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Immunology

Presentation of peptide antigens by mouse CD1 requires endosomal localization and protein antigen processing

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ABSTRACT **Mouse CD1(mCD1) molecules have been reported to present two types of antigens: peptides or proteins and the glycolipid α-galactosylceramide. Here, we demonstrate that a protein antigen, chicken ovalbumin (Ova), must be processed to generate peptides presented by mCD1 to $CD8^+$ T cells. The processing and mCD1-mediated presentation of chicken Ova depend on endosomal localization because inhibitors of endosomal acidification and endosomal recycling pathways block T cell reactivity. Furthermore, a cytoplasmic tail mutant of mCD1, which disrupts endosomal localization, has a greatly reduced capacity to present Ova to mCD1 restricted cells. Newly synthesized mCD1 molecules, however, are not required for Ova presentation, suggesting that molecules recycling from the cell surface are needed. Because of these data showing that mCD1 trafficks to endosomes, where it can bind peptides derived from exogenous proteins, we conclude that peptide antigen presentation by mCD1 is likely to be a naturally occurring phenomenon. In competition assays, α-galactosylceramide did not inhibit Ova presentation, and presentation of the glycolipid was not inhibited by excess Ova or the peptide epitope derived from it. This suggests that, although both lipid and peptide presentation may occur naturally, mCD1 may interact differently with these two types of antigens.**

CD1 molecules are a family of antigen-presenting molecules distantly related to major histocompatibility complex (MHC)-encoded class I and class II molecules. CD1 molecules have a number of distinct features, including a lack of polymorphism, nearly equal levels of homology with both class I and class II molecules (1), and expression that is independent of either the peptide transporter associated with antigen presentation (TAP) (2, 3) or the invariant chain (Ii). These distinct features, and the conservation of CD1 molecules through out much of the mammalian order, justify their categorization as a separate, third family of antigen-presenting molecules.

Two different T cell subsets reactive to wild-type mouse CD1.1 (mCD1) have been reported. T cells reactive with peptides or proteins that have a hydrophobic mCD1 binding motif have been described by our groups (4, 5). These T cells are TCR $\alpha\beta^+$, $CD8^+$ lymphocytes that exhibit cytolytic activity. The other subset of mCD1 specific T lymphocytes, which are either $CD4^+$ or double negative, have been shown to be mCD1 autoreactive (6, 7). More recently, a major population of these mCD1 autoreactive T cells, namely those that express the NK1.1 molecule and an invariant Vα14 TCR, have been shown to recognize the glycolipid α-galactosylceramide (α-GalCer) presented by mCD1 (8, 9). These so-called NK T cells rapidly secrete large amounts of cytokines, and they have been reported to play important immunoregulatory roles in a variety of situations (10, 11).

mCD1 molecules are unique in their ability to present chemically well defined peptide and nonpeptide antigens, although the nonclassical H-2M3 molecule also may be capable of this duality of function (12). It has been shown recently, however, that the great majority of mCD1 molecules purified from mammalian cells are bound to glycophosphatidyl inositol containing compounds and that bound peptides could not be detected (13). This raises the questions regarding whether peptide antigen presentation by mCD1 is a phenomenon that occurs naturally and, if so, whether mCD1 molecules are capable of acquiring peptide antigens intracellularly. The experiments described here demonstrate that mCD1 indeed can acquire peptide antigens from processed proteins intracellularly, and, furthermore, they suggest that peptide and nonpeptide antigens may bind to mCD1 differently.

MATERIALS AND METHODS

Mice, Cell Lines, and Hybridomas. C57BL/6, BALB/c, and C57BL/6 × BALB/c F1 (CB6F1) mice were obtained from The Jackson Laboratory or were bred in our animal facility. TAP $1^{-/-}$ mice were bred from stock provided by Luc van Kaer (Vanderbilt University, Nashville, TN). Mice 8–10 weeks old of both sexes were used. Transfectants expressing wild-type CD1, and transfectants expressing mouse CD1 with the cytoplasmic tail deleted (CD1.1TD), have been described elsewhere (5, 14).

