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Research Report

Anti–α-Fodrin Autoantibodies in Moyamoya Disease

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Background and Purpose—Moyamoya disease (MMD) is a rare entity that results in progressive occlusion of the arteries of the circle of Willis, but the pathogenesis of MMD is unknown.

Methods—MMD sera (n=32) were tested for anti-endothelial cell antibodies by enzyme-linked immunoassays and flow cytometric analysis. Apoptosis was induced in human umbilical vein endothelial cells by tumor necrosis factor-α.

Results—We found that a high proportion of MMD sera had anti-endothelial cell antibodies with apoptotic stimuli. Prominent reactivities of MMD sera (72%) with recombinant human α-fodrin were observed.

Conclusions—Our study demonstrates that MMD sera contain a high incidence of anti–α-fodrin autoantibodies, providing new insight into the mechanisms of occlusion of MMD arteries. (*Stroke*. **2003;34:e244-e246.**)

Key Words: α-fodrin ■ autoantibodies ■ moyamoya disease

Moyamoya disease (MMD) is a chronic cerebrovascular occlusive disease first reported by Japanese surgeons.[1] The disease is characterized by stenosis or occlusion of the terminal portions of the bilateral internal carotid arteries and an abnormal vascular network referred to as moyamoya vessels.[2] Although the cause of MMD remains undetermined, evidence supports an infectious origin, suggesting a role for bacterial and viral infections.[3,4] It was also reported that MMD itself has been associated with Sjögren's syndrome[5] and anti-phospholipid autoantibodies.[6]

It was demonstrated that a defined set of cytoskeletal and nuclear proteins, including α-fodrin and poly(ADP-ribose) polymerase (PARP), were selectively cleaved during apoptosis induced by various stimuli.[7] These findings suggest that different proteases act in apoptosis and that, although cell death processes result in selective cleavage of almost identical cellular proteins, they can be distinguished on the basis of their cleavage products. The purpose of the present study was to seek evidence for autoantibodies against apoptosis-related proteins in patients with MMD.

Subjects and Methods

Study Patients

This study included 32 MMD patients confirmed by cerebral angiograph, CT scans, or MRI scans (the Table). Comparative studies were performed with systemic sclerosis (SSc) patients (n=16).

Cell Culture and Induction of Apoptosis

Human umbilical vein endothelial cells (HUVECs) were purchased from Bio Whittaker. Apoptosis was induced in HUVEC by tumor necrosis factor (TNF)-α (100 ng/mL, R&D Systems) and determined by an EPICS flow cytometer (Coulter) with the Mitochondrial Apoptosis Detection Kit (Biovision).

Enzyme-Linked Immunosorbent Assay for Anti-Endothelial Cell Antibodies

Enzyme-linked immunosorbent assay (ELISA) for anti-endothelial cell antibodies (AECAs) was performed as described.[8] Optical density was measured at 495 nm in a Titertek Uniskan (Flow Labs). Absorbance values greater than the mean±3 SD in normal controls were considered positive.

Flow Cytometric Analysis for AECA With Apoptosis

Apoptotic HUVECs were incubated with sera diluted to 1:20 in bovine serum albumin/phosphate-buffered saline. Cells were analyzed on a EPICS flow cytometer (Coulter). Samples were recorded as positive if the binding index was greater than the mean+3 SD of the normal group.

Western Blot Analysis

Western blot analysis with mouse mAb to α-fodrin (AFFINITI, Mamhead), PARP (Transduction Laboratories), gelsolin (DAKO), and active caspase 3 (Transduction Laboratories) was performed and visualized with ECL Western blotting reagent (Amersham Corp). Recombinant caspase 3 was purchased from Biovision, and recombinant α-fodrin was constructed by inserting cDNA into the *Eco*RI site of pGEX-4Ts.[9]

Results

ELISA for AECAs

IgG AECAs were detected in 2 of the 32 MMD patients, not in 32 control subjects (Figure 1A). IgG AECAs were present in 8 of the 16 patients with SSc (50%) (*P*<0.0001).

Received March 25, 2003; final revision received June 10, 2003; accepted July 30, 2003.

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Stroke is available at http://www.strokeaha.org

DOI: 10.1161/01.STR.0000100479.63243.48

Frequency of α-Fodrin–Reactive Sera From MMD Patients and Age-Matched Healthy Control Subjects

	Patients, n	Mean Age (Range), y	Sex Ratio, F/M	Positive Sera With α-Fodrin, n (%)			
				JS-1	2.7A	3′DA	Any
MMD	32	33±17	27/5	9/32*	9/32	19/32†	23/32‡
		(12–66)	(5.4/1)	(28)	(28)	(59)	(72)
Control	32	30±11	27/5	1/32	3/32	2/32	4/32
		(13–53)	(5.4/1)	(3)	(9)	(6)	(13)

Statistically significant at *$P<0.01$, †$P<0.001$, and ‡$P<0.0001$ vs healthy control subjects (Mann-Whitney U test).

Flow Cytometric Analysis for AECAs With Apoptosis

Cytoplasmic staining was observed in a high proportion of SSc ($P<0.0001$) and MMD ($P<0.001$) patients positive for IgG AECAs with apoptosis (Figure 1B). Proteolysis of α-fodrin to 150- and 120-kDa breakdown products was detected in TNF-α–stimulated HUVECs (Figure 1C).

Anti-Human 120-kDa α-Fodrin Abs in MMD Sera

A high proportion of sera from MMD patients (72%) reacted with each recombinant α-fodrin compared with control subjects (13%) (Table). Serum reactivities with breakdown products of PARP were not observed. Strong reactivity of MMD sera with each recombinant human α-fodrin was observed, but not in sera from SSc patients (Figure 2A). A large proportion of MMD sera reacts with C-termini of recombinant α-fodrin protein (JS-1, 28%; 2.7A, 28%; 3′DA, 59%). Cleavage products (150 and 120 kDa) of rat brain α-fodrin were detected when treated with recombinant caspase 3, and MMD sera reacted with either 150- or 120-kDa but not with 240-kDa mature form (Figure 2B). Moreover, TNF-α–stimulated HUVECs were positive for active caspase 3 (Figure 2C), and the cleavage products of α-fodrin were entirely blocked by preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO) (Figure 2D).

