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Participation of cathepsin B in processing of antigen presentation to MHC class II

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Cellular and humoral immune responses to vaccines of hepatitis B type and rabies were inhibited by specific inhibitors of cathepsin B, specific synthetic substrates of cathepsin B and anti-cathepsin B antibody. Therefore the lysosomal cathepsin B of antigen presenting cells plays an essential role in processing of these antigens for presentation to MHC class II. One of the active sites of cathepsin B, $VN_{217\ 222}$ shares highly homologous sequences with a part of the desetope, a binding domain of antigenic peptides, $VN_{57\ 62}$ of MHC class II, β-chain. This evidence suggests that the peptides processed by the substrate specificity of cathepsin B exhibit a common affinity to bind with the desetope of MHC class II, β-chain.

Cathepsin B; Antigen processing; MHC class II; HBsAg; Revis vaccine

1. INTRODUCTION

Knowledge on the structures and properties of intralysosomal proteases has been accumulated recently, especially on cysteine proteases, such as cathepsin B, H, L, J, S and N [11,12]. On the other hand, increased awareness of important physiological roles in the intracellular protein degradation process has been paralleled by the increased attention to the different functional shares of individual cathepsins.

The determinant proteases responsible for individual protein degradation and processing appear to differ with different physiological purposes. Which lysosomal cathepsin is the determinant protease responsible for processing of specified antigens on the course of antigen presentation to MHC class II? T lymphocyte activation occurs only via interaction of antigen-specific clonally distributed T cell receptors (TCR) with structures on the surface of the antigen presenting cells (APC). The immunogenic ligands to T cells must be presented as a complex with major histocompatibility molecules class II (MHC class II) [1,2]. The processing of immunogenic fragments from the natural antigen is thought to be an intralysosomal event after endocytosis of antigens into macrophages, but the protease responsible for the antigen processing has not been determined. On the other hand, the primary structure and tertiary structure by X-ray crystallography of cathepsin B has been established by Katunuma et al. [18,19]. We investigated the protease responsible for the processing of hepatitis B vaccine (HBsAg) [3,4] and rabies vaccine [5] using various kinds of specific inhibitors of cysteine proteases, such as, E-64 [6,7], an inhibitor for all members of the cysteine protease family and CA-074 [8], a specific inhibitor of cathepsin B and also F(ab)′ of anti-cathepsin B antibody [9]. Herein, we report the mechanisms on antigen processing by cathepsin B and antigenic peptide presentation to MHC class II at the molecular level.

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Abbreviations. HBsAg, hepatitis B surface antigen; MHC-class II, major histocompatibility complex class II; RV, rabies vaccine, Rx-PBMC, irradiated autologous peripheral mononuclear cell; APC, antigen presenting cells; E-64a, *N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucine-4-aminobutylamide; E-64d, *N*-(L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl)-L-leucine-3-methylbutylamide; CA-074, *N*-(L-3-*trans*-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline); Z-RR-MCA, benzyloxycarbonyl arginyl arginyl methylcoumaryl amide.

2. MATERIALS AND METHODS

2.1. *Materials*

Male BALB/c mice were obtained from Clea Japan, Tokyo and were used at 8 to 10 weeks of age The ELISA kit for anti-HBs was purchased from Abbott Laboratories, North Chicago. Yeast-derived recombinant hepatitis B surface antigen (subtype adr, HBsAg) was purchased from Kaketsuken Pharmaceutical Co., Kumamoto, Japan For immunization, HBsAg was emulsified in aluminum hydroxide gel. Rabies vaccine (RV), peptides of rabies vaccine, $ER_{381\ 390}$; EECLDALESTMTTKSVSFR derived from rabies glyco-protein and $AP_{121\ 133}$; ALTGGMELTRDPTVP [5,15,16] from rabies nuclear-protein, and also human T-cell clones, namely 2C5 and B8, which respond specifically to rabies vaccine were a gift from Dr. E. Celis. The autologous peripheral blood mononuclear cells irradiated with 3,000 rd