Antigens and Antibodies. HPLC-grade chicken Ova was purchased from Sigma. HPLC-purified mCD1 binding peptides, p99 (YEHD*F*HH*I*RE*W*GNHWKNFLAVM) (4) and p18, containing amino acids 260–278 (IN*F*EK*L*TE*W*TSSNVCEER) of Ova, were purchased from Research Genetics (Huntsville, AL). The known (p99) or likely amino acids (p18) involved in mCD1 binding are in italics. p99A1 has the phenylalanine at position 5 of p99, which is an mCD1 anchor position, replaced with alanine. α-GalCer (15) was synthesized at Kirin Brewery (Gunma, Japan). The anti-CD1 mAb 1B1 was produced in our laboratory (16), and its isotype-matched mAb (IgG2b, κ, clone 49.2) and anti-CD8 antibodies were obtained from PharMingen.

Preparation of Plasmid DNA and Immunizations. The plasmids pACB-CD1, nCMV ova, and nCMV B7–1 have been described (5). Plasmid DNA was prepared by using EndoFree plasmid Maxi kits from Qiagen (Chatsworth, CA), and mice were immunized twice, at weekly intervals, with a combination

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Abbreviations: α-GalCer, α-galactosylceramide; APC, antigen-presenting cells; mCD1, wild-type mouse CD1.1; CD1.1TD, mouse CD1 with the cytoplasmic tail deleted; TAP, transporter associated with antigen presentation; MHC, major histocompatibility complex; IFN-γ, interferon γ.

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of the three plasmids. Immunizations consisted of 50–75 μg of each plasmid injected intradermally in the tail base.

Generation of Antigen-Specific T Cells. Typically, 7×10^6 splenic responders from DNA primed mice were incubated with 1×10^6 irradiated RMA-S/CD1.1 transfectants. These antigen-presenting cells (APC) generally were pulsed with 50 μg/ml of Ova. Mouse interleukin 2 (20 units/ml, PharMingen) was added 2 days after initiation of the cultures. After 6 days, the cells stimulated *in vitro* were harvested. For cytokine detection, 1×10^5 T cells/well in 96-well plates were cultured with 5×10^4 APC, which were either mCD1$^+$ transfectants, control parental lines, splenocytes, or thymocytes. APC were pulsed for 2–3 hr at 37°C with either Ova or peptide p18 and then were washed and irradiated before being added to the cultures. To obtain α-GalCer reactive T cells, fresh spleen cells from naive CB6F1 mice were cultured at 2×10^5 cells/well with 6×10^4 mCD1 transfected APC that had been pulsed with 100 ng/ml of α-GalCer. Supernatants were harvested 3 days later and were tested for IFN-γ production by using standard protocols.

Fixation and Inhibitor Treatment of APC. For APC fixation, cells were treated with 0.03% glutaraldehyde (Sigma) for 30 seconds with intermittent vortexing. One volume of 0.2 M L-lysine (pH 7.4) was added, the cells were incubated for an additional 2 min, and they then were washed. For the inhibitor experiments, Concanamycin A (Kamida Biomedicals, Ventura, CA), Bafilomycin A (Kamida Biomedicals), Wortmannin A (Sigma), and Brefeldin A (ICN) were used at the indicated concentrations. The inhibitors were added to the APC either 5 min before or 3 hr after pulsing with Ova. In either case, the inhibitors were left in the cultures for a total period of 4 hr. The APC then were fixed as described above and were used in cytokine detection assays. To test for the efficacy of the inhibitor treatment, inhibitors were added as described above to A20 cells, and these APC were added to cultures of the Ova-specific, class II-restricted T cell hybridoma D011.10. Supernatants were harvested from these cultures after 24 hr, and interleukin 2 production was measured by using a standard ELISA protocol.

Antibody Blocking Experiments. A20/CD1.1 transfected cells were pulsed with 50 μg/ml Ova and were seeded in the presence of the following mAbs at the concentrations indicated in the figures: anti-mCD1 mAb 1B1, anti-CD8 mAb 53–6.7, or the rat IgG2b isotype control. The T cells then were added, and cytokine release was measured as described above. For blocking of the glycolipid antigen response by fresh spleen cells, A20/CD1.1 transfected cells were pulsed with 0.1 mM α-GalCer, and anti-CD8 mAb (10 μg/ml) or anti-CD1 mAb (10 μg/ml) were added before the addition of spleen cells.