Discussion

A number of studies have suggested that endothelial cell injury results in an altered distribution of surface Ag and promotes active binding of immune complexes to these cells.[10] AECAs are reported to be closely correlated with the vasculitis in Kawasaki disease and Takayasu arteritis, suggesting that AECAs could contribute to the pathogenesis of vascular injury.[11]

The new information obtained here is the presence of AECAs with apoptotic stimuli in MMD patients. ELISAs performed with conventional AECAs in the MMD patients were almost negative, indicating that no antibodies directed against endothelial cells bind primarily to membrane-bound molecules. However, sera from MMD patients contain autoantibodies against cleaved product of 150- or 120-kDa α-fodrin derived from apoptotic HUVECs. In vitro study demonstrated that MMD sera react with either 150- or 120-kDa but not with 240-kDa mature-form α-fodrin, which was cleaved by the recombinant caspase 3. This is the first report that autoantibodies cleave products of α-fodrin derived from apoptotic endothelial cells in MMD patients. It was demonstrated that the fodrin α subunit is cleaved in association with apoptosis and that the 120-kDa fragment is a breakdown product of the mature form of 240-kDa fodrin α

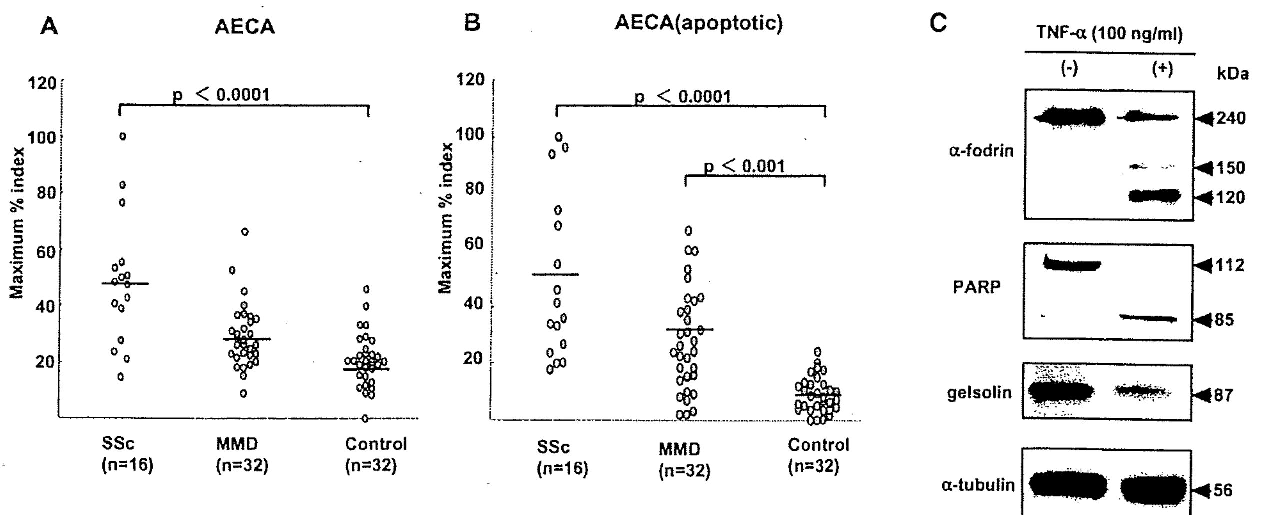

Figure 1. A, IgG AECA level was significantly higher in sera of SSC patients than control subjects ($P<0.0001$, Mann-Whitney U test). B, A high proportion of SSc and MMD patients were positive for IgG AECAs using apoptotic HUVECs compared with control subjects ($P<0.0001$ and $P<0.001$, respectively, Mann-Whitney U test). C, Proteolysis of α-fodrin to 150- and 120-kDa breakdown products was detected in TNF-α (100 ng/mL)–stimulated HUVECs. Treatment with TNF-α (100 ng/mL) affected breakdown of PARP (85 kDa) and gelsolin (cleavage product not detected).

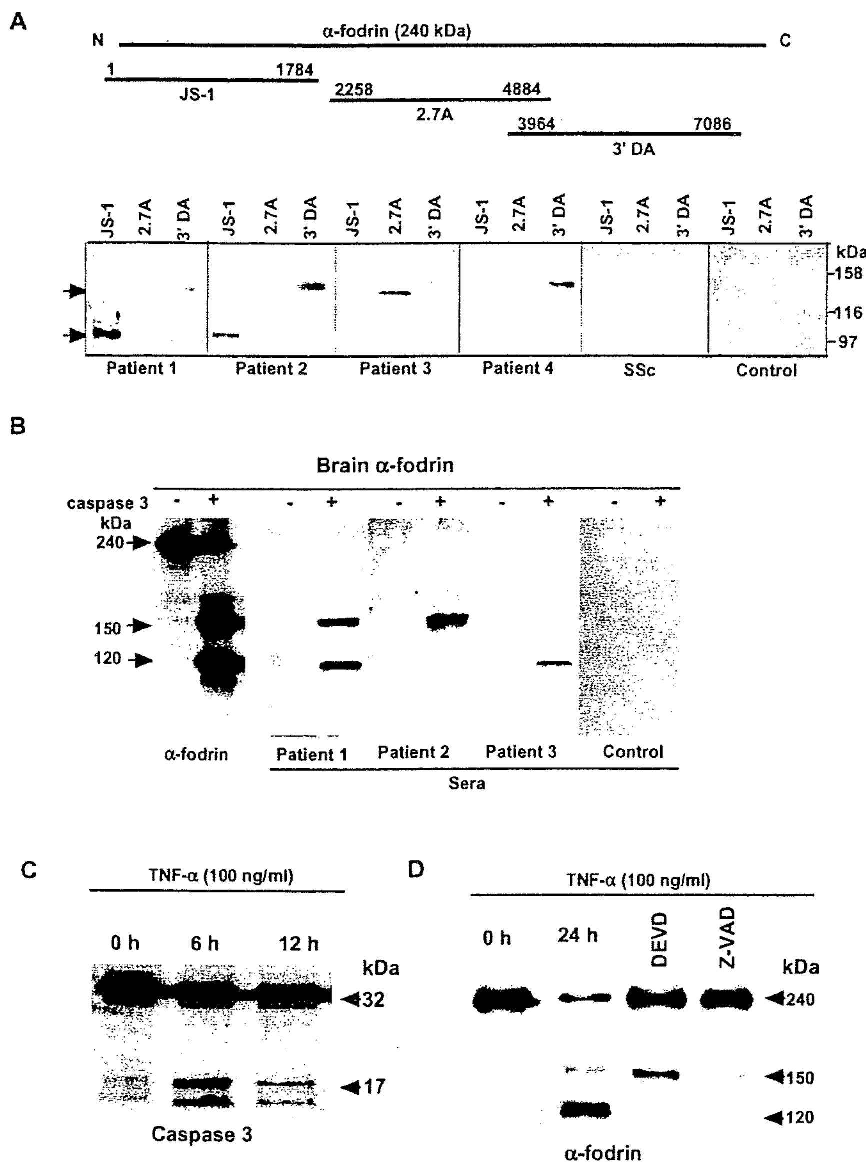

Figure 2. A, Map of cDNAs encoding human α-fodrin. MMD sera react mostly with C-termini of recombinant α-fodrin protein (JS-1, 28%; 2.7A, 28%; and 3'DA, 59%). B, MMD sera react with either 150- or 120-kDa but not with 240-kDa mature-form rat brain α-fodrin when cleaved by the recombinant caspase 3. C, TNF-α–stimulated HUVECs were positive for active-form caspase 3. D, Cleavage products of α-fodrin were entirely blocked by the preincubation with caspase inhibitors (z-VAD-fmk, DEVD-CHO).

subunit.[12] A higher proportion of MMD sera reacts with C-termini of α-fodrin containing caspase 3 cleavage sites. Indeed, we detected active caspase 3 in apoptotic HUVECs, and cleavage products of α-fodrin were blocked by caspase inhibitors. The activation and injury of endothelial cells induced by TNF-α and other proinflammatory cytokines may underlie the local effects of these mediators in vivo. These data suggest that anti–α-fodrin autoantibody could contribute in part to the pathogenesis of MMD and may provide new insight into the mechanisms of occlusion of the arteries.

