
**ENZYME CATALYSIS AND
REGULATION:**

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Involvement of Cathepsin E in Exogenous Antigen Processing in Primary Cultured Murine Microglia*

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We have attempted to elucidate an involvement of cathepsin E (CE) in major histocompatibility complex class II-mediated antigen presentation by microglia. In primary cultured murine microglia, CE was localized mainly in early endosomes and its expression level was markedly increased upon stimulation with interferon- γ . Pepstatin A, a specific inhibitor of aspartic proteases, significantly inhibited interleukin-2 production from an OVA-(266–281)-specific T helper cell hybridomas upon stimulation with native OVA presented by interferon- γ -treated microglia. However, pepstatin A failed to inhibit the presentation of OVA-(266–281) peptide. The possible involvement of CE in the processing of native OVA into antigenic peptide was further substantiated by that digested fragments of native OVA by CE could be recognized by OVA-specific Th cells. Cathepsin D also degraded native OVA into antigenic peptide, whereas microglia prepared from cathepsin D-deficient mice retained an ability for antigen presentation. On the other hand, the requirement for cysteine proteases such as cathepsins S and B in the processing of invariant chain (Ii) was confirmed by immunoblot analyses in the presence of their specific inhibitors. In conclusion, CE is required for the generation of an antigenic epitope from OVA but not for the processing of Ii in microglia.

Cathepsin E (CE,¹ EC 3.4.23.34) is an intracellular aspartic protease of the pepsin family, which is highly homologous to

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¹The abbreviations used are: CE, cathepsin E; CD, cathepsin D; Th, T helper; MHC, major histocompatibility complex; Ii, invariant chain; CL, cathepsin L; CS, cathepsin S; CLIP, class II-associated Ii peptide; OVA, ovalbumin; CD^{-/-}, CD-deficient; IFN, interferon; Tfr, transferrin receptor; CB, cathepsin B; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BMC, bone marrow cell; PEG, polyethylene glycol; IL, interleukin; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcriptase; APC, antigen presenting cell.

the lysosomal aspartic protease cathepsin D (CD, EC 3.4.23.5). In contrast to CD, CE has a limited distribution in tissues and cell types such as lymphoid tissues, gastrointestinal tracts, blood cells, and microglia (1–4). Furthermore, the intracellular localization and molecular form of CE in microglia contrast sharply with those in other tissues and cell types. CE is mainly localized in the endosomal structures possibly as the mature form in microglia (4), whereas this enzyme was found to be localized in various cellular compartments such as the plasma membrane, endoplasmic reticulum, and Golgi apparatus as the enzymatically inactive proform in other tissues and cell types (4–6). The localization of CE in the endosomal system has been also demonstrated in antigen-presenting B cells lymphoblasts (7). Thus it is conceivable that the mature form of CE is closely associated with endosomal localization of this enzyme because the proform of CE appears to be converted into mature form only after entering in intracellular acidic compartments. Microglia are known to interact with invaded CD4⁺ T helper (Th) cells in the central nervous system (8–10). Through this interaction, microglia may contribute to tissue damage and repair during autoimmune disease, viral infections, and chronic inflammatory disease (see reviews, see Refs. 11–13). The major histocompatibility complex (MHC) class II-mediated antigen presentation requires the participation of endosomal/lysosomal proteases in endocytic route to degrade exogenous antigens and invariant chain (Ii) associated with MHC class II (see reviews, Refs. 14 and 15). Despite the strategic localization of CE in microglia, no information is available about the involvement of CE in MHC class II-mediated antigen presentation by microglia.

There is increasing evidence that two classes of intracellular proteases, aspartic and cysteine proteases, are involved in proteolytic steps required for MHC class II-mediated antigen presentation. On the basis of studies utilizing specific inhibitors or cathepsin-deficient mice, cathepsin L (CL) and cathepsin S (CS), lysosomal cysteine proteases have been recently demonstrated to be responsible for the terminal degradation of Ii to class II-associated Ii peptide (CLIP) during maturation of MHC class II molecules in the thymus and the peripheral lymphoid organs, respectively (16–22). Furthermore, cathepsin F is also implicated in CLIP generation in peripheral macrophages (23). On the other hand, aspartic protease inhibitors such as pepstatin A have been shown to prevent antigen processing and Ii degradation (24, 25). In B cells, expression level of CE was

up-regulated upon cellular activation and specific inhibitor of CE blocked the presentation of ovalbumin (OVA) in these cells (7, 26). Furthermore, CD has been reported to generate antigenic peptides from OVA or hen egg lysozyme that could be presented to T cells (27–29). More recently, however, the experiments conducted with splenocytes and macrophages prepared from CD-deficient (CD^{-/-}) mice have concluded that CD is dispensable for degradation of Ii and processing of a number of exogenous and endogenous antigens (17, 30). These observations may suggest that CE plays a pivotal role in the MHC class II-mediated antigen presentation as an aspartic protease. To elucidate a specific involvement of CE and CD in the MHC class II-mediated antigen presentation by microglia, we conducted experiments utilizing specific inhibitors for cathepsins, purified enzymes and microglia prepared from CD^{-/-} mice.

