with LAMP1. This result is similar to that obtained with a tyrosine to alanine substitution mutant of CD1b expressed in HeLa cells (Jackman et al., 1998). Because some features of membrane protein internalization might not be normal in HeLa cells and because these cells of epithelial origin are not professional APC that would normally express CD1b, we also tested CD1b wt and CD1b YQ-AA-transduced THP.1 cells. The alteration in the steady-state distribution of CD1b was much more dramatic in these cells (Figures 2C and 2D). Intracellular CD1b labeling in the THP.1 CD1b YQ-AA-transduced cell lines was almost completely absent (Figure 2D). In the CD1b wt THP.1 cell line, however, there were many vesicular structures labeled with the anti-CD1b antibodies (Figure 2C).

Transduced cell lines were sorted to select for cells expressing similar surface levels of CD1b protein and tested in antigen presentation assays. Interestingly, CD1b YQ-AA-transduced HeLa cells presented GM1 as efficiently as APC transduced with wild-type CD1b (Figure 3A). Similar results were obtained with THP.1 cells transduced with the same wild-type and mutant CD1b gene constructs (Figure 3B). These data indicate that GM1 can associate with CD1b without being internalized.

This hypothesis was further tested by using dendritic cells pulsed with GM1 in different culture conditions that should inhibit endosomal uptake and/or processing of antigens. These conditions include: (1) antigen pulse for 2 hr at 18°C, a temperature that does not allow recycling of membrane proteins (Figure 3C); (2) culture in the presence of NH_4Cl or chloroquine, which prevent acidification of intracellular organelles (Figure 3C); or (3) culture in the presence of chlorpromazine and monodansylcadaverine, which inhibit recycling of membrane proteins (data not shown). All these treatments did not alter the GM1 antigen presentation capacity of the dendritic cells, while they affected presentation of PPD by the same APC to MHC class II–restricted and PPD-specific T cells (data not shown). Finally, dendritic cells were first fixed with glutaraldehyde and then pulsed either with GM1 or the control antigen PPD before testing in antigen presentation assays. Fixed dendritic cells efficiently presented GM1 (Figure 3D), while they did not stimulate HLA-DR-restricted PPD-specific T cells (data not shown). Furthermore, the kinetics of GM1–CD1b complex formation are comparable in living and fixed APC (data not shown), indicating that the association of GM1 with CD1b is independent of membrane fluidity and surface protein mobility. We also could exclude the possible involvement of carbohydrate-specific receptors in the uptake of GM1. Indeed, galactose, lactose, N-acetylgalactosamine, and mannan, which bind to surface lectins and block internalization of glycolipids (Prigozy et al., 1997), did not inhibit GM1 presentation (data not shown).

Intact GM1 Is Recognized without Being Processed

An important issue is whether GM1 associates as an intact molecule with CD1b, or instead if it is partially

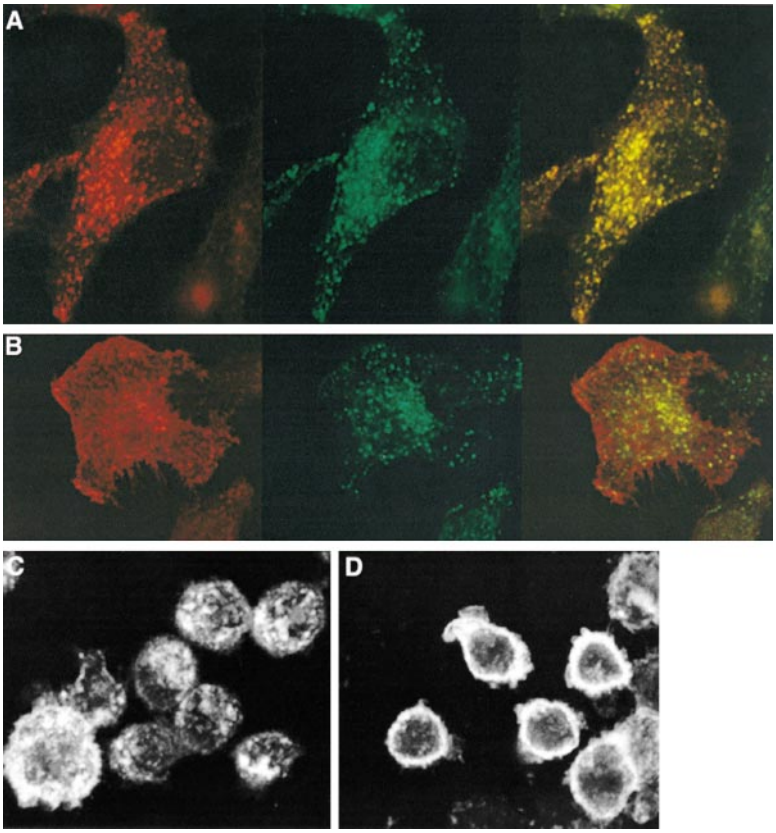

Figure 2. Altered Intracellular Distribution of CD1b in Cytoplasmic Tail Mutants

Immunofluorescently labeled cells were analyzed by confocal microscopy. HeLa–CD1b wt (A) and HeLa–CD1b YQ-AA (B) –transduced cell lines were labeled with anti-CD1b mAb (red, left panels) and anti-LAMP1 mAb (green, middle panels). The right panels display the overlay of red and green channels with the colocalization depicted by yellow signal. The CD1b-FITC labeling of THP.1 cells is shown for THP.1–CD1b wt (C) and THP.1–CD1b YQ-AA (D). The images were collected using the 60× objective with a 2.46 zoom.