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Biol. Chem., Vol. 384, pp. 883–890, June 2003 · Copyright © by Walter de Gruyter · Berlin · New York

Minireview

Insights into the Roles of Cathepsins in Antigen Processing and Presentation Revealed by Specific Inhibitors

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Eleven human cathepsins have been identified, however, the *in vivo* roles of individual cathepsins are still largely unknown. In this brief review we will summarize the functions of individual cathepsins in antigen processing and presentation, which are the initial steps of the immune response. Two general inhibitors of papain-like cysteine proteases, E-64 and pyridoxal phosphate, can completely suppress antigen presentation *in vivo*. To evaluate the contribution of individual cathepsins, specific inhibitors have been developed based on cathepsin tertiary structures: CA-074 for cathepsin B, CLIK-148 and -195 for cathepsin L, CLIK-60 for cathepsin S. Administration of CA-074, a cathepsin B inhibitor, suppresses the response to exogenous antigens, such as hepatitis B virus antigen, ovalbumin and *Leishmania major* antigen, and induces switching of the helper T cell responses from Th-2 to Th-1 of CD4⁺ T cells, thereby downregulating the production of IgE and IgG_1. Administration of the cathepsin S inhibitor CLIK-60 impairs presentation of an autoantigen, α-fodrin, in Sjogren's syndrome and suppresses the Th-1 response and autoantibody production.

Key words: Antigen presentation/Antigen processing/Autoantigen/Cathepsin/Cathepsin inhibitor/T cell response.

Introduction

Intracellular degradation of proteins is not always a random process and many biologically active peptides are generated by limited proteolysis, such as a number of hormones and growth factors. Cathepsins are the major proteases in lysosomes and more than ten different cathepsins have been identified. However, the reasons why so many different cathepsins are needed in humans are still unknown.

The immune response consists of the following four steps: antigen processing by cathepsins, peptide presentation on major histocompatibility class II complex (MHCII) molecules in macrophages, transduction of the information to the T cell receptors and, finally, production of the appropriate antibody in B cells. Structural analyses based on X-ray crystallography of MHC class II molecules (Allen *et al.*, 1987; Brown *et al.*, 1988; Srinivasan *et al.*, 1991) suggested that 15-mer peptides, corresponding to the epitopes of exogenous antigens, are processed by lysosomal proteases and presented to MHCII molecules. The 9-mer peptides for endogenous antigens are processed by the proteasome and presented in complex with MHC class I molecules (Spies *et al.*, 1992). However, the contribution of individual cathepsins to antigen presentation types has not been well defined. To identify the roles of cathepsins in antigen processing, we developed highly specific inhibitors for individual cathepsins: CA-074 for cathepsin B, CLIK-148 and CLIK-195 for cathepsin L, CLIK-60 for cathepsin S and CLIK-164 for cathepsin K (Murata *et al.*, 1991; Towatari *et al.*, 1991; Katunuma *et al.*, 1995, 1999). In addition, E-64, an epoxysuccinate peptide derivative which is a general cysteine protease inhibitor, was also developed in our group (Hashida *et al.*, 1982). These specific inhibitors were designed based on the tertiary structures of individual cathepsins.

Antigens processed by various cathepsins to antigenic epitopes are capable of binding to MHC molecules and the information is transduced *via* the T cell receptors to B cells, where specific antibodies are produced. In this process, T helper cells functionally differentiate into two subtypes, Th-1 or Th-2 of CD4⁺ T cells (Guagliardi *et al.*, 1990; Rudensky *et al.*, 1992), which produce different cytokines to support antibody production of different classes. Th-1 cells are responsible for IL-2 and IFN-γ production, needed for the generation of IgG_{2a}, while Th2 cells release IL-4, needed for IG_1 and IgE production (Mosmann *et al.*, 1989; Seder *et al.*, 1994).

Administration of the cathepsin B-specific inhibitor, CA-074, suppresses IgG_1 and IgE production and T cell

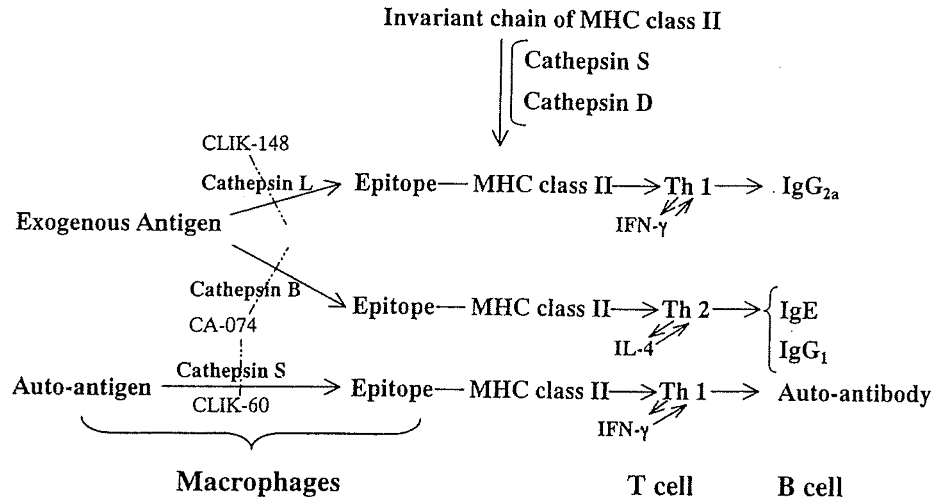

Fig. 1 Contribution of Different Cathepsins to Antigen Processing and T Cell Responses.
Dashed lines indicate inhibition of the respective enzymatic activity.

proliferation in response to exogenous antigens such as hepatitis B virus vaccine and rabies vaccine (Matsunaga *et al.*, 1993), ovalbumin (Katunuma *et al.*, 1998) and *Leishmania major* antigen (Maekawa *et al.*, 1998). CA-074 administration induces switching of the T helper cell differentiation from Th-2 to Th-1 in the *Leishmania major* and ovalbumin systems, suggesting that the alternation in antigen processing modulates the polarity of Th differentiation (Maekawa *et al.*, 1998). We also found that pyridoxal phosphate (PLP), a coenzyme form of vitamin B_6, inhibits both cathepsins B and L to the same extent (Katunuma *et al.*, 1998, 2000) and PLP was also used for the analysis of antigen processing. The *in vivo* administration of PLP or E-64 results in strong suppression of IgE and IgG_1 production and minor suppression of IgG_{2a} production (Katunuma *et al.*, 1994). The relationships between antigen presentation types and the participation of individual cathepsins are summarized in Figure 1.