Antigen Competition Experiments. mCD1-transfected A20 cells (APC) were pulsed for 2 hr with competitors: either 5.5 μM p99, 5.5 μM p99A1, or 25 μM α-GalCer. The APC then were pulsed for another 2 hr, either with 1 μM Ova protein or 1.25 μM p18. The APC then were washed and irradiated and then were added to cultures of the Ova-specific T cells. To assess peptide competition of lipid antigen recognition, A20/CD1.1 cells were preincubated for 2 hr with either 10 μM p99, 10 μM p18, or 1 μM Ova, then were pulsed with 0.1 μM α-GalCer for 1 hr, were washed and irradiated, and then were added to fresh spleen cells from CB6F1 mice for assay as described above.

RESULTS

Characterization of mCD1-Restricted, Ova-Specific T Cell Lines. Recently, the generation of Ova- and mCD1-specific T cells, by immunization of mice with naked plasmid DNA encoding B7–1, CD1.1, and chicken Ova, was reported (5). This protocol was repeated successfully numerous times in the present study. Spleen cells from the immunized mice were restimulated *in vitro* for 6 days with CD1.1-transfected RMA-S cells. Fig. 1*A* shows representative data, from one of four similar experiments, indicating that the Ova- and mCD1-specific T cells secrete IFN-γ after T cell stimulation, in addition to the previously reported cytotoxic activity of these cells (5). The response is dose-dependent, although the magnitude of the response varied in different T cell bulk cultures. The control responses of T cells alone, T cells cultured with CD1.1 transfectants but without antigen, or T cells given Ova antigen with parental APC that lack mCD1 were all minimal. Similar results were obtained in mice immunized with whole Ova protein plus adjuvant, suggesting that more conventional immunization protocols can generate this type of response, although the responses were lower. Therefore, all of the subsequent experiments were carried out with mice immunized with DNA. We also tested the ability of these T cells to secrete IFN-γ in response to p18, a peptide containing amino acids 260–278 of chicken Ova. This synthetic peptide was chosen because it contains a likely binding motif for mCD1 (4) and, based on our previous studies, was of the appropriate length for optimal mCD1 binding. As shown in Fig. 1*B*, p18 also was able to induce IFN-γ secretion from T cells in a dose-dependent fashion (Fig. 1*B*). The Ova-responding T cells have a phenotype characteristic of cytolytic, CD8$^+$ T lymphocytes. Typically, most of the T cells in the culture were TCRαβ$^+$, CD8$^+$ (75–86%, $n = 3$), similar to the mCD1-restricted, peptide-reactive cells characterized earlier (4).

Recognition of Ova Is Not Restricted by MHC Class I Molecules. Although it is unlikely that the p18 peptide would

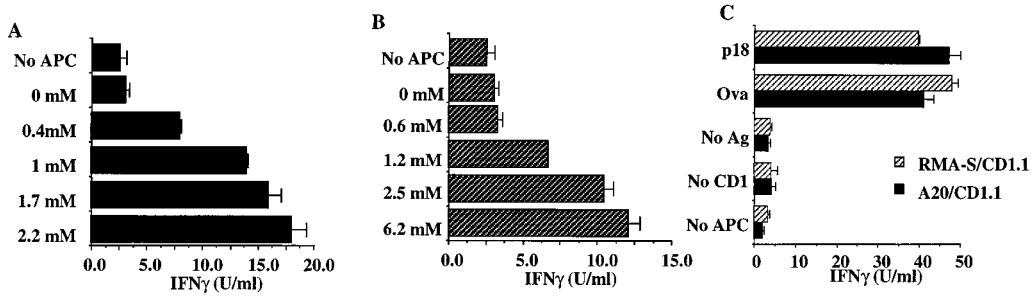

FIG. 1. Cytokine release by mCD1-restricted T cells in response to Ova or Ova peptide. The response of T cells to APC pulsed with various concentrations of Ova (*A*) or p18 peptide (*B*) are shown. Reactive T cells were obtained from groups of four C57BL/6 mice immunized with 50–70 μg of plasmids encoding B7.1, Ova, and CD1.1. Spleen cells were harvested, were stimulated *in vitro*, and were restimulated with RMA-S/CD1.1 cells pulsed with indicated concentrations of antigen. The levels of IFN-γ were determined by ELISA. The IFN-γ produced by untransfected RMA-S APC pulsed with Ova or p18 was <2.5 units/ml. (*C*) The response of T cells from TAP$^{-/-}$ mice, generated and stimulated as described above, to Ova or p18 peptide presented by either A20 or RMA-S transfectants is shown. Representative data from one of three TAP$^{-/-}$ responder mice are shown.