(Rx-PBMC) was prepared as APC (antigen presenting cells) Complete Freund's adjuvant was from Difco Laboratories. Cysteine protease inhibitors; E-64a, E-64d, and CA-074 were a gift from Taisho Pharmaceutical Co., Tokyo E-64a was dissolved in saline, and E-64d and CA-074 was in dimethylsulfoxide at a concentration of 10 mg/ml, respectively, and diluted in saline at the indicated concentrations before use. The specific substrate for cathepsin B, Z-RR-MCA was purchased from the Institute for Protein Research, Peptide Inst. Inc., Osaka [^{3}H]Thymidine was purchased from New England Nuclear, Boston, MA. RPMI 1640, metrizamide and heparin were purchased from Sigma Chemicals, St Louis. A series of synthetic peptides, corresponding to antigenic domain of HBsAG (adr), $SR_{64\ 79}$, $SL_{64\ 94}$ and $FG_{93\ 112}$ were synthesized by Applied Biosystem Model A 431 peptide synthesizer F(ab)' of specific antibody to cathepsin B, was prepared by the method of Kominami et al. [14].

2.2. *Immunization with HBsAG or rabies vaccine and treatment of cathepsin inhibitors*

Each group of this study consisted of 10 male BALB/c mice. Mice were injected once intraperitoneally with different doses of HBsAg (0.4 μg, 0 8 μg and 4 0 μg/mouse) with or without a cathepsin inhibitor, E-64a, E-64d or CA-074 The cathepsin inhibitor was injected twice intraperitoneally at 100 μg/mouse before and after 2 h of immunization BALB/c mice were injected in the base of the tail and hind footpads with 5 μg/mouse of rabies vaccine or 30 μg/mouse of synthetic peptide $ER_{281\ 296}$ in complete Freund's adjuvant with/without 300 μg/mouse of cysteine inhibitors, such as E-64a, E-64d or CA-074

2.3 *Assay of primary antibody production for HBsAg in vivo*

The amounts of IgG-class anti-HBsAg in the pooled mice sera on days 7, 10, 12, 14, 21 and 42 after immunization were titrated in triplicate using the ELISA Kit Results were presented as mean ± 1 S.D (mU/ml).

2.4. *Proliferation assay of primed splenocytes by rechallenge of antigenic proteins or peptides*

At the 14th day after priming by HBsAg, splenocytes (3×10^6 cells/ml) of the primed BALB/c mice were prepared and incubated on a 96-well culture plate with various concentrations of HBsAg or synthetic peptides with or without cathepsin B inhibitor (25 μg/ml) for 72 h in triplicate in RPMI 1640 medium supplemented with 3% mouse serum, 2-mercaptoethanol (5×10^{-6} M), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cultures were pulsed during the last 8 h with 1 μCi/well of [^{3}H]thymidine and harvested onto glass fiber filters. Radioactivity was quantified by liquid scintillation counting and the results of the cell proliferation were expressed as counts per minute (cpm). The results are presented as mean cpm ± 1 S D. Cell proliferation was considered significant when the ratio of the cpm obtained in the presence of antigen to that in the absence of antigen was ⩾ 2 5.

2 5. *Cell proliferative response of B8 and 2C5 clones for rabies vaccine or synthetic peptides $AP_{121\ 135}$ and $ER_{281\ 296}$, respectively in vitro*

T cell clone 2C5 or B8 (5×10^4 cells/well) in complete medium (RPMI 1640 plus 10% human AB serum) was incubated with rabies vaccine or synthetic peptide, $ER_{281\ 296}$ or $AP_{121\ 135}$ at concentrations ranging from 0 1 μg/ml to 0.4 μg/ml, respectively, in the presence of 5×10^4 cells/well of Rx-PBMC plus various concentrations (1.25, 2.5 and 5 μg/ml) of cysteine protease inhibitors E-64d, E-64a, or CA-074 for 72 h at 37°C, in 5% CO_2 in a final volume of 200 μl/well in complete medium, and 1 μCi/well of [^{3}H]thymidine were pulsed for the final 16 h and the incorporation of [^{3}H]thymidine was assayed The Rx-PBMC were preincubated with various concentrations of rabies vaccine for 12 h as a control, and after washing with medium, 2C5 or B8 cells were incubated with the antigen-pulsed Rx-PBMC in the presence of 5 μg/ml of inhibitor E-64d or CA-074, and then [^{3}H]thymidine incorporation was assayed