EXPERIMENTAL PROCEDURES

Materials—Pepstatin A, leupeptin, E-64d, and CA074Me were purchased from Peptide Institute Inc. (Osaka, Japan). CLIK-060 and CLIK-148 were synthesized as described previously (31). Recombinant *Ascaris* pepsin inhibitor was kindly provided from Dr. T. Kageyama (Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Aichi, Japan). Recombinant mouse interferon (IFN)- γ was purchased from Genzyme Corp. (Cambridge, MA). OVA grade V was purchased from Sigma. OVA-(266–281) peptide (TEWTSS-NVMEERKIKV) was purchased from Sawady Technology (Tokyo, Japan). F4/80 was purchased from Serotec LTD. (Bicester, UK). In-1 (anti-mouse Ii antibody), anti-mouse Lamp-2 antibody, and anti-mouse transferrin receptor (TfR) antibody were purchased from PharMingen (San Diego, CA). Polyclonal antibodies against purified rat spleen CE and CD were raised in rabbits and purified by affinity chromatography as described previously (32–34). CE and CD were purified from rat spleen according to the previously described methods (32, 33). Cathepsin B (CB) was purified from rat kidney as described (35). Papain and DNase were purchased from Worthington Biochemical (Freehold, NJ). Eagle's minimum essential medium was purchased from Nissui (Tokyo, Japan). Iscove's modified Dulbecco's medium, Dulbecco's modified Eagle's medium (DMEM), and RPMI 1640 were purchased from Invitrogen (Grand Island, NY).

Animals—C57BL/6 mice were purchased from Seac Yoshitomi (Fukuoka, Japan). Heterozygous (+/-) mice (36) were transferred to the Institute of Experimental Animal Sciences, Kyushu University Faculty of Dental Sciences, and kept in conventional facilities. Selection of CD^{-/-} mice from littermates obtained by heterozygous coupling was performed according to the method as previously reported (37, 38).

Cell Culture—Microglia were isolated from a primary culture of mouse brain as described previously (4, 39). Briefly, the whole brain from 3-day-old C57BL/6 mice was minced and treated with papain (90 units/ml) and DNase (2000 units/ml) at 37 °C for 30 min. The mechanically dissociated cells were seeded into plastic flasks at a density of 10⁷ pre-300 cm² in Eagle's minimum essential medium, 0.3% NaHCO₃, 2 mM glutamin, 0.2% glucose, 5 μ g/ml insulin, and 10% fetal calf serum (FCS, Mi medium), and maintained at 37 °C a 10% CO₂, 90% air atmosphere. Subsequent medium replacement was carried out every 3 days. After 10–14 days in culture, floating cells and weakly attached cells on the mixed primary cultured cell layer were isolated by gentle shaking of the flask for 10 min. The resulting cell suspension was transferred to Petri dish (Falcon 1001, Lincoln Park, NJ) and allowed to adhere at 37 °C. Unattached cells were removed after 30 min, microglia were isolated as strongly adhering cells. About 90% of these attached cells were positive for F4/80, makers for macrophage/microglial cell types. In some experiments, microglia prepared from CD^{-/-}, wild-type and hetero-type littermate mice were also used.

Peritoneal macrophages were obtained from 10-week-old male C57BL/6 mice injected peritoneally with 4.05% thioglycollate (Nissui) (2 ml/mouse) 3 days before harvesting cells. Thioglycollate-elicited macrophages were isolated by peritoneal lavage with DMEM followed by adherence to Petri dishes in the presence of 10% FCS in DMEM. Adherent macrophages were washed vigorously with phosphate-buffered saline (PBS) and scraped off the dish. Primary cultures were used for experiments immediately after their isolation.