have epitopes for presentation by both b and d haplotype class I molecules, certainly the whole Ova protein has such epitopes. Therefore, several experiments were carried out to rule out the possibility that an mCD1-mediated response was providing help for a class I-restricted response to Ova. Fig. 1*C* shows the cytokine response of T cells from DNA-immunized $TAP^{-/-}$ mice that were restimulated once *in vitro* with mCD1 transfectants of TAP-deficient RMA-S cells. Although $TAP^{-/-}$ mice should have very few T cells selected by classical class I molecules, the *in vitro* response by T cells from these mice is robust. Furthermore, the *in vitro* response shows no MHC restriction. Reactive T cells obtained from $TAP^{-/-}$ mice responded approximately as well to $mCD1^{+}$ d haplotype A20 cells as they did to the RMA-S transfectants, although the responding T cells had no contact with d haplotype class I molecules before the cytokine release assay. In addition, mAbs specific for the K, D, and L molecules expressed by the A20/CD1.1 transfectant, or the K and D molecules expressed at low levels by the RMA-S transfectant, did not block the Ova response significantly in CB6F1 immunized mice (data not shown). By contrast, an anti-CD8 mAb blocked the CD1-dependent Ova response nearly completely (Table 1).

Antigen Processing Is Required for Ova Presentation by mCD1. To determine whether antigen processing is essential for generating an mCD1-restricted Ova response, $mCD1^{+}$-transfected cells were fixed with 0.03% glutaraldehyde at various time points either before or after antigen pulse. As shown in Fig. 2*A*, fixation of RMA-S/CD1.1 cells (APC), 5 min before adding antigen, diminished the T cell reactivity by 88%. By contrast, fixation did not have any effect on the presentation of the synthetic peptide p18. This suggests that antigen processing is a prerequisite for Ova presentation by mCD1 but not for peptide presentation. Furthermore, this experiment shows that antigen processing and the appearance of mCD1 peptide complexes on the cell surface are essentially complete by 3 hr (Fig. 2*A*) because fixation after a 3-hr antigen incubation was not inhibitory.

Endosomal Acidification and Recycling Are Required for Ova Presentation by mCD1. Endosomal acidification has been shown to be necessary for the presentation of lipid antigens to T lymphocytes by human CD1b molecules (17–20). To determine which compartment(s) in the APC is essential for Ova presentation, we incubated APC with various chemical inhibitors. Bafilomycin A and Concanamycin A both have been shown to inhibit endosomal acidification (21, 22). Concanamycin A blocks transport from early to late endosomes whereas Bafilomycin A is particularly effective at blocking the transport from late endosomes to lysosomes. Brefeldin A blocks transit of newly synthesized molecules from the trans-Golgi network (23), and Wortmannin A has been shown to inhibit endosomal recycling pathways, such as internalization of molecules from the cell surface (24–26), without effecting endosomal acidification. CD1.1-transfected RMA-S cells were incubated with the inhibitors for 5 min before or 3 hr after addition of antigen. The APC then were fixed to prevent any further endosomal trafficking or antigen processing events and were added to the Ova-reactive T cells for measurement of cytokine release.

As seen in Fig. 2*B*, although Bafilomycin did not inhibit presentation, Concanamycin A inhibited mCD1-mediated, Ova-specific T cell reactivity by 83%, and Wortmannin inhibited it by 79%. Brefeldin A did not have any affect on cytokine secretion by the Ova reactive T cells, suggesting that newly synthesized mCD1 molecules are not required for Ova presentation (Fig. 2*B*). Similar effects of these four inhibitors were seen when A20/CD1.1 cells were used as antigen-presenting cells (data not shown). A toxic effect of the inhibitors on presentation was ruled out because addition of the inhibitors 3 hr after adding the antigen did not decrease T cell stimulation.