3. RESULTS AND DISCUSSION

3.1. *Cathepsin B inhibitors suppress the primary antibody response to HBsAg in vivo*

A single immunization with HBsAg to BALB/c mice induced a sufficient increase in the anti-HBs titer (IgG) to 200 mIU/ml on the 10th day after immunization and reached a maximum level of 800 mIU/ml on the 21st day as shown in Fig. 1 [10], whereas the titers of antibody by priming with less than 1.0 μg of HBsAg were significantly lower (below 100 mIU/ml throughout 21 days).

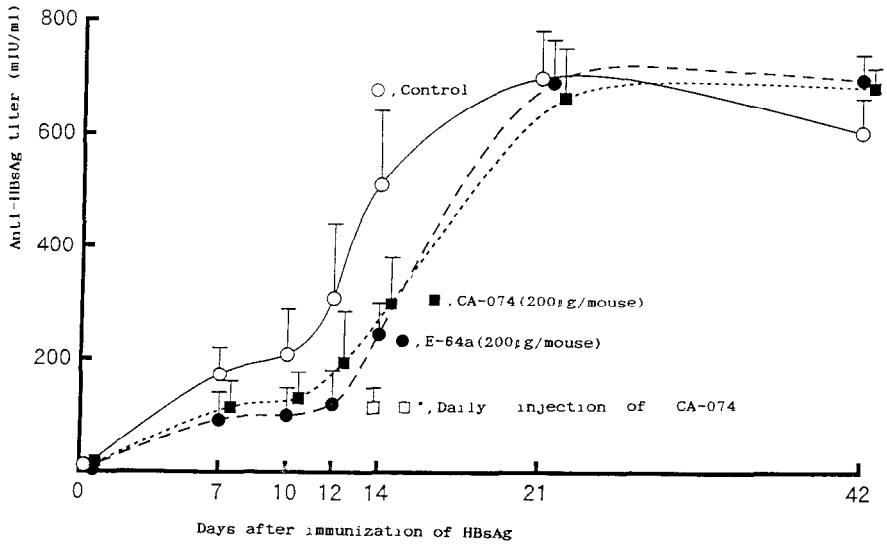

Fig 1. Effect of cathepsin B inhibitors on production of primary antibody for HBsAg in vivo BALB/c mice were immunized with 4 μg/head of HBsAg intraperitoneally Cathepsin B inhibitors were administered twice intraperitoneally at 2 h before and after immunization ●, E-64a (200 μg/mouse); ■, CA-074 (200 μg/mouse), ○, control (saline); □, daily injection of CA-074 (200 μg/mouse) for 14 days after priming Anti-HBs IgG titers in sera were titrated by ELISA at the indicated time Data are expressed as mean ± S.D

Treatment of E-64a or CA-074 caused a marked suppression of anti-HBsAg production and delay in the response. Maximum suppression by the inhibitors was observed on the 14th day after immunization. That is, 500 mIU/ml in the control and 250 mIU/ml by a single treatment with CA-074 or E-64a. Furthermore, daily treatment with CA-074 for 14 consecutive days after immunization caused strong suppression of the antibody production to 120 mIU/ml level on the 14th day.

3.2. *Cathepsin B inhibitors suppress primary T cell sensitization and also proliferative responses of primed splenocytes by rechallenge of whole vaccine or the antigenic peptide of HBsAg*

BALB/c mice were primed with 4 μg/mouse of HBsAg and then 14 days after priming, the primed splenocytes were rechallenged with HBsAg in vitro in the presence or absence of 5 μg/ml of cathepsin B inhibitors and the proliferative response was assayed by [^{3}H]thymidine incorporation. The primed splenocytes showed a proliferative response by rechallenge with HBsAg, while in the presence of cathepsin B inhibitors such as E-64a or CA-074, the responses were markedly suppressed to 30–50% of that without inhibitor as shown in Exp. 1 of Table I. Splenocytes primed by HBsAg in the presence of CA-074 or E-64a showed a very weak response, but also this proliferative response was further suppressed by administration of E-64a or CA-074 to 40–45% of that without the inhibitor.