degraded before binding to CD1b. As fixed APC efficiently present GM1, intracellular processing is not required. However, processing could occur on the cell surface through the action of enzymes present in the serum. This possibility was tested with two approaches. First, APC were fixed and then pulsed in serum-free conditions to avoid potential GM1 processing by serum components. In these conditions, efficient presentation

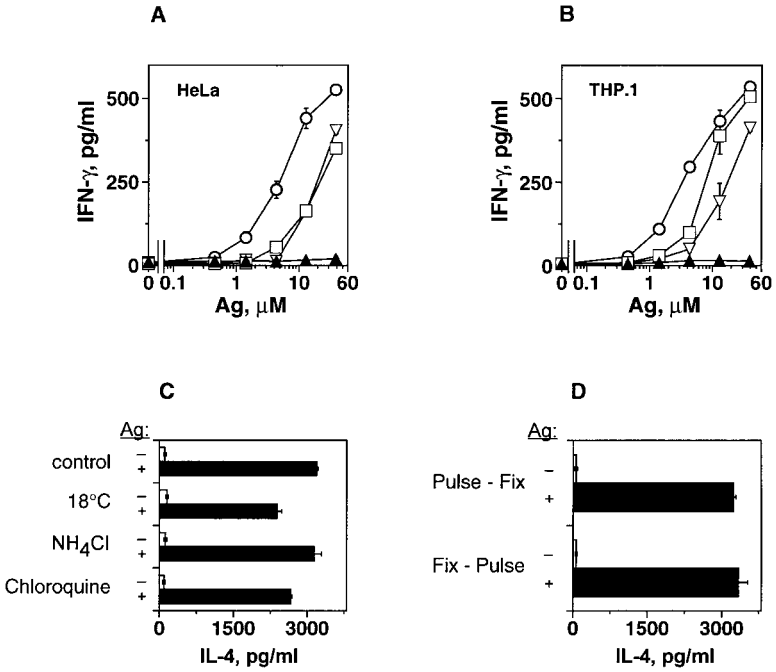

Figure 3. GM1 Internalization and Endosomal Acidification Are Not Required for Activation of GM1-Specific T Cells

(A and B) HeLa cells (A) or THP.1 cells (B) expressing CD1b wild-type (inverted open triangles), YQ-AA mutant (open squares), or mock transfected controls (closed triangle) were incubated with GM1 before addition of T cells. Dendritic cells were used as positive control APC (open circle). Figures are representative of four experiments with two T cell clones. Similar results were obtained by measuring release of TNFα (data not shown). Both wild-type and mutant CD1b transfectants expressed similar levels of CD1b on cell surface.

(C) Dendritic cells were incubated at 37°C in the presence of NH_4Cl or chloroquine or at 18°C for 1 hr and then pulsed with GM1 (10 μM). After extensive washing, these dendritic cells were used to stimulate T cells.

(D) Dendritic cells were pulsed with GM1 (10 μM) at 37°C (pH 7.0) for 2 hr before or after fixation, and after extensive washing they were used to stimulate T cells.

(C and D) Similar results were obtained by measuring release of IFNγ and TNFα. Results are representative of three separate experiments for each condition performed with two T cell clones.