Maturation of cathepsin L from procathepsin L in spleen was strongly induced in response to ovalbumin immunization, probably being mediated by IFN-γ, suggesting an important role of cathepsin L in immune regulation in peripheral lymphoid tissues (Zhang *et al.*, 2001).

In autoimmune disorders, the immune system fails to distinguish between self and foreign proteins. However, the underlying recognition mechanisms are not well understood. The effects of cathepsin S inhibition on autoantigen presentation in a mouse model of Sjogren's syndrome (SS) were studied. Administration of a specific inhibitor of cathepsin S (CLIK-60) to salivary gland cells from SS mice markedly impaired presentation of an organ-specific autoantigen, 120-kDa α-fodrin, and suppressed the surface expression of MHCII-antigenic peptide complexes. Autoantigen-specific T cell responses were suppressed by incubation with CLIK-60 in a dose-dependent manner, but not with inhibitors of cathepsins B or L (Saegusa *et al.*, 2002).

Cysteine Proteases in Macrophages Are Essential for Antigen Processing

Antigens are processed to epitope peptides for presentation to MHC molecules in antigen presenting cells (APC) such as macrophages, Kupffer cells and dendritic cells. Administration of E-64 *in vivo* suppresses antibody production. PLP and pyridoxal propionate also inhibit the activities of both cathepsins B and L *in vitro* and *in vivo* non-selectively, pyridoxal-5-propionate being a stronger inhibitor (Katunuma *et al.*, 2000). Based on a modeled structure of the cathepsin B-PLP complex, the aldehyde moiety of PLP was suggested to bind to the reactive site -SH group of the cysteine proteases to form a thiosemiacetal bond. The phosphate group seems to be essential for fixing PLP in the active site of cathepsin B. When administered, both compounds suppress the immune response, including antibody and cytokine production (Katunuma *et al.*, 1994, 1998). Moreover, these pyridoxal derivatives show the same effects on antigen presentation as E-64. This is in agreement with other results indicating that cysteine proteases play an important role in antigen processing and antigen presentation (Turk *et al.*, 2001).

Role of Cathepsin B in Antigen Presentation

Specific Inhibition of Cathepsin B by CA-074

Cathepsin B is the major papain-like protease in lysosomes of macrophages, Kupffer cells and dendritic cells (Kominami *et al.*, 1985). It is active at slightly acidic pH in lysosomes and cleaves protein substrates as an endopeptidase or as a dipeptidyl carboxypeptidase. CA-074 and CA-030, developed by the Katunuma group, show high specificity for cathepsin B with K_i values of 1×10^{-9} M, compared to that for cathepsin L (2.3×10^{-5} M;

Murata *et al.*, 1991; Towatari *et al.*, 1991; Katunuma *et al.*, 1995). The high specificity of CA-030 was explained by the X-ray structure of cathepsin B complexed with CA-030 (Turk *et al.*, 1995). Its analog CA-074 can also be used *in vivo* and showed the same level of specificity for cathepsin B.

Inhibitory Effect of CA-074 on Antibody and Cytokine Production

The different contributions of cathepsins to antigen processing were studied using ovalbumin (OVA) (Katunuma *et al.*, 1994, 1998) and *Leishmania major* (Maekawa *et al.*, 1998; Zhang *et al.*, 2000) as antigens. In the OVA antigen system, administration of CA-074 strongly suppresses production of IgE and IgG_1, whereas production of IgG_{2a} is slightly increased. Similarly, production of IFN-γ and IL-2 cytokines is increased, whereas that of IL-4 is significantly decreased. These results suggest that CA-074 induces preferential suppression of the Th-2 response and an increased Th-1 response (Figure 2).

Analysis of Cathepsin B Function at the Cellular Level

The role of cathepsin B in antigen processing and antigen presentation to MHC class II molecules in macrophages, including signal transduction to the T helper cells, was studied in a cell culture system. X-ray irradiated macrophages were used as APCs, and the sensitive clones of human T cells for rabies vaccine or primed splenocytes with hepatitis B vaccine for the immune kinetic analysis (Matsunaga *et al.*, 1993; Katunuma *et al.*, 1994).

Suppression Mechanisms of Immune Responses to Hepatitis B Virus Vaccine (HBV) by the Cathepsin B Inhibitor CA-074 *in vivo* Immunizing mice with HBV vaccine induced a substantial increase in anti-HBV vaccine antibody (IgG) production. Coadministration of E-64 or CA-074 2 h before and 2 h after immunization suppressed IgG production by 40%. Moreover, daily treatment with CA-074 for 14 days suppressed IgG production for 70%. Suppression of cell proliferative responses by CA-074 was examined for HBV vaccine primed splenocytes with rechallenge with either HBV antigen or the epitope peptide, S_{64}-R_{79}, derived from the HBV antigen.The [^{3}H]thymidine incorporation assay was used and results were expressed as the blastogenesis stimulation index. The synthetic 16-mer peptide, SCPPICPGYRWMCLRR (S_{64}-R_{79}), which can be generated from HBV antigen by cathepsin B cleavage, also induced a strong response. However, the latter could not be inhibited by treatment with CA-074 (Figure 3A).

Suppression by CA-074 of Antigen Processing of Rabies Vaccine in Macrophages and the Proliferative Response of Clonal T Cells The rabies-specific T cell clones 2C5 and B8 showed high levels of proliferation in response to rabies vaccine and its derivative, EECDALESTMTTKSVSFR (E_{281}-R_{299}, generated by cathepsin B cleavage), *in vitro* and the responses could be sup-