Splenocytes and thymocytes were obtained from 10-week-old male C57BL/6 mice. The spleen and thymus were excised and cells dispersed into tissue culture dishes containing Hank's balanced salt solution. Cell suspensions were filtrated through cell strainer (Falcon 2350). Erythro-

cytes were lysed by incubation with 1.66% NH₄Cl for 5 min at room temperature. The splenocyte and thymocyte were washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

Bone marrow cells (BMCs) were obtained from 10-week-old male C57BL/6 mice femur and tibia. Using a 25-gauge needle, ice-cold PBS was forced through the bone cavity. Cells were drawn several times through the syringe to break up clumps. Cell suspensions were filtrated through cell strainer. Erythrocytes were lysed by incubation with 1.66% NH₄Cl for 5 min at room temperature. The bone marrow cells were washed and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 50 μ M 2-mercaptoethanol, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

Hepatocytes isolated from 10-week-old male C57BL/6 mice using a collagenase-perfusion procedure (40). The hepatocytes were washed and resuspended in DMEM supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin and 100 μ g/ml streptomycin, seeded on collagen-coated tissue culture dishes.

Generation of OVA-(266–281)-specific I-A^b Restricted Th Cell Hybridomas—OVA-specific Th cell hybridomas were prepared from C57BL/6 mice which were immunized with protein emulsified in complete Freund's adjuvant (100 μ g, subcutaneously). After 10 days, draining lymph nodes cells were cultured (4 \times 10⁶ cells/ml) with OVA (50 μ g) for 7 days. Viable cells were collected by centrifugation over Ficoll-Hypaque (Amersham Biosciences Inc., Piscataway, NJ) and cultured (10⁶ cells/ml) for another 4 days with irradiated syngenic splenocytes as APC (7 \times 10⁶ cells/ml) in the presence of OVA (10 μ g). Th cell blasts were collected by centrifugation over Ficoll-Hypaque and used for fusion. Th cell blasts were fused with BW5147, a NFAT-LacZ⁺, ab-TCR-AKR thymoma line (a kind gift from Dr. Kenneth Rock, Harvard Medical School, Boston, MA) using polyethylene glycol (PEG 1500, Roche Molecular Biochemicals). Briefly, Th cell blasts and BW5147 fusion partner cells were mixed with the same ration (10⁶-10⁷ cells) in a 50-ml conical centrifuge tube, and washed once in pre-warmed serum-free medium. The supernatant was aspirated off and the pellet loosened by gentle tapping. Then, 1 ml of 50% PEG was slowly added over the course of 1 min and 45 s. PEG was then diluted out with 10 ml of warm serum-free medium and the tube placed in a 37 °C water bath. After 8 min, cells were centrifuged and resuspended in complete medium to a density of 2–3 \times 10⁵/ml, and 0.1 ml of this suspension was added to yield a final concentration of 1.36 mg/ml hypoxanthine (Sigma), 1.76 mg/ml aminopterin (Sigma), and 388 mg/ml thymidine (Sigma). The hypoxanthine/aminopterin/thymidine-resistant Th cell hybrids were generally observed within 2 weeks of culture. Individual clones were expanded and initially screened for expression of CD3. CD3⁺ hybridomas were then screened for OVA and OVA-(266–281) peptide with I-A^b specificity in the interleukin (IL)-2 production assay described below. The hybridoma clone was established by subcloning a hybridoma clone which showed both OVA and OVA-(266–281) peptide specificity. Stock OVA-(266–281)-specific Th cell hybridomas were cultured in Iscove's modified Dulbecco's medium containing 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 50 μ M 2-mercaptoethanol.

Antigen Presentation Assay—Microglia or macrophages were seeded in 96-well flat-bottom tissue culture plates at a density 3 \times 10⁴ per well. After 18 h, the culture medium was replaced with fresh medium without or with IFN- γ (100 units/ml), and cells were incubated at 37 °C for 24 h. Immediately before the addition of OVA-specific Th cell hybridomas, microglia, or macrophages were gently washed three times with medium to completely remove IFN- γ . Th cells were added at a density 5 \times 10⁴ in Iscove's modified Dulbecco's medium, 10% FCS to wells containing the microglia in presence of native OVA (1 μ M) or OVA-(266–281) peptide (0.3 μ M). For analysis of Th cell-derived IL-2, supernatants from triplicate cultures were harvested after 24 h. IL-2 was quantified by using mouse IL-2 enzyme-linked immunosorbent assay (ELISA) kit (BioSource International Inc.).