To verify that the different inhibitors that did not block mCD1-mediated antigen presentation were active at the concentrations used on A20 APC, they also were tested for their ability to block interleukin 2 production by D011.10, an A^{d} class II-restricted, Ova-specific T cell hybridoma. Bafilomycin inhibited antigen-specific T cell hybridoma interleukin 2 production by 86%, and Brefeldin A did so by 60%, demonstrating that these inhibitors were active. In the same experiment, Concanamycin A inhibited the class II-mediated Ova response by 71%.

mCD1 Trafficking to Endosomes Is Required for Presentation of Ova. To elucidate the importance of endosomal trafficking of mCD1 in peptide presentation, we tested a mutant of mCD1 that lacks the YQDI endosomal localization sequence in the cytoplasmic tail (14). We have demonstrated previously, by using confocal microscopy, that A20 cells transfected with this tail mutant (A20/CD1.1TD) did not have detectable mCD1 molecules in endosomal compartments (14), although the surface level of mCD1 expression was similar to that in wild-type mCD1 transfectants. As seen in Fig. 2*C*, Ova-pulsed A20/CD1.1TD transfectants poorly stimulated IFN-γ secretion, compared with the wild-type A20/CD1.1 transfectants. The A20/CD1.1TD and A20/CD1.1 transfectants, however, were able to present the Ova peptide (p18) equally well, suggesting that there is no inherent antigen binding-defect in the tail-deleted mCD1 molecules. Identical results were obtained when we used EL4 cells transfected with the wild-type or the tail-deleted form of mCD1 (data not shown), indicating that the requirement for endosomal trafficking of mCD1 is not restricted to one cell type or transfectant.

The Level of mCD1 Expression by Normal Cells Is Sufficient for Ova Presentation. Because the transfected cells express relatively high levels of mCD1, we also determined whether normal $mCD1^{+}$ cells are capable of Ova presentation. Fig. 2*D* shows the results from an experiment in which presentation of Ova and p18 by transfected cells, splenocytes, and thymocytes are compared. Although the A20 transfectants gave the highest stimulation of the T cells, splenocytes from either normal or from $TAP^{-/-}$ mice also could present both Ova protein and p18 peptide efficiently.

Peptide and Glycolipid Antigens Do Not Compete for mCD1 Presentation. The data presented above indicate that mCD1 molecules located in endosomal compartments can acquire peptide antigens from processed, exogenous proteins. mCD1 also can bind and present glycolipids, but it remains to be determined whether both types of antigens bind to the same site on mCD1. Of interest, the anti-mCD1 mAb 1B1 only could inhibit partially the Ova-specific T cell reactivity, even at the highest concentrations used. Data from a representative experiment are presented in Table 1, showing 43% inhibition of the T cell response with antibody at 50 μg/ml and a suboptimal concentration of peptide antigen. By contrast, anti-CD8 mAb could block the reactivity of these Ova-reactive T cells by 88%.

Table 1. CD1 antibodies more effectively block glycolipid antigen recognition

	C, 10 μg/ml	αCD1, 10 μg/ml	αCD1, 20 μg/ml	αCD1, 50 μg/ml	αCD8, 10 μg/ml
A20CD1.1 + Ova	1.3 ± 0.4	37.2 ± 1	38.3 ± 0.6	43.1 ± 2.3	88.2 ± 5.7
A20CD1.1 + α-GalCer	1.2 ± 0	91.4 ± 4.3	Not determined	Not determined	11.3 ± 2.4

The data are expressed as percentage inhibition and are representative of three different experiments for the Ova response and two experiments for the α-GalCer response. C, control antibody.