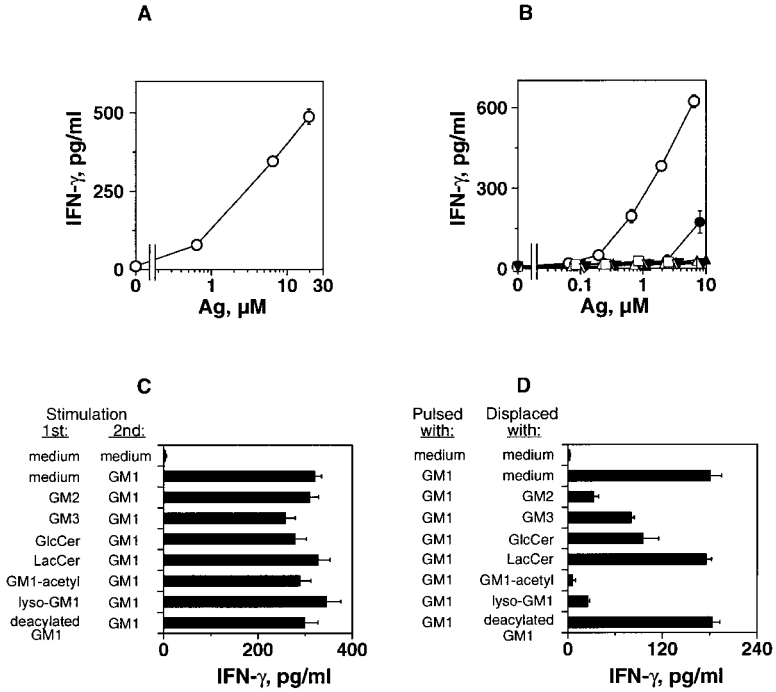

Figure 4. GM1 Does Not Require Processing for Presentation

(A) Fixed dendritic cells were pulsed in serum-free medium with indicated doses of GM1 (open circle) for 2 hr and then used to stimulate GM1-specific T cells.

(B) Dendritic cells were incubated for 2 hr with GM1 (open circle), asialo-GM1 (closed circle), GM2 (open triangle), asialo-GM2 (closed triangle), GM3 (open square), lactosylceramide (LacCer, inverted open triangle), or glucosylceramide (GlcCer, inverted closed triangle) before addition of T cells. Similar results were obtained with TNFα ELISA (data not shown).

(C) Stimulation with nonantigenic GSLs does not induce a state of T cell unresponsiveness. T cells were first incubated with APC pulsed with indicated nonstimulatory GSLs (40 μM) and after 6 hr with GM1-pulsed (20 μM) APC.

(D) GM1 is displaced by other glycolipids. Dendritic cells were fixed, then pulsed with GM1 (4 μM), extensively washed, and incubated with 40 μM of GM2, GM3, LacCer, GlcCer, or deacylated GM1. After 2 hr, dendritic cells were extensively washed and used to stimulate T cells. Results are representative of four experiments with two T cell clones.

of GM1 also did occur (Figure 4A). Second, we tested a set of gangliosides with identical ceramide tails but having carbohydrate head groups smaller than GM1. These compounds represent all the possible products of the putative processing of GM1 carbohydrates, including asialo-GM1, GM2, asialo-GM2, GM3 (Table 1, #2–#5), lactosylceramide (#6), and glucosylceramide (#7). None of these compounds stimulated either of the two GM1-specific T cell clones (Figure 4B). This effect is not the result of anergic state induced in T cells by small GSLs. Indeed, when T cells were first stimulated with APC pulsed with small GSLs and after 6 hr with GM1-pulsed APC, they were fully reactive as control cells (Figure 4C). Taken together, these findings indicate that GM1 is the minimal structure recognized by these T cells and that the terminal galactose IV, N-acetylgalactosamine, and lateral sialic acid all contribute to the GM1 stimulatory epitope.

Reversible Binding of Gangliosides to CD1b

To assess whether nonstimulatory GSLs smaller than GM1 bind to CD1b, two types of displacement assays were established. In the first assay, a soluble recombinant CD1b refolded in the presence of GM1 was used. The appropriate refolding of the molecule was confirmed by its capacity to activate only GM1-specific and not T cells with irrelevant specificities (Figures 5A and 5B). The soluble molecule was used to test displacement of GM1 by GM2. ^{3}H-labeled GM1 bound to soluble CD1b was displaced by cold GM2 (Figure 5C). In addition, binding of ^{3}H-labeled GM2 was detected after displacement of cold GM1 (Figure 5D). These experiments demonstrate that GM1 and GM2 gangliosides bind to CD1b and are readily substituted, although GM2 does not stimulate the GM1-reactive T cell clones.

To extend this information to the other GSLs, we performed a second displacement assay in which fixed APC were loaded with GM1, washed, and then pulsed with a 10-fold excess of the nonstimulatory small gangliosides before culture with T cells. Using these doses, no alteration of T cell viability was observed, thus excluding the possibility that these compounds exert toxic or detergent-like effects. Deacylated GM1, which was used as negative control (Figure 1A), did not displace GM1, while all tested compounds were capable of inhibiting the GM1-specific response, although with different efficiencies (Figure 4D). One exception was lactosylceramide, which never stimulated the T cell clones, and neither did it displace GM1, even after careful solubilization of two preparations from different sources. A possible explanation for these findings is that both lactosylceramide preparations contained large fractions (~75%–85%) of C22–24 acyl chains, which, as described above (Figure 1B), is not optimal for T cell stimulation. Thus, it is likely that, with the exception of lactosylceramide, all tested gangliosides bind to CD1b and displace GM1. The lack of antigenicity therefore most likely is determined by the inability of their carbohydrates to stimulate the TCR of the tested clones.