The most susceptible bond in the HBsAg sequence [13] to cathepsin B [11,12] is considered to be the bond between 79th and 80th peptide, and this most susceptible bond is practically cleaved by purified cathepsin B in vitro. The synthesized 16-mer peptide, SR_{64-79} [14] of HBsAg, the extention peptide in the direction of the N-terminus before RR, showed the strongest proliferative response to primed splenocytes as shown in Exp. 2 of Table I, and the other domain in the HBsAg molecule showed a much weaker response in the same system. The response by this active peptide was not inhibited by cathepsin B inhibitors. Therefore, it is possible to conclude that the SR_{64-79} sequence is the most powerful antigenic peptide of HBsAg and the fragment is expected to be naturally formed by limited proteolysis of cathepsin B in situ.

3.3. *Inhibitory effect of cathepsin B inhibitors, specific substrate of cathepsin B and F(ab)' of anti-cathepsin B antibody on the proliferative responses of human T cell clones with antigenic peptides*

Human T cell clones, 2C5 and B8 [5,15,16] in the presence of Rx-PBMC showed a high proliferative response to 0.2 μg/ml of rabies vaccine. The responses were suppressed by E-64d and CA-074 in a dose-dependent manner as shown in Fig. 2(a)-1 and (b)-1. When Rx-PBMC prepulsed with rabies vaccine was used as the APC, this response was not suppressed by cathepsin B inhibitors as the dotted line shows. 2C5 and B8 in the presence of Rx-PBMC was also stimulated by $ER_{281\ 299}$ and $AP_{121-135}$, respectively. However, the pro-

Table I

Effect of cathepsin B inhibitors on proliferative response of primed splenocytes by rechallenge of HBsAg or antigenic peptides of HBsAg

	In vivo sensitization with	[^{3}H]Thymidine incorporation into splenocytes (cpm) by in vitro rechallenge of:			
		None	HBsAg	HBsAg + E-64a	HBsAg + CA-074
Exp. 1	None	142 ± 48 (1)	412 ± 57 (2.9)	213 ± 65 (1.5)	190 ± 50 (1.4)
	HBsAg	440 ± 64 (1)	4,092 ± 540 (9.3)	2,112 ± 660 (4.8)	1,980 ± 360 (4.5)
	HBsAg + E-64a	214 ± 58 (1)	550 ± 124 (2.3)	481 ± 143 (2.0)	410 ± 185 (1.7)
	HBsAg	183 ± 41 (1)	5,475 ± 439 (30)	1,336 ± 328 (7.3)	1,841 ± 210 (10)
	HBsAg + CA-074	113 ± 38 (1)	464 ± 85 (4.1)	184 ± 23 (1.6)	206 ± 58
		None	$SR_{64\ 79}$		$SL_{64\ 94}$	$FG_{93\ 112}$
			− CA-074	+ CA-074		
Exp. 2	HBsAg	428 ± 56 (1)	2,568 ± 410 (6.0)	2,482 ± 550 (5.8)	1,626 ± 359 (3.7)	556 ± 66 (1.2)

BALB/c mice were immunized as described in Fig. 1. 14 days after immunization, 1×10^5 cells of splenocytes were incubated for 72 h with 4 μg/ml of HBsAg and 1 μg/ml of cathepsin B inhibitor, E-64a or CA-074. Cells were pulsed with 1 μCi of [^{3}H]thymidine for the last 12 h and [^{3}H]thymidine incorporations were assayed. Data are shown as mean ± S.D. cpm. $SR_{64\ 79}$: SCPPI CPGYRWMCLRR. $SL_{64\ 94}$: SCPPI CPGYRWMCLRRFI I FI FI LLLCLI FL. $FG_{93\ 112}$: FLLVLLDYQGMLPVCPLLPG. Each value indicates the mean ± S.D of 5 observations. $P < 0.05$; significant difference from control group (*Student's t*-test).