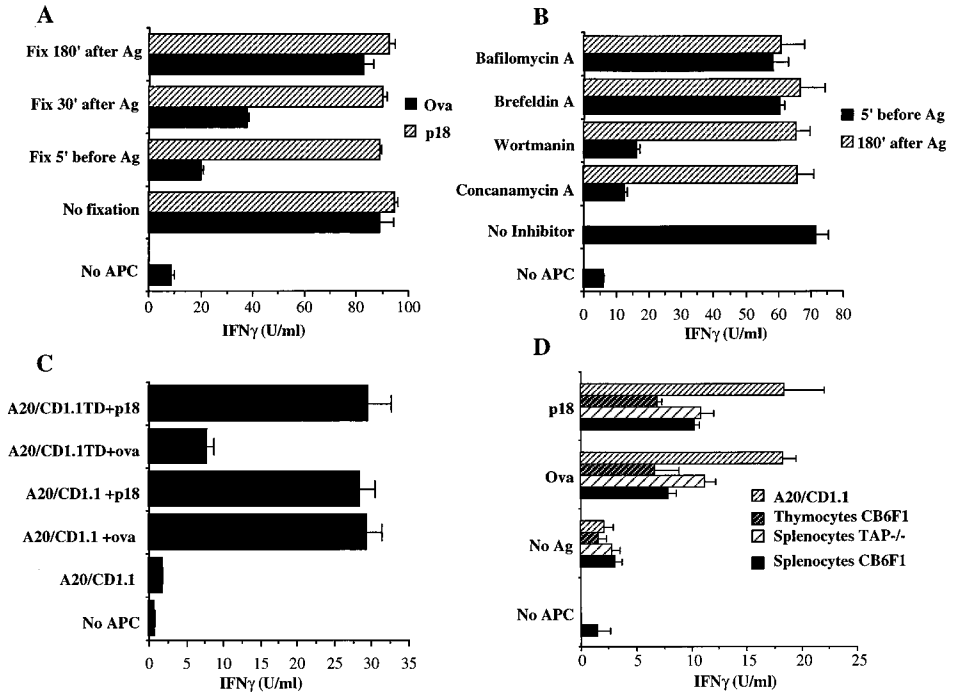

FIG. 2. Antigen processing and endosomal localization are required for Ova presentation by mCD1. (*A*) Fixed cells can present p18 peptide but not Ova. RMA-S/CD1.1 transfectants were fixed with 0.03% glutaraldehyde either before, or at different time points after, addition of antigens. The antigens were either 50 μg/ml Ova or 4 μg/ml p18 peptide. (*B*) RMA-S/CD1.1 transfectants were incubated with the indicated inhibitors: 20 nM Concanamycin A, 50 nM Bafilomycin, 200 nM Wortmannin, or 10 μg/ml Brefeldin A. Inhibitors were added either 5 min before, or 180 min after, addition of 50 μg/ml Ova. After 4 hr in the presence of the inhibitors, the APC were fixed and tested for their ability to stimulate Ova reactive T cells. (*C*) A20/CD1.1 and A20/CD1.1TD transfectants were pulsed either with 50 μg/ml Ova or 4 μg/ml p18 peptide for 3 hr, were irradiated, and were added to T cell stimulation assays. ELISAs were used to measure the IFN-γ levels after 3 days. In the case of *A* and *B*, the *in vitro* activated CTLs were derived from C57BL/6 mice whereas in *C* the CTLs were derived from CB6F1 mice. The control production of cytokine from untransfected cells pulsed with Ova, or T cells alone, in each case was <6 units/ml. These data are representative of one of six experiments. (*D*). Normal APC can present Ova to mCD1-restricted T cells. Ova-reactive T cells were generated from CB6F1 DNA immunized mice and were restimulated with Ova plus mCD1 RMA-S transfectants as described above. The representative data from one of six animals analyzed in this way is shown.

It has been reported that Vα14$^+$, mCD1-restricted, and α-GalCer-specific T cells can be obtained after a brief pulse of fresh spleen cells with glycolipid antigen (9). This α-GalCer response could be inhibited by 91% when the mCD1 antibody 1B1 was used at concentrations as low as 10 μg/ml (Table 1), with a greater-than-optimal concentration of glycolipid antigen (100 ng/ml). The 1B1 mCD1 mAb was similarly effective at blocking the response of Vα14$^+$ T cell hybridomas to α-GalCer (data not shown). Although indirect, these data suggest that, when the peptide is bound to it, mCD1 is in a different conformation or that the 1B1 epitope is partially blocked.