The results from the GM1 displacement experiments suggest that GM1 is in binding equilibrium with CD1b and that the binding can be reversed in the presence of competitors. We used the displacement assay to determine if analogs with one lipid tail, which are not antigenic (Figure 1A), also are capable of binding to CD1b. Lyso–GM1 and GM1–acetyl, two GSLs containing only one acyl chain, completely displaced GM1 from CD1b on cell surface, while deacylated GM1 did not displace GM1 (Figure 4D). Cell viability also was not impaired under these experimental conditions. Furthermore, ^{3}H-labeled GM1–acetyl displaced cold GM1 and bound to recombinant soluble CD1b (Figure 5E). Therefore, while GSLs with two acyl chains form immunogenic complexes with CD1b, GSLs with one acyl chain displace gangliosides, bind to CD1b, but are not stimulatory for the tested T cell clones.

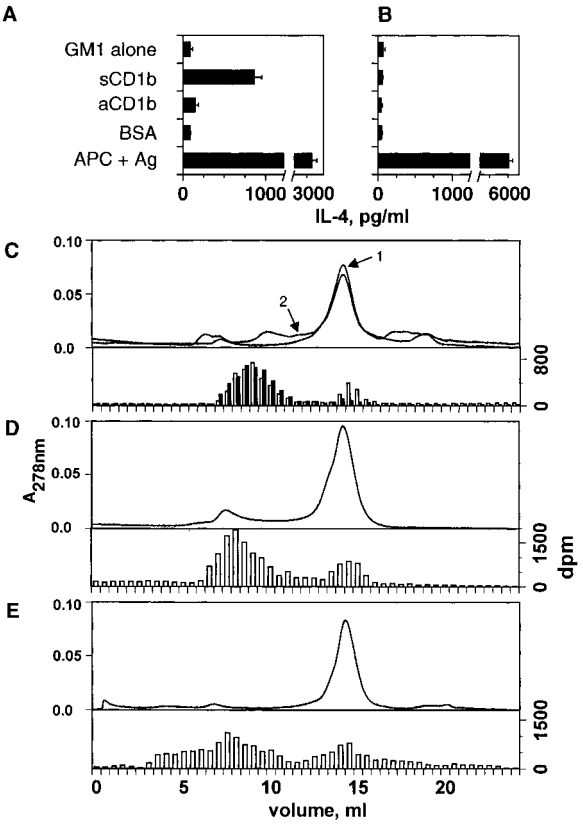

Figure 5. GM1 Bound to Soluble CD1b Stimulates T Cells and Is Displaced by Other GSLs

sCD1b refolded in the presence of GM1 stimulated GM1-specific T cells (A) and not a NK T cell clone recognizing CD1d-α-GalCer complexes (B). Aggregated denatured soluble CD1b (aCD1b), BSA, and GM1 were used as negative control. Similar results were obtained by measuring released TNFα. (C) ^{3}H-labeled GM1 bound to soluble CD1b is displaced by cold GM2. White and black bars indicate radioactivity associated with sCD1b before and after displacement, respectively. Curves 1 and 2 show sCD1b elution profiles before and after displacement, respectively. (D) ^{3}H-labeled GM2 and (E) ^{3}H-labeled GM1–acetyl bound to sCD1b after displacement of cold GM1. ^{3}H-labeled GSLs eluting between 3–13 ml represent micelles not binding to CD1b. Results are representative of two separate experiments for each condition.

GM1-Specific T Cells Cross-React with a Variety of Self-GSLs with Complex Oligosaccharides

As the oligosaccharidic portion of GM1 is the minimal structure required for forming the epitope recognized by the T cell clones, we asked whether gangliosides with hydrophilic head groups even bigger than GM1 also can be recognized without processing. We tested GD1a (Table 1, #16), GD1b (#17), GT1b (#18), and GQ1b (#19), which all differ from GM1 by the presence of additional sialic acid residues added to the external galactose IV or to galactose II. GT1b, GD1b, and GD1a were slightly more stimulatory than GM1, while GQ1b was as active as GM1 (Figure 6A). These gangliosides were also efficiently presented by fixed APC pulsed in the absence of serum (Figure 6B), thereby excluding the possibility that intracellular or serum-dependent processing is required for their recognition. We conclude that the additional sialic acid residues, generating gangliosides with up to eight carbohydrates, do not prevent the CD1b-mediated interaction of these intact compounds with the TCR.

To further define the epitope stimulating the TCR of the tested cells, the responses to three additional GM1 analogs, including GM1–alcohol (#14), GalNAc–GD1a (#15), and GM1–Neu5Gc (#13) were tested. All these gangliosides have single monosaccharides or chemical groups added to the GM1 stimulatory core epitope. GM1–alcohol and GalNAc–GD1a were more stimulatory than GM1, while GM1–Neu5Gc was as stimulatory as GM1 (Figure 6C). As with the other GSLs, the same results were observed using APC pulsed after fixation and in the absence of serum (Figure 6D), showing that these active ligands also are not subjected to intracellular or serum-dependent processing. These data exclude an important role in T cell interaction of the carboxylic and methyl groups present on sialic acid as well as of the hydroxyls in C3 and C4 in the terminal galactose IV.