In Vitro Digestion of OVA and Antigen Presentation Assay—OVA was dissolved in 100 mM citrate buffer, pH 5.5, at a final concentration of 10 mg/ml. CD and CE were added at a final concentration of 200 units/ml, and control sample contained 1 μ M pepstatin A. The sample sterilized by passing through 0.2- μ m filters, and incubated overnight at 37 °C. For CB digestions, OVA was dissolved at 10 mg/ml in 250 mM citrate buffer at pH 5.5 containing 1 mM EDTA and 2 mM dithiothreitol. The 20 μ g/ml CB was added, and control sample contained 1 μ M leupeptin. Microglia cells at a density 1 \times 10⁵ per well were fixed for 5 min at room temperature in 0.5% paraformaldehyde before assay. Fixed microglia cells were incubated with 1 \times 10⁵ per well of OVA-specific Th cell hybridomas in the presence of the digested OVA (final concentration 1

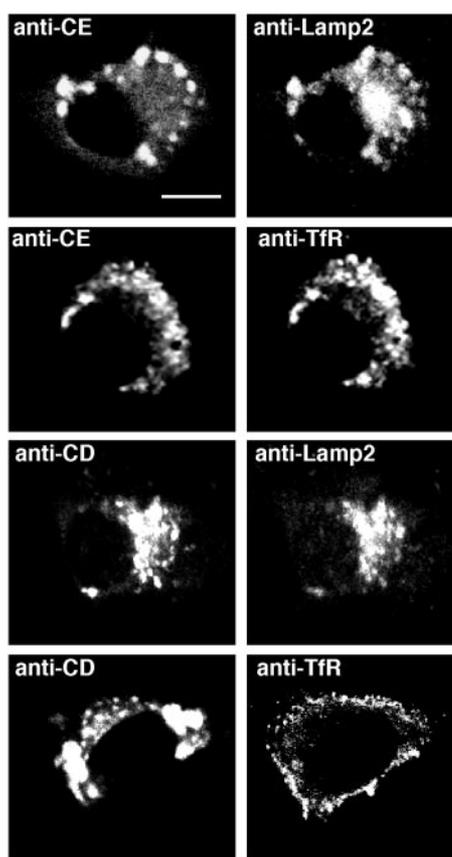


FIG. 2. **Differential intracellular localization of CE and CD in the primary cultured murine microglia.** Confocal laser scanning microscopic images showing localization of CE and CD in IFN- γ -treated microglia. The early endosomes and lysosomes were identified by anti-Lamp-2 antibody and anti-TfR antibody, respectively. It was noted that CE was mainly localized in the early endosomes, whereas CD was mainly localized in lysosomes. Scale bar = 10 μ m.

treated microglia failed to activate Th cell hybridomas even after treatment with the optimal concentration of native OVA or OVA-(266–281) peptide. These results indicate that microglia treated with IFN- γ , but not untreated cells, show strong antigen presenting ability for both native OVA and OVA-(266–281) peptide.

Effects of Protease Inhibitors on Antigen Presentation by Microglia—To assess roles of cathepsins in antigen presentation by microglia, IFN- γ -activated microglia pretreated with various inhibitors for 24 h were cultured with OVA-specific Th cell hybridomas. Upon stimulation with native OVA or OVA-(266–281) peptide, the amounts of IL-2 released from OVA-specific Th cell hybridomas into the culture medium were normalized by the control value. As shown in Fig. 4, pepstatin A (10 μ M), a specific inhibitor of aspartic proteases, significantly inhibited the IL-2 production from OVA-specific Th cells upon stimulation with native OVA presented by IFN- γ -activated microglia. However, OVA-specific Th cells produced IL-2 upon stimulation with OVA-(266–281) peptide presented by IFN- γ -activated microglia in the presence of pepstatin A. These observations were interpreted as that aspartic proteases, CE and CD, were involved in the degradation of OVA into antigenic peptide but not in the degradation of Ii chain in microglia.

CLIK-060, a specific inhibitor of CS, and CA074Me, a specific inhibitor of CB, significantly inhibited the IL-2 production from OVA-specific Th cell hybridomas upon stimulation with both native OVA and OVA-(266–281) peptide (Fig. 4). Leupeptin and E64-d, broad spectrum cysteine protease inhibitors, also almost completely suppressed the production of IL-2 upon stim-

ulation with both native OVA and OVA-(266–281) peptide (data not shown). However, CLIK-148, a specific inhibitor of CL, had no significant effect on the IL-2 production upon stimulation with either native OVA or OVA-(266–281) peptide. These results indicate that CS and CB are involved in the degradation of Ii chain or in both proteolytic processes for antigen and Ii chain in microglia.