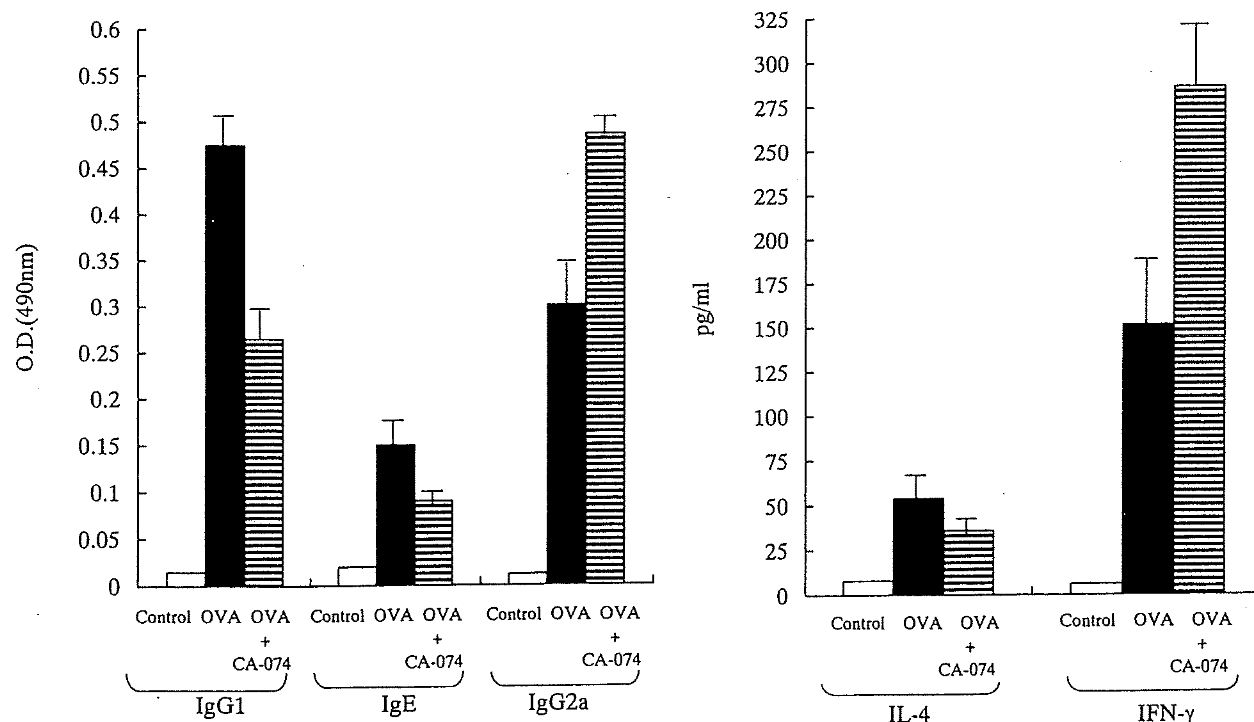

Fig. 2 Effect of CA-074 on the Immune Response to Ovalbumin.
Black bars: ovalbumin immunization; stippled bars: ovalbumin plus CA-074; white bars: control. ELISA readout was performed at 490 nm (O.D.490).

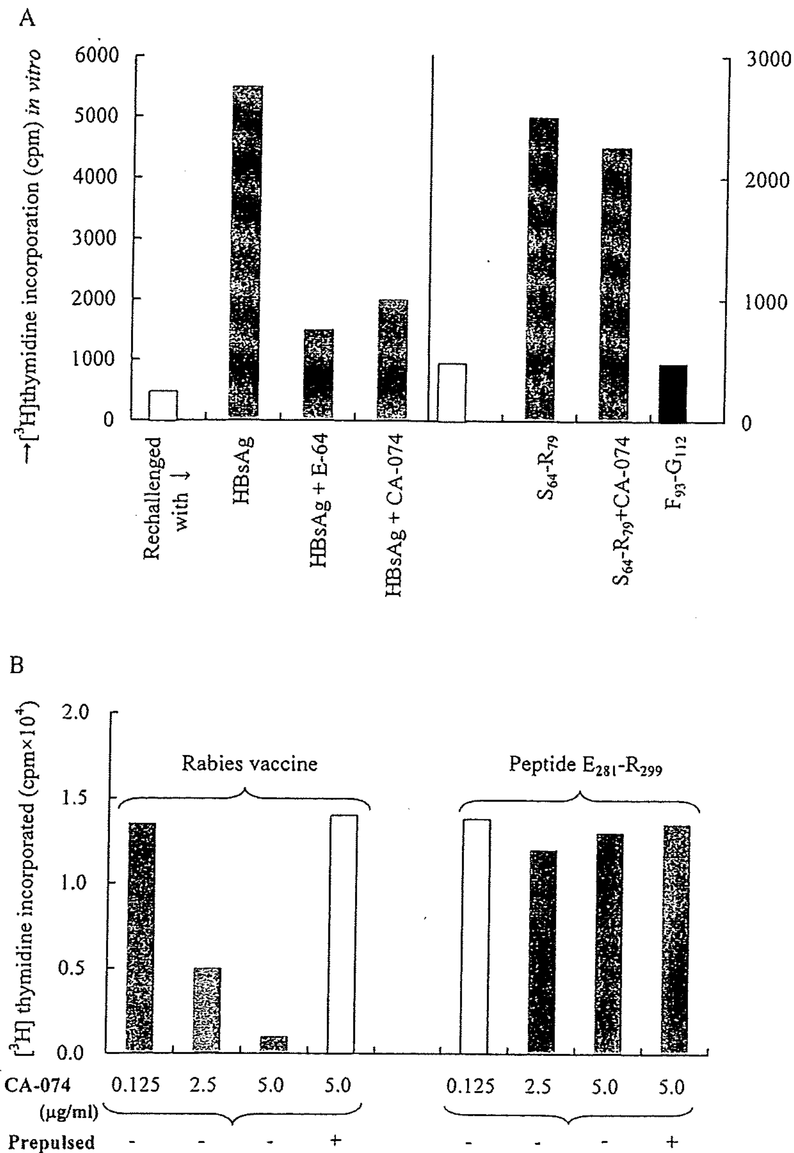

Fig. 3 T Cell Response to Virus Antigens and Its Suppression by Cathepsin B Inhibitors.
(A) Suppression of [^{3}H]thymidine incorporation into primed splenocytes by CA-074 or E-64 on the response to rechallenge of HBs antigen or the antigenic peptide (S_{64}-R_{79}) derived from HBs antigen. S_{64}-R_{79}: active antigenic peptide of HBs antigen. F_{93}-G_{112}: non antigenic peptide of HBs antigen. (B) Inhibitory effects of CA-074 on the proliferation of clonal T cells (2C5) in the response to a rabies vaccine or the antigenic peptide (E_{281}-R_{299}) derived from rabies antigen. 2C5 is a clonal T cell line that responds specifically to rabies antigen. Antigenic peptide sequence of the rabies vaccine (E_{281}-R_{299}): EECLDALESTMTTKSVSFR.

pressed by CA-074 and E-64 in a dose-dependent manner. When APC were pre-pulsed with the vaccine or E_{281}-R_{299} peptide, this response was not affected by CA-074 (Figure 3B). This suggests that cathepsin B plays an important role in antigen processing prior to presenting the antigenic peptides to the MHCII molecules.