Discussion

We have carried out a detailed characterization of the CD1b-mediated recognition of self-GSLs. The recognition of gangliosides by T lymphocytes may have implications for the pathogenesis of autoimmune diseases such as multiple sclerosis (Shamshiev et al., 1999). Additionally, the availability of a large set of purified and synthetic gangliosides has permitted us to carry out a more precise analysis of the structural basis for lipid antigen recognition than has been possible previously. Three main novel features of the CD1b-mediated recognition of self-GSLs have been described in this report. First, we have found that several self-GSLs can readily bind to CD1b molecules on the cell surface at neutral pH, and that glycolipid–CD1b complexes formed in this way can stimulate autoreactive T cells. There is no evidence that internalization into APC augments the T cell response to GM1 or related compounds. Furthermore, soluble GM1–CD1b complexes are formed at physiological pH in the absence of other proteins, and these complexes stimulate antigen-specific T cells. These data, together with the evidence from assays carried out using APC pulsed in serum-free medium suggest that self-GSLs can bind to CD1b directly without a need for chaperones and/or specific capture proteins. These properties are in stark contrast to those described for glycolipids of bacterial origin. Indeed, presentation of LAM, GMM, and mycolic acids by CD1b occurs only after internalization (Sieling et al., 1995; Jackman et al., 1998), which for LAM requires uptake by the mannose receptor (Prigozy et al., 1997) and transport to acidified endosomes where the encounter with CD1b and antigen processing may occur. Furthermore, microbial glycolipid binding to soluble CD1b molecules derived from insect cells occurred efficiently only at acidic pH (Ernst et al., 1998).

The predominant localization of CD1b to late endosomes, in contrast to the localization of CD1a to recycling endosomes, has led recently to the hypothesis that different CD1 molecules are important for the surveillance of different intracellular compartments (Sugita et al., 1999). While this is likely to be true, our data show that the binding of some lipid antigens to CD1b does not require acidic pH, and also, that CD1b molecules are more versatile than previously expected, as they can

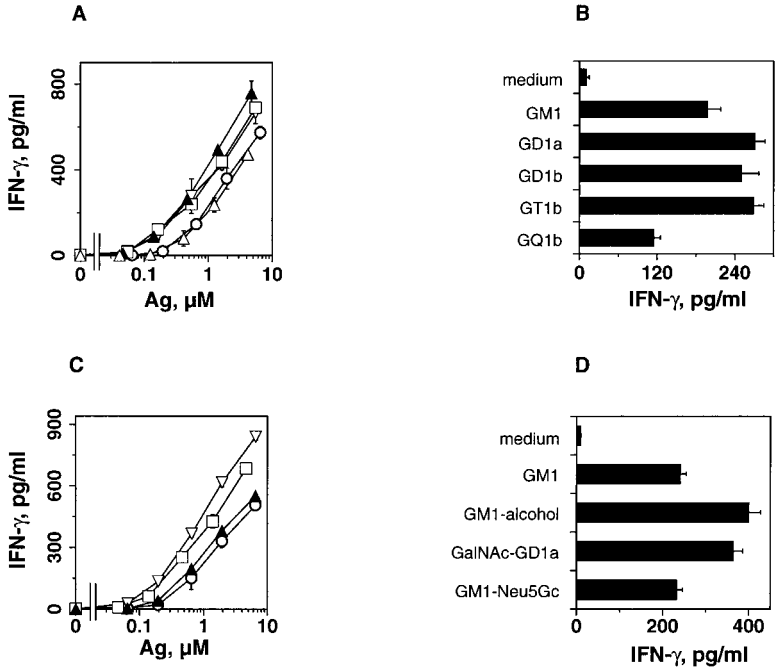

Figure 6. GM1-Specific T Cells Cross-React with GD1a, GD1b, GT1b, GQ1b, GM1–Alcohol, GalNAc–GD1a, and GM1–Neu5Gc

(A) Dendritic cells were pulsed with GM1 (open circle), GD1a (inverted open triangle), GD1b (open square), GT1b (closed triangle), or GQ1b (open triangle) before addition of T cells or (C) with GM1 (open circle), GM1–alcohol (inverted open triangle), GalNAc–GD1a (open square), and GM1–Neu5Gc (closed triangle). (B and D) Dendritic cells were fixed, then pulsed with indicated gangliosides (10 μM) for 2 hr at 37°C in serum-free medium, washed extensively, and plated to stimulate T cells. Data are representative of the response of two T cell clones each tested three times with all compounds. Each time release of TNFα was also assessed and showed similar results (data not shown).

acquire antigens on the cell surface as well as in late endosomes.