We also examined effects of protease inhibitors on activation of OVA-specific Th cell hybridomas upon stimulation with phorbol myristate acetate (10 ng/ml) and ionomycin (1 μ M). Protease inhibitors used in the present study did not affect the activity of Th cell hybridomas quantitated by measuring activity of β -galactosidase with the concentration up to 10 μ M (data not shown). Although *Ascaris* pepsin inhibitor, a specific inhibitor of CE, with the concentration of 10 μ M inhibited the antigen presentation of both native OVA and OVA-(266–281) peptide, we omitted this observation from the present results because *Ascaris* pepsin inhibitor with the concentration of 10 μ M significantly reduced β -galactosidase activity.

Similar effects of those protease inhibitors were also obtained when IFN- γ -activated peripheral macrophages were used as APCs. Pepstatin A (10 μ M) significantly inhibited the IL-2 production from OVA-specific Th cell hybridomas upon stimulation with native OVA but not with OVA-(266–281) peptide (Fig. 5). CLIK-060 significantly inhibited the IL-2 production from OVA-specific Th cell hybridomas upon stimulation with native OVA and OVA-(266–281) peptide. On the other hand, CLIK-148 had no significant effect on the IL-2 production upon stimulation with either native OVA or OVA-(266–281) peptide.

Effects of Protease Inhibitors on the Degradation of Ii Chain in Microglia—To further elucidate the role of aspartic and cysteine proteases on proteolytic processing of Ii chain in microglia, effects of protease inhibitors on degradation of Ii chain were examined by 15% SDS-PAGE under the reduced condition and immunoblotting by using the Ii chain cytoplasmic tail-specific monoclonal antibody In-1. IFN- γ -activated microglia were cultured for 24 h in the absence and presence of protease inhibitors. In cell extracts from non-treated microglia, two bands corresponding to p31 and p41 isoforms of intact Ii chain were detected (Fig. 6). The 10-kDa proteolytic fragment corresponding to Ii-p10, an NH₂ terminal fragment of Ii chain, was slightly detectable. On the other hand, there was a marked accumulation of Ii-p10 in leupeptin-treated microglia (Fig. 6). The accumulation of Ii-p10 was also detected in microglia treated with CLIK-060 or CA074Me. On the other hand, CLIK-148 showed no detectable effect on the accumulation of Ii-p10 (data not shown). These results indicate that CB and CS play a pivotal role in the degradation of Ii chain in microglia.

In contrast, accumulation of Ii-p10 was not detected in microglia treated with pepstatin A (Fig. 6). To further assess the requirement of aspartic proteases in the initial stages of Ii chain processing, microglia were treated with leupeptin in the presence of pepstatin A. Inhibition of total aspartic protease activity by pepstatin A did not prevent the accumulation of Ii-p10 in leupeptin-treated microglia indicating that aspartic proteases were not required for even in the initial stages of Ii chain processing.

Requirement of Proteolytic Processes by Aspartic Proteases to Generate Antigen Peptide from Native OVA—To directly assess the requirement of aspartic proteases, CE and CD, for generation of antigenic peptide from native OVA, native OVA was digested with, respectively, purified CE or CD *in vitro* for 24 h at the relatively mild acidic condition similar to the early endosome (*i.e.* pH 5.5). The digested fragments were added to the co-culture system consisting of fixed microglia and OVA-

FIG. 3. Abilities of primary cultured murine microglia in processing native OVA and antigenic peptide. OVA-specific Th cell hybridomas were cultured together with graded numbers of IFN- γ -treated (●) or untreated (○) microglia in the presence of native OVA or OVA-(266–281) peptide. After 24 h, supernatants from triplicate cultures were harvested, and Th cell-derived IL-2 was measured by ELISA. The data shown are from a representative experiment of three performed.