Antigenic Peptides of Rabies Vaccine and HBV Vaccine Processed by Cathepsin B Are Capable of Binding to the MHCII Molecules

It has been shown that the antigenic peptides from HBV vaccine, rabies vaccine and ovalbumin generated by cathepsin B form epitopes with -RR or -FR at the C-termini (HBV: -YRWMCLRR; rabies vaccine: -TTKSVSFR; ovalbumin: -AASVSEEFR). Furthermore, these fragments have been demonstrated to bind to the desetope of the β-chain of the MHCII molecule. The question is, however, why these epitope sequences could bind to the desetope of MHCII molecules? It is intriguing to suggest that the -VANSWNT- (V_{217}-N_{222}) region of cathepsin B, which is located within the active site region and shows 86% homology with a part of the desetope of MHCII β-chain, -VAESWNS- (V_{57}-N_{62}) of HLA DR 7 (Young *et al.*, 1987; Musil *et al.*, 1991; Matsunaga *et al.*, 1993; Marsh *et al.*, 1993), is responsible for the binding. This region is different in all other cathepsins, where Ala218 is replaced by Arg/Lys and Asn222 by a conserved Gly. It is quite likely

that the recognition sequence of the T cell receptor has a homologous sequence to both V_{217}-N_{222} of cathepsin B and V_{57}-N_{62} of MHCII β-chain. We suggest that this could be the role of cathepsin B in antigen processing and antigenic epitope presentation to MHCII molecules in macrophages.

Role of Cathepsin L in Antigen Presentation

We developed specific inhibitors of cathepsin L, CLIK-148 and CLIK-195, which showed complete inhibition of cathepsin L at a concentration of 10^{-7} M, but no inhibition of cathepsin B at 10^{-5} M or cathepsins K, C and H at 10^{-6} M. From the crystal structure of the CLIK-148/papain complex (Tsuge *et al.*, 1999), which served as a model for cathepsin L, it is evident that CLIK-148 is bound into the substrate binding pocket and the epoxysuccinate binds to the active site Cys25 of papain.

Both inhibitors also showed high specificity for cathepsin L *in vivo*. Intraperitoneal injection of 3.0 mg/kg of CLIK-148 thus caused specific inhibition of hepatic cathepsin L (Katunuma *et al.*, 1999). When mice were immunized with OVA and CLIK-148, production of IgG_{2a} and IgE antibodies was decreased by 40%, whereas production of IgG_1 antibodies was not affected with the control (OVA only) (Zhang *et al.*, 2001). Administration of CLIK-195 showed the same type of immunosuppression as that with CLIK-148. Maturation of splenic procathepsin L to active cathepsin L was significantly enhanced with OVA immunization, but not with mouse albumin immunization as the control. These results suggest that cathepsin L plays an important role in the immune regulation in peripheral lymphoid tissues.

Table 1 Pathological Symptoms of Sjogren's Syndrome Model Mice and Their Suppression by CLIK-060.

Pathological legion grade		
	Grade of legions	
	Lacrimal G.	Submandibular
non-Tx SS	0.3	0.3
Tx	3.8	3.0
SS + CA-074	2.8	2.5
SS + CLIK-148	3.5	2.8
SS + CLIK-060	0.9	1.0
Secretion volume of saliva and tears		
	Saliva	Tears
non-Tx SS	9.0	3.0
Tx	2.0	1.0
SS + CLIK-060	7.0	2.9

Tx-SS: Thymectomized Sjogren's model mice. Secretion volume given in µl/20 min.

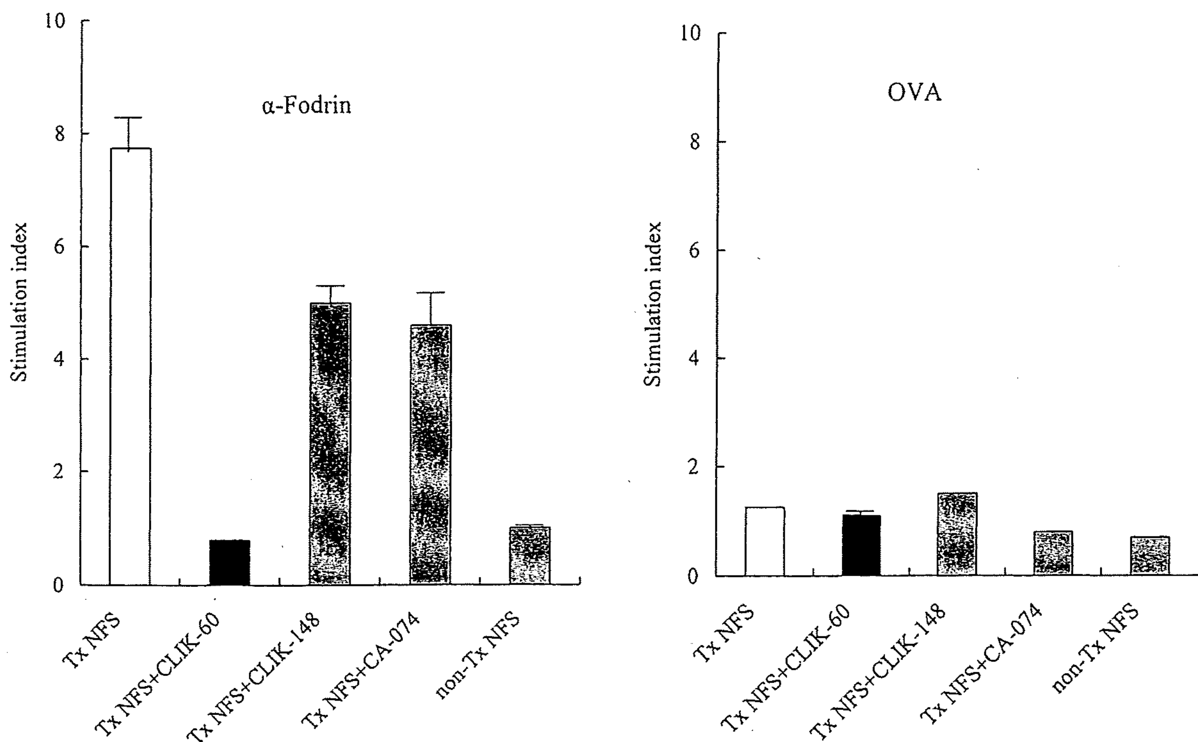

Fig. 4 Inhibition of T Cell Proliferation by CLIK-60 in Sjogren's Syndrome (SS) Model Mice.
Response to autoantigen α-fodrin (left panel) and OVA (right panel). TxNFS: thymectomized SS model mice at 3 days after birth as a model mouse of Sjogren's syndrome. Stimulation indexes: accelerated [³H]thymidine incorporation in T cells of spleen from SS model mice.