Our studies also show how the structure of the lipid tails may influence CD1b binding and presentation. In addition to the number of lipid tails, the length of the fatty acid is important, with the GM1 compound with stearic acid having the highest T cell stimulatory capacity. In the GMM model, by contrast, the natural GMM from mycobacteria containing C80 mycolic acid, or synthetic GMM containing C32 mycolic acid, both activate T cells (Moody et al., 1997). The C80 compound is too long to fit into the CD1b groove, and partial degradation of the chain after uptake into APC might occur. An additional requirement for proper structure of one lipid chain is suggested by the results obtained with the GM1 analog containing sphinganine instead of sphingosine. The double bond present in the sphingosine base might confer a more favorable conformation to the ceramide, likely facilitating anchoring to CD1b.

The second novel finding that we report is that self-GSLs with relatively complex oligosaccharides, including those with up to eight sugars, are presented without being processed. In fact, the minimal epitope required for antigen recognition by the clones analyzed in this study is the GM1 pentasaccharide, with the external galactose IV required. The core epitope is not present in gangliosides smaller than GM1, which indeed are not stimulatory, although they bind to CD1b. Instead, the same epitope is present in other GSLs that are bigger than GM1 and that stimulate the two T cell clones used in this study. This is in agreement with the known GM1 and GD1a conformations, detected by NMR, which show a high degree of three-dimensional similarity in both molecules (Acquotti et al., 1994). Furthermore, similar conformations are also suggested by the structure models of these gangliosides (our unpublished data).

Given all these similarities in the structure relevant for T cell activation, the important question remains as to how the TCR of the reactive clones interacts with ganglioside–CD1b complexes. It is most likely that the lipid tails of the ceramide are inserted into the CD1b hydrophobic pockets (Zeng et al., 1997) with much of the presented ganglioside antigen exposed above the CD1b groove. In light of this, there are several possible models as to how the TCR interaction occurs. First, specificity could result because the TCR recognizes mostly carbohydrate, with relatively little contact with the CD1b α helices. We consider this unlikely, because the TCRs of the same T cell clones used in this study require cognate interaction with CD1b, as shown by mutagenesis of residues in the CD1b α helices that point toward the TCR (A. Melian et al., submitted). Second, the TCR CDR3 loops might form a large cavity that can accommodate the relatively complex carbohydrate part of the gangliosides. Third, the ganglioside sugars might assume a lateral conformation perpendicular to the CD1 α helices, thus making difficult the interaction with the CDR3 loops. An additional possibility is that the complex oligosaccharide present in GM1 and other stimulatory gangliosides protrudes out of the CD1b binding groove. This model is similar to TCR recognition of long peptides that bind to MHC class II molecules and that may extend beyond the groove (Stern et al., 1994) and might explain the conserved contact of TCR with monosaccharides in the GM1 core epitope present in all stimulatory gangliosides.

Whatever the mechanism, a remarkable observation from our experimental data is that the same TCR recognizes carbohydrate epitopes shared by a variety of self-GSLs. The GM1 epitope recognized by the autoreactive T cells is also present in bacterial carbohydrates (Prendergast et al., 1998), suggesting a possible cross-reactivity between self- and microbial glycolipid antigens. Based upon these considerations, we propose that antigen mimicry, which is a potentially important mechanism for autoimmune disease pathogenesis (Albert and Inman, 1999), also may apply to the CD1-mediated recognition of glycolipid antigens.

The third important finding is that the interaction of the hydrophobic acyl chains of ceramide with CD1b is

a very dynamic or highly reversible process, in contrast to the more stable complexes that characterize the binding of peptides to MHC class I and class II molecules. This suggests that, compared to peptides, a relatively high concentration of self-GSL antigen may be required for T cell stimulation. However, the significant precursor frequency of self-GSL-reactive T cells in normal donors and its increase in multiple sclerosis patients (Shamshiev et al., 1999) suggest that this concentration of antigen is likely to occur in vivo. Interestingly, a variety of nonantigenic glycolipids, containing either one or two acyl chains, can displace GM1 molecules previously bound to CD1b. This implies that the groove of CD1b can be easily accessed by exogenous glycolipids, even when GM1 is already bound to CD1b, and also that GM1 analogs with only one acyl chain interact with CD1b. This single chain interaction does not generate TCR-stimulatory complexes because it is not stable enough or, alternatively, because the presence of only one acyl chain prevents the appropriate positioning of the carbohydrate core epitope. Regardless of the mechanism, presentation by CD1 molecules could be subject to fine regulation by the levels of circulating ligands. It remains to be investigated whether this particular regulatory system, carried out by competing self-compounds, is adequate to displace self-GSLs, thereby preventing presentation of self-antigens, but not microbial ligands.