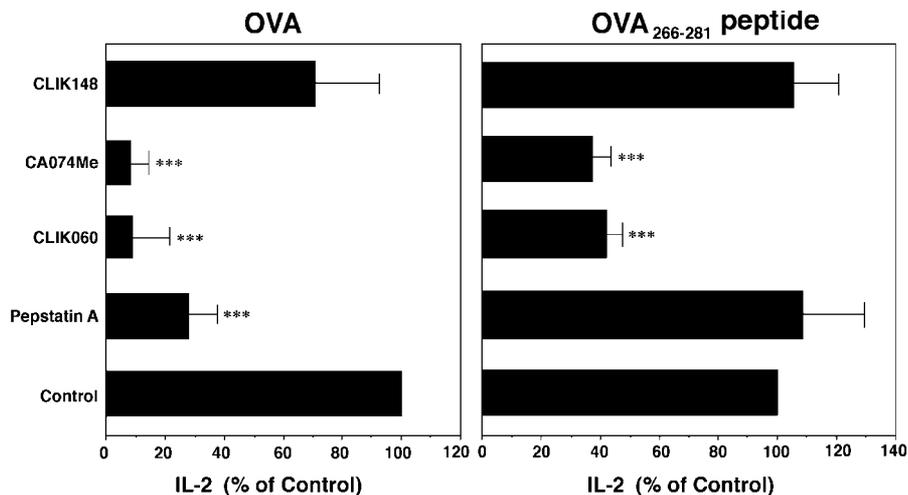
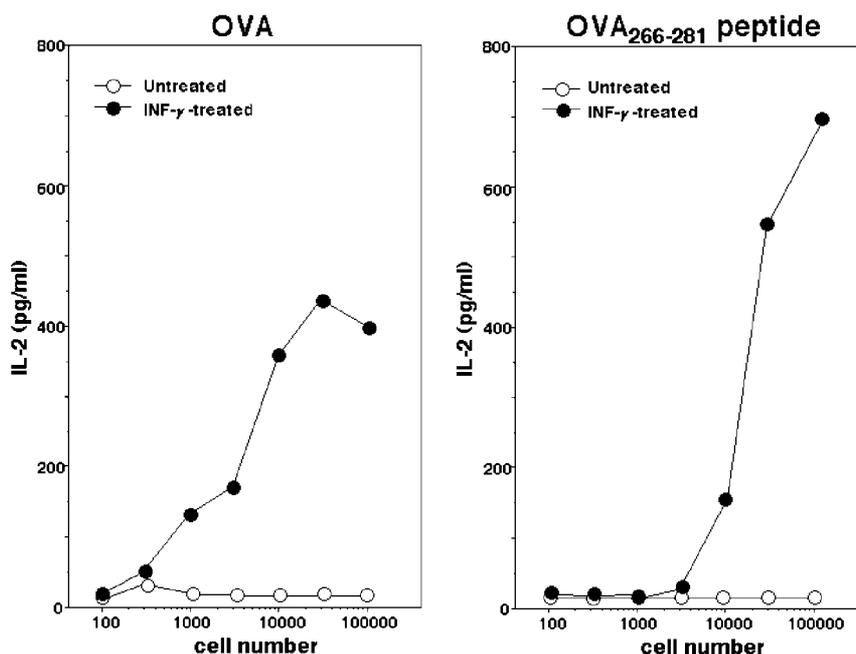
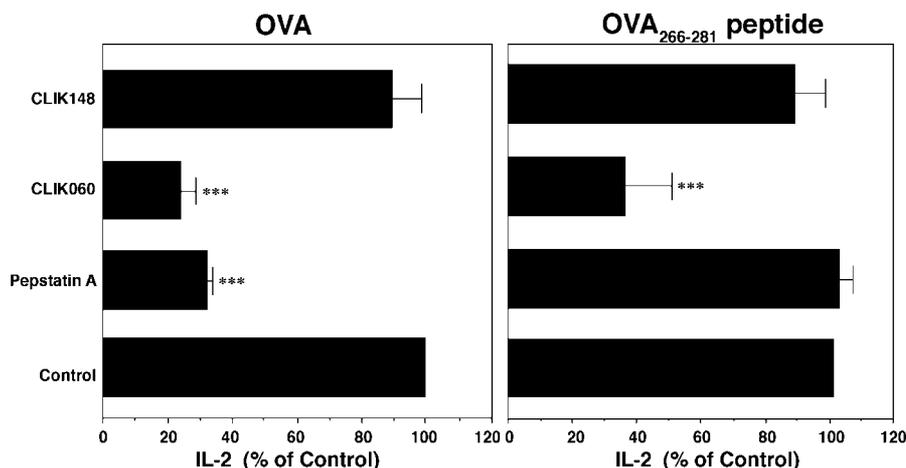


FIG. 5. Effects of protease inhibitors on antigen presentation of native OVA and antigenic peptide by primary cultured murine peripheral macrophages. Each protease inhibitor (10 μ M) was applied to culture medium 24 h before adding OVA-specific Th cell hybridomas and native OVA or OVA-(266–281) peptide. Supernatants from triplicate cultures were harvested, and Th cell-derived IL-2 was measured by ELISA. Each column and bar represents the mean \pm S.D. of three experiments, respectively. Each value is expressed as a percentage of the amount to normalize the values with respect to the amount of IL-2 in the absence of inhibitors (control). ***, $p < 0.001$ versus control.