To determine more directly whether peptide and lipid antigens are bound to the same site on mCD1, competition assays were carried out. A20/CD1.1 cells were incubated with 4-fold molar excess of either p99 mCD1 binding peptide, p99A1, which is a variant of p99 that does not bind to mCD1, or a >20-fold molar excess of α-GalCer and then were pulsed with either whole Ova or p18. As seen in Fig. 3*A*, p99 totally abrogated the T cell reactivity to Ova peptide whereas the

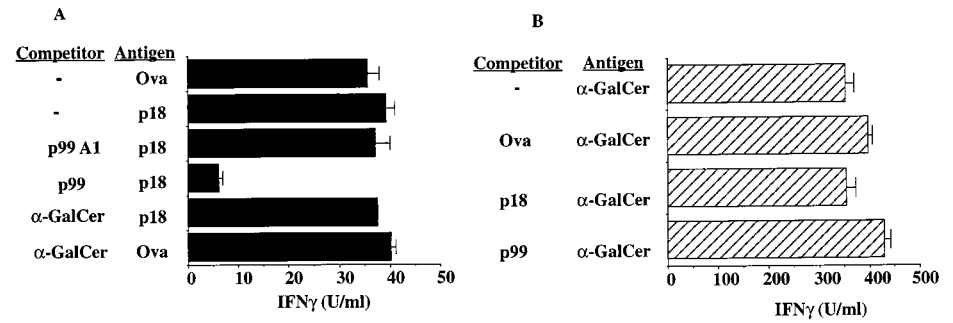

FIG. 3. Lack of mutual competition between peptide and lipid antigens. (*A*) A lipid antigen does not compete for peptide recognition. A20/CD1.1 transfectants were pulsed with either 5.5 μM p99, 5.5 μM p99A1, or 25 μM α-GalCer and then were pulsed either with 1 μM Ova or 1.25 μM p18 peptide. After washing out the antigen, APC were irradiated and cultured with *in vitro* activated T cells, and cytokine detection assays were carried out as described above. The production of IFN-γ from untransfected cells pulsed with Ova, or T cells alone, was <8 units/ml. Representative data are from one of three experiments. (*B*) Peptide antigens do not compete for lipid recognition. A20/CD1.1 cells were pulsed either with 10 μM p99, 10 μM p18, or 1 μM Ova; they then were pulsed further with 0.1 μM α-GalCer and were handled as described in *A*. These APC then were cultured together with spleen cells from CB6F1 mice. After 3 days of coculture, ELISAs for IFN-γ detection were carried out.

p99A1 peptide did not. Of interest, α-GalCer did not have any effect on T cell presentation of either whole protein or the peptide epitope. It should be noted that the amount of glycolipid competitor added is >250-fold more than the amount required for an optimal α-GalCer response (9).

In the reverse competition experiment, we determined whether peptide antigens could inhibit the glycolipid antigen response. A20/CD1.1 cells were pulsed for 2 hr with a 100-fold excess of mCD1 binding peptide, either p99 or p18, or 10-fold molar excess of whole Ova, after which α-GalCer (0.1 μM) was added, the APC were irradiated, and they were tested for their ability to induce IFN-γ secretion from fresh spleen cells in response to α-GalCer. As seen in Fig. 3*B*, neither the peptides nor whole Ova protein competed for the α-GalCer induced secretion of IFN-γ by T cells. In additional experiments, even a 1,000-fold excess of peptide did not compete for α-GalCer recognition (data not shown). These data suggest that peptide and glycolipid antigen do not compete effectively for the same site on mCD1 molecules.

DISCUSSION

mCD1 molecules are unique in that they are known to present peptide- and lipid-containing antigens. The data presented here and elsewhere (4, 5) demonstrate that the properties of the peptide-reactive T cells in different experimental systems are quite similar because they are TCR $\alpha\beta^+$, $CD8^+$, and $NK1.1^-$ and are cytolytic and capable of IFN-γ secretion. In addition, excess p99 competes for Ova presentation, indicating that different peptides bind to the same site on mCD1. Although the presentation of peptides by mCD1, therefore, is well established, we carried out experiments to demonstrate that the response to Ova protein is not caused by help from mCD1-reactive T cells for Ova-specific and class I-restricted lymphocytes. Data consistent with mCD1 presentation of antigen derived from Ova protein include the efficient generation of Ova reactive T cells from $TAP^{-/-}$ mice, the ability to stimulate the reactive T cells with $mCD1^+$ $TAP^{-/-}$ APC, the lack of MHC restriction for the reactive T cells when tested with different $mCD1^+$ APC, and the inability of K-, D-, and L-specific mAbs to block this response.