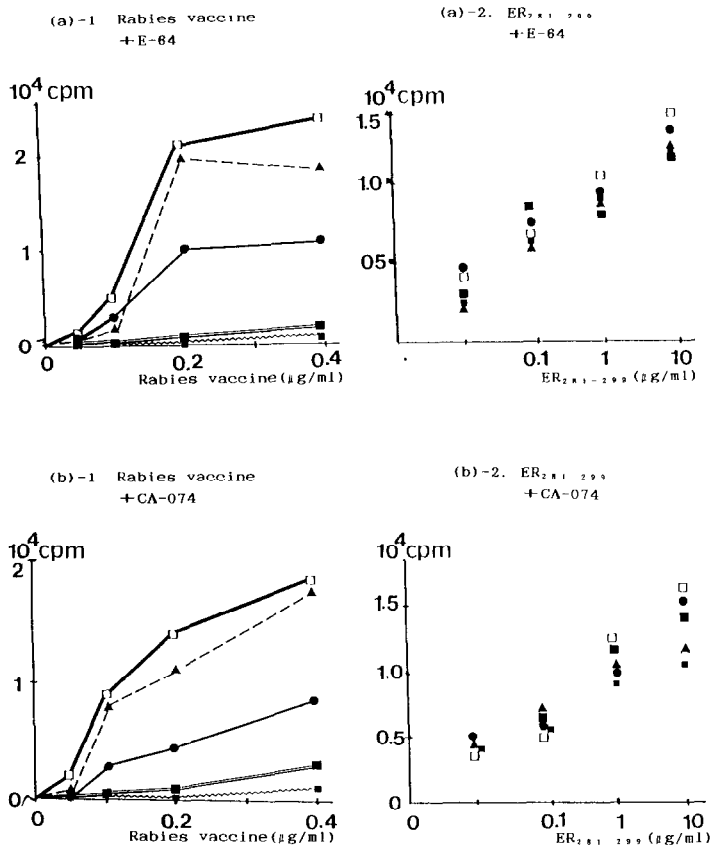

Fig. 2. Inhibitory effects of E-64d and CA-074 on proliferative response of human T cell clones by rechallenge of rabies vaccine or antigenic peptide. 3×10^4 cells of 2C5 clone were incubated for 72 h with rabies vaccine ((a)-1, (b)-1) or synthetic peptide, $ER_{281\text{-}299}$ ((a)-2, (b)-2) in the presence of 1×10^5 cells of Rx-PBMC plus various concentrations of cathepsin B inhibitors. E-64d or CA-074. E-64d was used in (a)-1 and (a)-2, and CA-074 was used in (b)-1 and (b)-2. (▲ ▲) Prepulsed Rx-PBMC + 5 μg/ml of inhibitor; (●——●) Rx-PBMC + antigen + 1.25 μg/ml of inhibitor; (■===■) Rx-PBMC + antigen + 2.5 μg/ml of inhibitor; (▪---▪) Rx-PBMC + antigen + 5 μg/ml of inhibitor, (□——□) Rx-PBMC + antigen

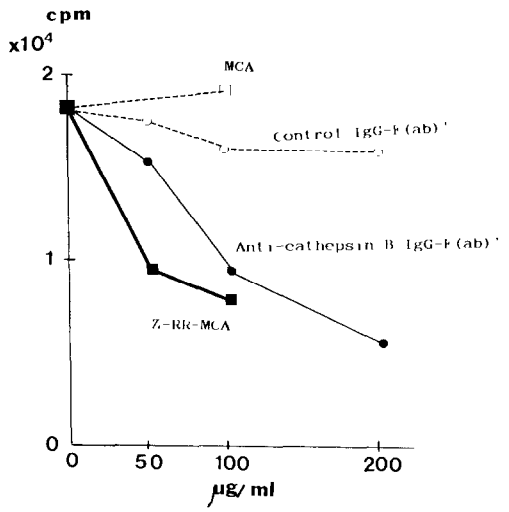

Fig. 3. Inhibition of T cell proliferation by F(ab)' of anti-cathepsin B antibody and specific substrate of cathepsin B, Z-RR-MCA. 3×10^4 cells of 2C5 clone were incubated for 72 h with 1×10^5 cells of Rx-PBMC and 0.2 μg/ml of rabies vaccine in the presence of various concentrations of F(ab)' of anti-cathepsin B antibody or control IgG. Cells were pulsed with 1 μCi/well of [^{3}H]thymidine for the last 12 h and harvested.