specific Th cell hybridomas. In this system, OVA-specific Th cell hybridomas can be activated to produce IL-2 only in the presence of antigenic peptide, because microglia were lightly fixed with 0.5% paraformaldehyde to prevent further processing of native OVA through endocytic uptake (Table I). Upon stimulation with OVA digested by purified CE, OVA-specific

FIG. 4. Effects of protease inhibitors on antigen presentation of native OVA and antigenic peptide by primary cultured murine microglia. Each protease inhibitor (10 μ M) was applied to culture medium 24 h before adding OVA-specific Th cell hybridomas and native OVA or OVA-(266–281) peptide. Supernatants from triplicate cultures were harvested, and Th cell-derived IL-2 was measured by ELISA. Each column and bar represents the mean \pm S.D. of three experiments, respectively. Each value is expressed as a percentage of the amount to normalize the values with respect to the amount of IL-2 in the absence of inhibitors (control). ***, $p < 0.001$ versus control.



Th cell hybridomas could produce a significant amount of IL-2 (Table I). A similar result was also obtained upon stimulation with OVA digested by purified CD. When OVA was digested by purified CE or CD in the presence of pepstatin A, digested fragments failed to produce a significant amount of IL-2 (data not shown). On the other hand, there was no significant pro-

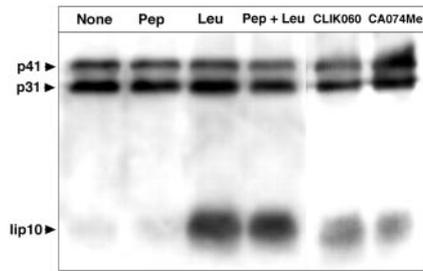


FIG. 6. Effects of protease inhibitors on the Ii-chain processing in primary cultured murine microglia. Immunoblot analysis of the cell extract of microglia by the Ii chain cytoplasmic tail-specific monoclonal antibody In-1. The cell extracts from microglia treated with protease inhibitors were analyzed by SDS-PAGE under reduced condition following immunoblotting. *Leu*, leupeptin; *Pep*, pepstatin A.

TABLE I
Antigen presentation of OVA digested with CE, CD, and CB by fixed microglia

Data are mean \pm S.D. values of three experiments.

	IL-2 (pg/ml)
Native OVA	1.64 \pm 1.60
OVA-(266-281)peptide	9.74 \pm 4.91 ^a
OVA digested with CE	6.70 \pm 2.29 ^a
OVA digested with CD	4.78 \pm 0.24 ^a
OVA digested with CB	0.54 \pm 0.37

^a $p < 0.05$ versus the amount of IL-2 obtained by native OVA.

duction of IL-2 upon stimulation with OVA digested by purified CB. These results indicate that CE and CD are able to digest native OVA into antigenic peptide.

Generation of Antigenic Epitope in Microglia Prepared from CD^{-/-} Mice—Finally we conducted experiments utilizing microglia prepared from CD^{-/-} mice to better characterize the role of aspartic proteases in the generation of OVA antigenic epitope. Primary cultured microglia prepared from CD^{-/-}, wild-type, and hetero-type littermate mice were stimulated with IFN- γ for 24 h. After co-culture with OVA-specific Th cell hybridomas in the presence of native OVA for 24 h, IL-2 released in the culture medium was measured. Although the efficiency for presenting OVA antigenic peptide to Th cell hybridomas in microglia prepared from CD^{-/-} mice was slightly reduced as compared with those from their wild- and hetero-type littermates, the difference did not reach the statistical significance (Fig. 7). These results strongly suggest that CD is dispensable for presenting OVA-derived peptide to OVA-specific Th cell hybridomas by microglia.