Despite this consistent set of data on peptide presentation, the results from other studies suggest that mCD1 is most adapted for the presentation of lipid antigens. mCD1 has a narrow antigen-binding groove that is blocked at one end (27) and that therefore cannot easily accommodate long peptides. Furthermore, the results from a recent study indicate that the predominant material obtained from purified mCD1 molecules is a glycophosphatidyl inositol-containing compound (13). Based on these results, one might conclude that mCD1 molecules do not bind peptides intracellularly and that peptide presentation by mCD1 is perhaps not a frequent or physiologic event. In a publication demonstrating an mCD1-mediated response to Ova, no antigen processing was shown, and reactivity to an Ova-derived peptide was not demonstrated (5). In this manuscript, however, we clearly have demonstrated that intracellular mCD1 located in endosomes can bind processed peptides from internalized antigenic proteins, strongly suggesting that intracellular mCD1 might normally be associated with peptides and therefore that peptide presentation may be a true physiologic function of mCD1. Consistent with this conclusion, we demonstrate that the level of mCD1 expression on APC from thymus and spleen is sufficient to support a vigorous *in vitro* Ova response by the reactive T cells. There is no difference in the efficacy of presentation when RMA-S or EL4 as opposed to A20 transfectants are used, indicating that TAP, H-2M, and Ii are not required for efficient processing or presentation of peptides by mCD1.

Studies using different chemical inhibitors implicate mature, cell-surface mCD1 molecules, which are recycling from the cell surface into early endosomes, as the critical antigen-presenting molecules for Ova-derived peptides. The significant degree of inhibition of Ova presentation obtained after Wortmannin treatment supports this conclusion. Consistent with this view, T cell reactivity was unaffected by treatment of the APC with Brefeldin A, which prevents newly synthesized mCD1 molecules from reaching the plasma membrane. The inhibitor Bafilomycin A did not have any effect on Ova presentation, although it also has been used as an endosomal acidification inhibitor (21) similar in function to Concanamycin A. This could be caused by the fact that Concanamycin A is a more stable inhibitor of vacuolar ATPases and that it has a broader ATPase specificity than Bafilomycin (28, 29). Alternatively, by contrast to Concanamycin A, Bafilomycin A may be specifically potent at inhibiting transport from late endosomes to lysosomes (30, 31). In summary, the results from the inhibitors are consistent with Ova uptake and processing in early and late endosomes and with the binding of peptides derived from these processing events to mCD1 molecules recycling through endosomal compartments to the cell surface. Consistent with this, by using Ova conjugated to fluorochromes, several groups have demonstrated that Ova can traffick through early and late endosomes (32, 33) in which mCD1 also is found (14). Furthermore, the results from ongoing experiments suggest that mCD1 molecules do recycle efficiently (S.T., unpublished observations). Because of the requirement for all three plasmids in the DNA immunization, it is most likely that the cells expressing CD1 from the injected DNA also are expressing Ova and are presenting it. For class II molecules, there are ample precedents for the presentation of peptides derived from endogenously expressed, secreted proteins (34), and, therefore, it is not so surprising that peptides derived from such proteins also reach endosomes containing CD1.

Complete inhibition of Ova presentation could not be achieved by using any one of the inhibitors or the cytoplasmic tail mutant, suggesting the possibility of an additional, relatively minor pathway for Ova presentation by mCD1. There are several possible explanations for this, including the presence of Ova peptides in the initial antigen because of degradation, extracellular processing of Ova in the tissue culture medium, or regurgitation of Ova peptides.

The means by which mCD1 binds both to peptide and to lipid-containing antigens remain to be determined. The strongest evidence against binding of lipid and protein to the same site on mCD1, which most likely would be the antigen-binding groove, comes from competition studies. The inability to compete in these experiments could be caused by the inability of the competitors to saturate the available sites on mCD1 rather than the presence of different sites. We consider this unlikely, however, because, in parallel experiments, p99 could inhibit Ova peptide presentation completely whereas a greater molar excess of α-GalCer did not compete at all. A possible model to explain the lack of competition is that one of the antigens, either peptide or α-GalCer, binds outside the groove. It remains possible, therefore, that, compared with peptide binding to the groove of MHC class I and class II molecules, the binding of peptides to mCD1 is unconventional. Alternatively, because of posttranslational modification or some other factor, there could be two different populations of mCD1 molecules, one that can bind to peptide and one that can bind to lipid.

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