liferative response by these antigenic peptides was not suppressed by cathepsin B inhibitors, E-64 and CA-074 as shown in Fig. 2(a)-2 and (b)-2. This response of the 2C5 clone to rabies vaccine was also suppressed by coincubation with F(ab)' of cathepsin B-specific antibody as shown in Fig. 3. Z-RR-MCA, a specific substrate of cathepsin B [17], strongly suppressed the response, and MCA itself shows no effect. The same results were obtained in these proliferative responses mentioned above on the B8 clone (data not shown). It was reconfirmed that cathepsin B is the determinant protease responsible for antigen processing of vaccines of HBsAg and rabies presented with MHC, class II.

3.4. *Inhibitory effect of CA-074 and E-64d on the priming of mice in vivo and rechallenge in vitro with rabies vaccine or the antigenic peptides*

Splenocytes from mice primed with the rabies vaccine showed a high proliferative response to rechallenge with the rabies vaccine and also to antigenic peptide $ER_{281\text{-}299}$ in vitro. Since the priming with rabies vaccine in the presence of CA-074 or E-64d was suppressed strongly, the splenocyte response to the rechallenge with the vac-

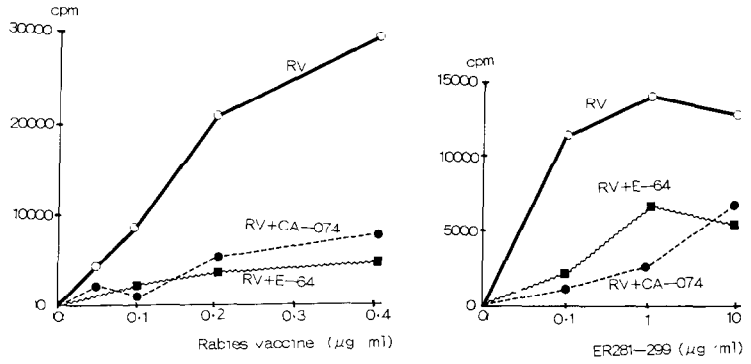

Fig. 4. Inhibitory effect of E-64d and CA-074 on priming of BALB/c mice with rabies vaccine. Splenocytes from BALB/c mice primed with rabies vaccine in vivo in the presence or absence of E-64d or CA-074 were incubated with the vaccine or $ER_{281\ 299}$ and the proliferative responses were assayed.

cine or $ER_{281\ 299}$ was suppressed as shown in Fig. 4. However, when mice were primed with $ER_{281\ 299}$ with adjuvant in the presence of CA-074 or E-64d, the priming was not suppressed by these inhibitors (data not shown). These findings indicate that cathepsin B inhibitors cannot interfere with the binding of the already processed peptides to MHC class II and any other processes of the immune responses except the proteolytic processing of antigens.

3.5. *Antigenic peptides processed by limited proteolysis of cathepsin B show common affinity to bind with MHC class II*

Since cathepsin B shows substrate specificity for particular susceptible sequences, the processed peptides by cathepsin B contain a particular common sequence in the ragged C-terminus sequences, such as -XYRR etc. It is possible to speculate that the fragments formed by selective proteolysis of cathepsin B can bind commonly with the desetope of MHC class II β-chain. The synthetic peptides, MCLRR and YRWMCLRR, ragged C-end sequences of the antigenic peptide of HBsAg, SR_{64-79}, inhibited the immune responses to rabies vaccine. When the 2C5 clone with Rx-PBMC was immunized with rabies vaccine in the presence of MCLRR or YRWMCLRR, the proliferative response of [^{3}H]thymidin incorporation was suppressed to 75% or 60% of that in the absence of these peptides, respectively. It is possible to speculate that these peptides inhibit antigen processing by cathepsin B and also inhibit the binding of processed peptides to the desetope of MHC class II.