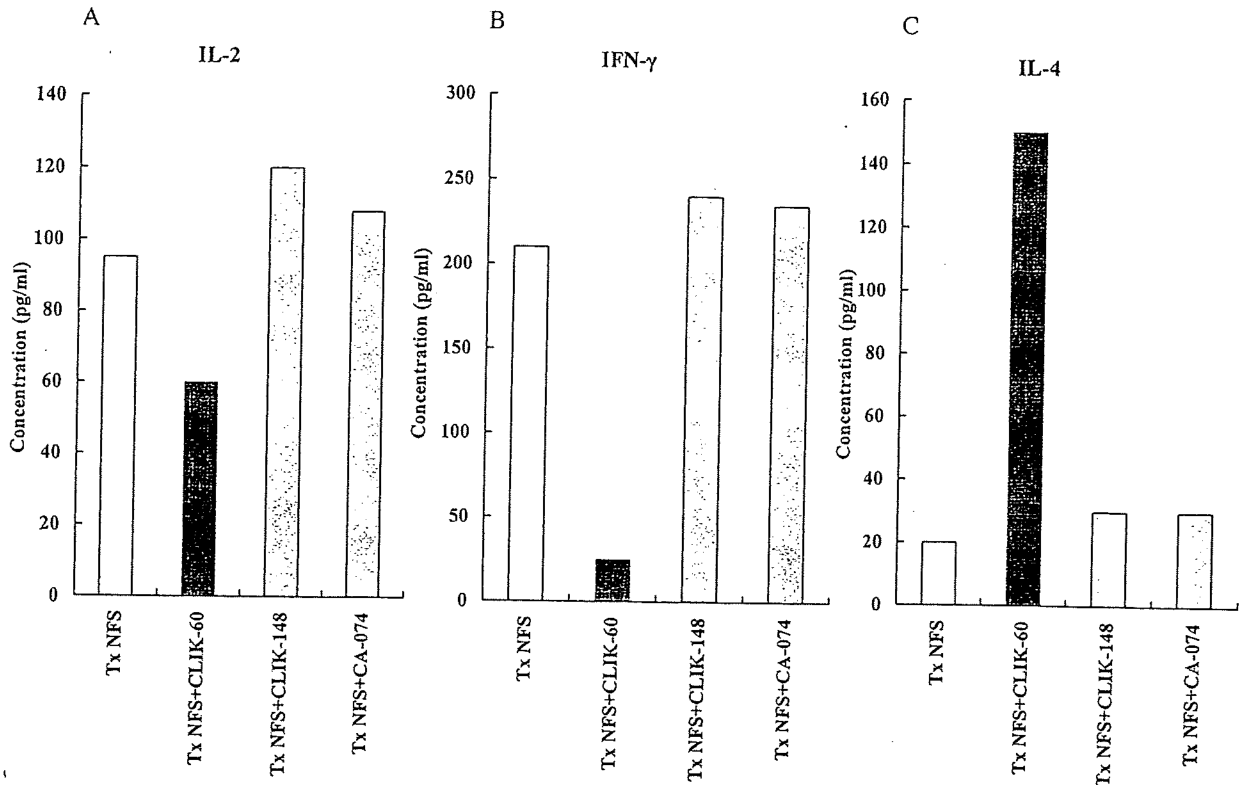

Fig. 5 Downregulation of Th-1 Type Cytokine Production by Splenic T Cells from SS Model Mice (TxNSF) following Anti-CD3mAb Stimulation.
(A) IL-2 production; (B) IFN-γ production; (C) IL-4 production.

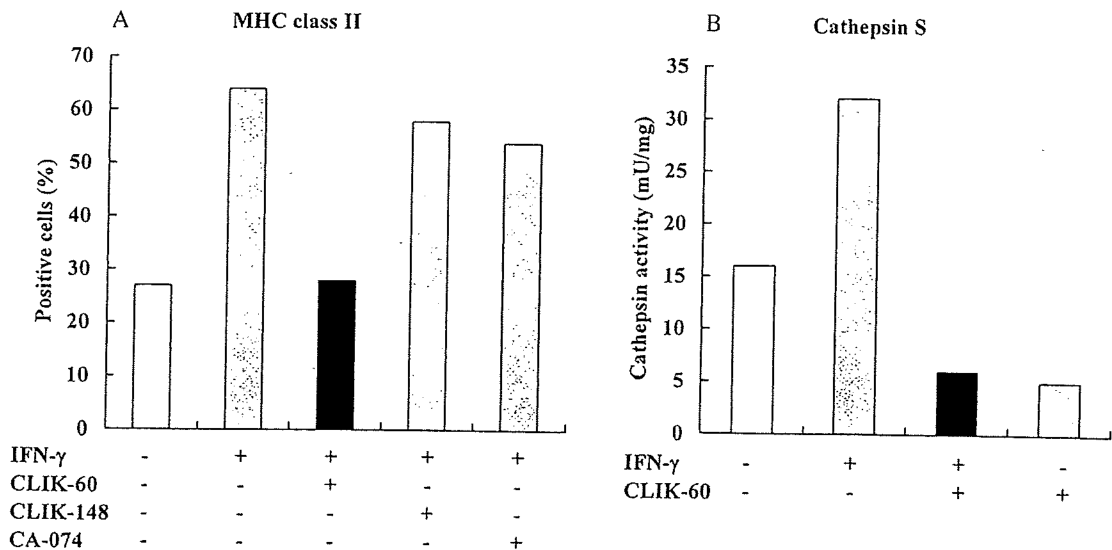

Fig. 6 Inhibition of MHCII Surface Expression by CLIK-60 in Salivary Gland Cells of TxNFs and Suppression of Cathepsin S Production by CLIK-60 in Lymph Nodes of TxNFs.
(A) Surface expression of MHCII molecules induced by IFN-γ in salivary gland epithelial cells from Tx-SS model mice and specific inhibition by CLIK-60. The expression levels were determined by flow cytometry. (B) Induction of cathepsin S by IFN-γ in mouse salivary gland epithelial cells of TxNFs and its inhibition by CLIK-60.

Participation of Cathepsin S in the Presentation of Autoantigen in Autoimmune Diseases

The mechanisms of mis-discrimination between self protein and not-self protein in autoimmune diseases are not well defined. We found that the mechanisms of autoantigen processing are completely different from those of processing of exogenous antigens. Autoantigen processing is completely dependent on cathepsin S, and other cathepsins do not participate in the processing. We de-

veloped a specific inhibitor of cathepsin S to clarify its role in autoantigen processing. CLIK-60 inhibited cathepsin S at 10^{-8} M, while cathepsin B, L, K, C and H were not inhibited at all at 10^{-7} M. CLIK-60 exhibited considerable specificity for cathepsin S *in vitro* at 10^{-5}–10^{-4} M, as well as *in vivo* upon administration at 0.1–0.2 mg/mouse (Katunuma *et al.*, 1999; Saegusa *et al.*, 2002).

Participation of Cathepsin S in Sjogren's Syndrome

The therapeutic effects of CLIK-60 on Sjogren's syndrome (SS) model mice were evaluated in terms of the severity of the pathological lesion and volumes of secreted saliva and tears (Table 1). Intraperitoneal treatment of the model mice with CLIK-60 dramatically prevented progression of the symptoms. CA-074 and CLIK-148, however, were not effective.