DISCUSSION

Our observations here show that CE exists in murine microglia/macrophages as the mature enzyme, whereas this enzyme exists in other peripheral APCs such as splenocytes and BMCs as enzymatically inactive proform. The difference in molecular form of CE among APCs is considered to reflect the difference in its intracellular localization, because CE was found to be converted into the mature form in an autocatalytic manner only after entering acidic compartments such as endosomes (4). Furthermore, the expression level of CE mRNA in microglia was found to be markedly increased upon stimulation with IFN- γ which is well known to induce MHC class II expression as well as co-stimulatory molecule which are required for Th cell activation (43, 44). These unique properties of CE in microglia/macrophages prompted us to further investigate the role of CE in the MHC class II-mediated antigen presentation by microglia. The involvement of CE in antigen presentation of OVA by microglia was first assessed by using pepstatin A, a specific inhibitor of aspartic proteases. Pepstatin A inhibited

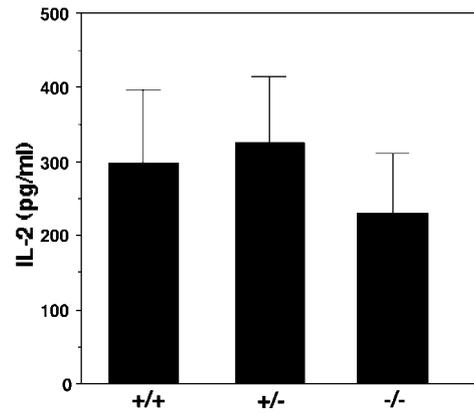


FIG. 7. Ability of antigen presentation of OVA by primary cultured murine microglia defected of CD. OVA-specific Th cell hybridomas were cultured together with IFN- γ -treated microglia prepared from CD^{-/-} mice as well as their wild (+/+) and hetero (+/-) type littermates in the presence of native OVA. After 24 h, supernatant from triplicate cultures were harvested and Th cell-derived IL-2 was measured by ELISA. Each column and bar represents the mean \pm S.D. of three experiments, respectively.

the processing of OVA, but not inhibited the presentation of OVA antigenic peptide. The possible involvement of pepstatin A-sensitive aspartic proteases in the processing of antigenic peptide of native OVA was further substantiated by the present observations that digested fragments of native OVA by CE or CD could be recognized by OVA-specific Th cell hybridomas. In the present study, we could obtain no evidence indicating the involvement of aspartic proteases in the processing of Ii chain. However, there are several discrepant reports showing that aspartic protease inhibitors effectively prevented the processing of Ii chain (24, 25). One reason for this discrepancy may be that aspartic proteases are involved in antigen presentation by microglia in a different way from that by peripheral APCs such as B cells. Additional experiments are necessary to elucidate this deduction.

Our observations here further showed that microglia isolated from CD^{-/-} mice retained an ability for antigen presentation. This is consistent with recent studies utilizing peripheral APCs prepared from CD^{-/-} mice indicating that CD is not involved in degradation of Ii chain or generation of antigenic peptides (17, 30). The present finding that pepstatin A inhibited the antigen presentation of OVA by microglia suggest that CE is essentially involved in processing of OVA because CD and CE are only known for pepstatin A-sensitive endosomal/lysosomal aspartic proteases in mammalian cells. Recent evidence has also shown that CE is closely linked with the proteolytic processing of tetanus toxin (45). There is increasing evidence that agrees with a role for CE in antigen processing. CE is expressed in APCs such as Langerhans cells and B cells (26, 46). Furthermore, it has been reported that CE is responsible for the processing of OVA in A20 cells (7). Although CE in B cells is found in the endoplasmic reticulum, Golgi, and endosome-like compartment (26), the distinct subcellular localization and molecular form is unclear. We have previously reported that CE was found to be localized in the early endosome as well as endoplasmic reticulum and Golgi of primary cultured rat microglia (4). Thus it is likely to consider that the expression of CE in activated microglia is mainly associated with processing of endocytic antigen into the endosomal compartments. The requirement of CE for the processing of exogenous antigens in microglia is to be further elucidated in future studies utilizing CE^{-/-} mice that have been under generation.

Although cysteine proteases are considered to be critical for the terminal step of Ii breakdown, their exact role varies among

different APCs (14, 18, 22, 23). In B cells and dendritic cells, CS exclusively mediates the degradation of Ii to CLIP. For this purpose, thymic cortical cells and macrophages use CL and cathepsin F, respectively. Microglia have been suggested to use CS or CL for the degradation of Ii to CLIP, because microglia express CS and CL, but not cathepsin F (47, 48). To address this suggestion, we conducted experiments utilizing specific inhibitors for cysteine proteases and found that the degradation of Ii to CLIP in microglia was mediated by both CS and CB but not CL. Similar results were also obtained when peripheral macrophages were used as APCs. Although there is increasing evidence that microglia is distinct from peripheral macrophages in various aspects, microglia were found to closely resemble peripheral macrophages as APCs.

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