One of the active sites of cathepsin B [18,19], $VN_{217\text{-}223}$, -VANSWNT-, shows high homology with part of the binding domain (desetope) of MHC class II, β- chain, $VN_{57\ 63}$, -VAESWNS- [20]. Furthermore, within the common homologous sequences, alanine and asparagine are the same amino acids on cathepsin B and MHC class II, while the alanine and the asparagine in the same active domain of the other cathepsins are commonly substituted by lysine and glycine, respectively, as shown in Fig. 5. This is one of the important reasons why only the cathepsin B specifically makes antigenic peptides to bind to MHC class II.

Here, we propose the hypothesis as illustrated in Fig. 6. The C-terminal domain sequences in antigenic peptides showing common affinity for MHC class II are selected by the substrate specificity of cathepsin B. The sequences of antigenic peptides isolated from MHC class II molecules has been reported [23–25]. The majority of these peptides contain affinity sequences for cathepsin B, although some of the peptides reported do not contain the affinity sequences for cathepsin B. Many explanations for this problem may be possible. Some additional proteolytic modifications may follow during the antigen presentation, or other lysosomal proteases than cathepsin B may participate in some other antigen processing, and the antigenic peptides are affinity-linked to another domain of the desetope of MHC class II. We conclude that the antigenic fragments of HBsAg and rabies vaccines are processed by cathepsin B and show common affinity to bind to the desetope of MHC class II, β-chain.

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			57						63			
MHC-II, β-chain	R	P	V	A	E	S	W	N	S	Q	K	D
Cathepsin B	W	L	V 217	A	N	S	W	N	T 223	D	W	G
Cathepsin L	W	L	V	K	N	S	W	G	K	E	W	G
Cathepsin H	W	I	V	K	N	S	W	G	S	N	W	G
Cathepsin S	W	L	V	K	N	S	W	G	L	H	F	G
Cathepsin J	W	I	V	K	N	S	W	G	S	Q	W	G
Papain	I	L	I	K	N	S	W	G	T	G	W	G

Fig. 5. One of active sites of cathepsin B shares homology with a part of antigenic peptide binding domain (desetope) of MHC class II.

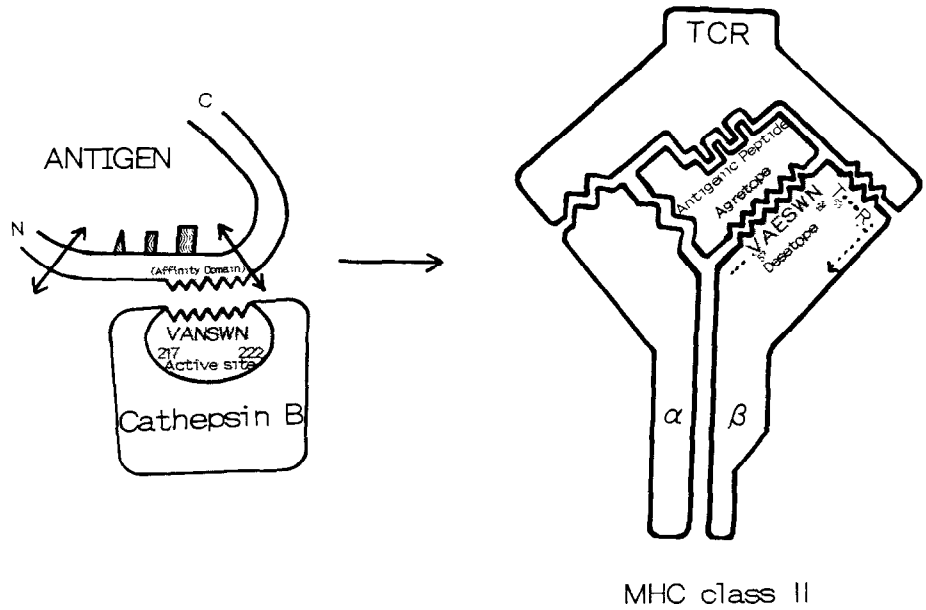

Fig. 6. Hypothesis on the processing and presentation mechanisms of antigenic peptide by cathepsin B (by N. Katunuma).

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