Experimental Procedures

Antigens

GSLs were purchased from different companies or purified as reported. In some cases, the same GSLs obtained from different sources were tested to exclude the presence of active contaminants. From Matreya Inc. (Pleasant Gap, PA), we purchased the following GSLs purified from bovine or porcine brain: GM1 (#1061), asialo-GM1 (#1064), GM2 (#1502), GM3 (#1503), GD1a (#1062), GD1b (#1501), GT1b (#1063), GQ1b (#1516), lyso–GM1 (#1518), lactosylceramide (#1507 and #1500, with ~84% and ~75%, respectively, of C20–24 acyl chains). The following purified gangliosides purified from bovine brain were purchased from Sigma (Buchs, Switzerland): GM1 (#G7641), asialo-GM1 (#G3018), GM2 (#G8397), and asialo-GM2 (#G9398). GM1, GM2, and asialo-GM2 were >95% pure, and asialo-GM1 was >98% pure. Glucosylceramide (#49108, >98% pure, from bovine brain) was purchased from Fluka (Buchs, Switzerland). The following gangliosides were purified from bovine brain and in some cases modified in our laboratory (S. Sonnino): GD1a and GalNAc–GD1a (Acquotti et al., 1994), GM1–Neu5Gc (containing N-glycolylneuraminic acid instead of N-acetylneuraminic acid) (Sonnino et al., 1988), deacylated-GM1 (GM1 without the lipid tails) (Wiegandt and Bucking, 1970), GM1–sphinganine (GM1 with sphinganine instead of sphingosine) (Sonnino et al., 1985), GM1–alcohol (sialic acid with CH_2OH instead of COOH) (Li et al., 1984). Semisynthetic GM1–acetyl and GM1–C24 (containing an acetyl group and a lignoceric acid as acyl moiety, respectively) were prepared as described (Sonnino et al., 1985, 1990). ^{3}H-labeled GM1, GM2, and lyso–GM1 were prepared as described (Sonnino et al., 1996). GM1 and other GSLs were 98%–99% pure according to TLC analysis. The critical micellar concentrations of GM1, GM2, GM3, GD1a, GD1b, GT1b, and GQ1b are similar (Sonnino et al., 1994) and therefore are not critical parameters responsible for the different results we obtained with these compounds.

T Cell Clones and Antigen-Presenting Cells

Ganglioside GM1-specific and CD1b-restricted T cell clones GG33A and GG123 were used in all experiments (Shamshiev et al., 1999). Dendritic cells were used as APC in most experiments and were derived from peripheral blood mononuclear cells as described (Porcelli et al., 1992). Before each experiment, the percentage of dendritic cells was monitored by immunofluorescence analysis using mAbs specific for CD1b (WM-25, Immunokontakt, Lugano, Switzerland), CD1a (OKT6, ATCC CRL8019), and CD1c (L161, Instrumentation Laboratory, Schlieren, Switzerland). APC were always >90% positive for these markers. Immunofluorescence was performed as described (Shamshiev et al., 1999) using a FACScan flow cytometer (Becton Dickinson).

Plasmids, Mutagenesis, and Generation of Stable CD1b Cell Lines

The CD1b wild-type (wt) and CD1b YQ-AA coding regions were generated by PCR amplification using a common 5′ oligonucleotide primer, and two different 3′ primers. In the mutant, cytoplasmic tyrosine (Y)-311 and glutamine (Q)-312 were replaced with alanines (AA). The PCR products were subcloned into the pCR3.1 vector (Clontech, San Diego, CA) and sequenced. PmeI-restricted inserts were subcloned into the SnaBI site of the pBabe-puro retroviral expression plasmid (Morgenstern and Land, 1990). Stable cell lines expressing CD1b wt and CD1b YQ-AA were generated by retroviral transduction as described (Soneoka et al., 1995). The pBabe-puro CD1b wt or YQ-AA plasmids were cotransfected into 293T cells with pHit60 (gag-pol expression plasmid) and pCG (VSV-g expression plasmid) by calcium phosphate–DNA coprecipitation. The transfected cells were shocked for 12 hr with sodium butyrate followed by the addition of fresh media for 16 hr. The virus-containing supernatants were filtered through 0.45 μm filters and retroviruses titered by transducing HeLa cells and drug selecting with puromycin. To generate stable cell lines expressing CD1b wt and CD1b YQ-AA, HeLa and THP.1 cells were seeded in 24-well plates at 2×10^5 cells/well. High-level expression was generated by transducing cells in the presence of polybrene (4 μg/ml) with 1 ml of retrovirus three times in succession. After selection, the high-expressing cells were selected by fluorescence-activated cell sorting using the WM25 anti-CD1b mAb.

Immunofluorescent Labeling and Confocal Imaging

For immunofluorescent labeling, cells were seeded in 8-well permanox chamber slides at 2×10^5 cells/well and 1×10^5 cells/well for THP.1- and HeLa-transduced cell lines, respectively. After culturing overnight, the cells were rinsed with PBS and fixed with 4% formaldehyde in PBS. After quenching with 0.2 M glycine, the cells were permeabilized with 0.1% saponin for 8 min. The cells were incubated in 10% donkey serum for 20 min prior to the addition of the anti-CD1b mAbs. Double labeling was performed by incubating with goat-anti mouse Fab (Jackson Immunoresearch, West Grove, PA) as previously described (Prigozy et al. 1997). LAMP1 molecules were detected with a biotinylated anti-LAMP1 mAb (PharMingen, San Diego, CA) followed by FITC-conjugated streptavidin. Cells were analyzed using a Nikon microscope 60× objective and an MRC 1024 ES confocal system (Bio-Rad, London, UK). A minimum of 16 0.5 μm optical z sections were collected and vertically projected using the Lasersharp image processing software. For double labelings, the colocalization was depicted by yellow signal.

Antigen Presentation Assays

Dendritic cells, CD1b wild-type, CD1b YQ-AA, or mock transfected HeLa and THP.1 cells (6×10^4/well in triplicate), in RPMI-1640 medium containing 10% FCS, were preincubated for 2 hr at 37°C with sonicated antigen (0.01–30 μM) before addition of T cells (6×10^4/well). Supernatants were harvested after 48 hr, and cytokine levels were measured by ELISA.

Fixation of APCs was performed using 0.05% glutaraldehyde (Fluka) for 30 s at 37°C with vortexing. Further fixation was immediately blocked by addition of an equal volume of 0.2 M lysine (Fluka). Cells were washed three times and then used in antigen presentation assays. The efficiency of fixation was controlled by ^{3}H-thymidine incorporation of fixed cells and by PPD stimulation of PPD-specific and MHC class II–restricted T cells.

To investigate the kinetics of internalization, dendritic cells were incubated at 37°C with GM1 (20 μM), washed at different time points, fixed as above, and cocultured with T cells. In some experiments, APC were first fixed, then washed and pulsed with GSLs for 2 hr before further washing and addition of T cells. In other experiments, dendritic cells were incubated at 18°C, or at 37°C in the presence

of NH_4Cl (40 mM), chloroquine (80 μM), chlorpromazine (10 μg/ml), or monodansylcadaverine (500 μM) (all purchased from Sigma) for 1 hr before addition of GM1 (10 μM).

Generation of Soluble CD1b

cDNA coding for soluble CD1b was amplified by RT-PCR using a 3′ primer starting at position 939 before the transmembrane region. After sequencing, it was subcloned in the expression vector PGMT7. A similar construct was generated to express the full-length cDNA of human β2m. Recombinant proteins were expressed in BL21 LysS bacteria and recovered in inclusion bodies. In vitro refolding of CD1b, β2m, and GM1 was done at pH 7.2 by dilution as described (Garboczi et al., 1992). After concentration on Amicon 10K, the refolded complex (sCD1b) was purified from aggregated proteins (aCD1b) on a HiLoad 16/60 Superdex 200 column (Pharmacia). The purity of soluble protein was >90% as assessed by Coomassie staining. The correct association with β2m and refolding was confirmed by sandwich ELISA in which anti-β2m and conformational dependent anti-CD1b mAbs (WM25) were used as capture and revealing reagents.

Stimulation of T Cells with sCD1b and Displacement of Bound GM1

The sCD1b or control proteins were immobilized at 10 μg/ml and used to stimulate T cells (8×10^4/well) for 48 hr in the presence of 1 ng/ml PMA. Released lymphokines were measured by ELISA. For displacement assay, sCD1b was refolded in the presence of ^{3}H-labeled GM1 and then purified as above. After concentration, sCD1b was incubated with 10 μM cold GM2 for 16 hr at 4°C using the refolding buffer at pH 7.2. This material was then run on Superdex 200 HR 10/30 column, and radioactivity was measured in collected fractions. In other experiments, sCD1b was refolded in the presence of cold GM1. After purification and concentration, it was incubated with ^{3}H-labeled GM2 or ^{3}H-labeled GM1–acetyl and subjected to gel filtration. Soluble CD1b-associated radioactivity was measured as above. Control experiments showed that hot GM1, GM2, and GM1–acetyl do not associate with β2m alone or with the aggregated CD1b.

Cell Surface GM1 Displacement

Fixed dendritic cells were pulsed with GM1 (4 μM) for 1 hr at 37°C, washed and incubated with GM1 analogs (40 μM) for 2 hr, washed three times, and then plated (8×10^4/well) with GM1-specific T cell clones (8×10^4/well). Released lymphokines were measured by ELISA.

Detection of Released Lymphokines

TNFα and IFNγ were detected using sandwich ELISA kits according to manufacturer's instruction (Instrumentation Laboratory, Schlieren, Switzerland). IL-4 was detected as described (Shamshiev et al., 1999). The data are expressed as mean pg/ml ± SD of triplicates.

Acknowledgments

We thank M. Degano, P. Dellabona, and our colleagues in the Experimental Immunology lab for helpful discussions and careful revision of the manuscript. We also thank V. Cerundolo for the gift of PGMT7 vector. This work was supported by grant N. 31-55698.98 from Swiss National Fund, by grants from the Swiss Multiple Sclerosis Society, the Velux, Desireé Niels Yde, and Ciba Foundations (G. D. L.), and by NIH grant AI40617 (M. K.).

Received February 22, 2000; revised July 5, 2000.

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