Processing of Autoantigens by Cathepsin S and Presentation to MHCII Molecules

Haneji *et al.* (1997) reported that a cleavage product of α-fodrin (120 kDa) is an important autoantigen in the SS model mouse, NFS/sld. The autoantigen (recombinant α-fodrin, JS-1)-stimulated T cell response of regional lymph node cells using salivary gland cells of thymectomized SS mice (TxNFs) was significantly inhibited by CLIK-60, but not CLIK-148 or CA-074 (Figure 4). However, these mice were not stimulated by OVA. Culture supernatants of anti-CD3 mAb-stimulated splenic T cells from TxNFs contained high levels of IL-2 and IFN-γ, but low levels of IL-4. The production of IL-2 and IFN-γ decreased and IL-4 production increased significantly upon CLIK-60 treatment. The cytokine profile indicated the downregulation of Th-1 in SS model mice (TxNFs) (Figure 5). Supernatants from splenic T cells stimulated with anti-CD3 mAb in CLIK-60 treated SS model mice contained low levels of INF-γ and IL-2 and a high level of IL-4 (Figure 5). Flow cytometric analysis of surface expression of MHCII molecules induced by INF-γ in mouse salivary gland cells from SS model mice (TxNFs) clearly demonstrated inhibition of the expression with CLIK-60, but not with other inhibitors (Figure 6A).

Unique Behavior of Cathepsin S in Lymph Nodes of SS Model Mice

Cathepsin S and L activities in the lymph nodes of SS model mice (Tx-SS) are three to four times higher than those in non-Ts-SS mice and in C57BL/6 mice, which were used as the control. However, the level of cathepsin B activity in all three mouse strains was the same. Furthermore, cathepsin S activity in macrophages was induced by INF-γ and all cathepsin S activities, including endogenous and induced activities, were inhibited by treatment with CLIK-60 at 10^{-5} M (Figure 6B).

Auto-Antibody Production against an Autoantigen and Its Inhibition by CLIK-60

Although a serum antibody to 120 kDa α-fodrin was produced, production was suppressed by treatment with CLIK-60 in SS model mice (TxNFS) but not by treatment with specific inhibitors of cathepsins L (CLIK-148) or B (CA-074), suggesting a major role of cathepsin S (Saegusa *et al.*, 2002).

In conclusion, the role of cathepsins in antigen processing and presentation can also be assessed by specific inhitiors. Cathepsins B, L and S were thus found to have specific and different roles in various mouse models, in agreement with other studies (Watts, 2001).

Acknowledgments

We thank Prof. Vito Turk, Prof. Hans Fritz and Prof. Boris Turk for their useful advice on the preparation of the manuscript.

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Cathepsin S inhibitor prevents autoantigen presentation and autoimmunity

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The cysteine endoprotease cathepsin S mediates degradation of the MHC class II invariant chain Ii in human and mouse antigen-presenting cells. Studies described here examine the functional significance of cathepsin S inhibition on autoantigen presentation and organ-specific autoimmune diseases in a murine model for Sjögren syndrome. Specific inhibitor of cathepsin S (Clik60) in vitro markedly impaired presentation of an organ-specific autoantigen, 120-kDa α-fodrin, by interfering with MHC class II–peptide binding. Autoantigen-specific T cell responses were significantly and dose-dependently inhibited by incubation with Clik60, but not with inhibitor s of cathepsin B or L. Clik60 treatment of mouse salivary gland cells selectively inhibited autopeptide-bound class II molecules. Moreover, the treatment with Clik60 in vivo profoundly blocked lymphocytic infiltration into the salivary and lacrimal glands, abrogated a rise in serum autoantibody production, and led to recovery from autoimmune manifestations. Thus, inhibition of cathepsin S in vivo alters autoantigen presentation and development of organ-specific autoimmunity. These data identify selective inhibition of cysteine protease cathepsin S as a potential therapeutic strategy for autoimmune disease processes.

J. Clin. Invest. 110:361–369 (2002). doi:10.1172/JCI200214682.

Introduction

Sjögren syndrome (SS) is an autoimmune disorder characterized by lymphocytic infiltrates and destruction of the salivary and lacrimal glands, and systemic production of autoantibodies to the ribonucleoprotein particles SS-A/Ro and SS-B/La (1–3). We have investigated an animal model for SS in NFS/*sld* mutant mice thymectomized 3 days after birth (3d-Tx) (4–9). All 3d-Tx NFS/*sld* mice develop autoimmune lesions in the salivary and lacrimal glands, starting at 3 weeks of age, and the disease mediated by CD4$^+$ T cells is chronic and progressive (4, 5). Previously, we reported a 120-kDa α-fodrin autoantigen in the salivary gland tissues from SS model mice and identified autoantigen-specific T cell responses associated with Th1 cytokine production of IL-2 and IFN-γ (10). However, the role of antigen-presenting cells (APCs) in organ-specific T cell activation in this model has not yet been analyzed.

MHC class II molecules encounter and bind antigenic peptides as class II–peptide complexes on the cell surface of APCs for recognition by CD4$^+$ T cells (11–13). The molecular mechanisms leading to formation of class II–peptide complexes and presentation of antigen on the cell surface begin with synthesis of class II αβ heterodimers in the endoplasmic reticulum. These class II αβ heterodimers associate early during biosynthesis with a type II membrane protein, the invariant chain (Ii) (14, 15). Inhibition of Ii degradation in B lymphoblastoid cells and murine spleen cells induces accumulation of class II–associated Ii fragments and inhibition of class II–peptide formation (16–19). Selective inhibition of the proteases responsible for both these degradative processes is a potential mechanism for modulating the immune response. Several lysosomal proteases have been implicated in the processing of Ii and antigenic peptides. Cathepsin B, the most abundant lysosomal cysteine protease, has been tied to Ii degradation using purified class II–Ii complexes (20). Cathepsin L, a potent cysteine-class endoprotease, is specifically inhibited by a fragment of the alternatively spliced Ii form p41 (21). Cathepsin S containing potent endoproteolytic activity is highly expressed in the spleen and professional APCs and other class II–positive cells and is inducible by IFN-γ (22, 23). In mouse splenocytes, inhibition of cathepsin S also induces buildup of Ii breakdown products and attenuation of class II–peptide association, although the extent of this effect appears to be haplotype-dependent (24). We have developed specific inhibitors of cathepsin B (CA074), cathepsin L (Clik148), and cathepsin S (Clik60), in vivo as well as in vitro (25–27). Matsunaga et al. first reported that CA074 suppresses immune responses (28), suggesting that cysteine proteases in

Received for publication November 19, 2001, and accepted in revised form June 17, 2002.

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Conflict of interest: No conflict of interest has been declared.

Nonstandard abbreviations used: Sjögren syndrome (SS); thymectomized 3 days after birth (3d-Tx); nonthymectomized (non-Tx); antigen-presenting cell (APC); invariant chain (Ii); lymph node cell (LNC); ovalbumin (OVA); concanabalin A (ConA); mouse salivary gland (MSG); propidium iodide (PI).

lysosomes play an important role in the functional differentiation of MHC class II-restricted $CD4^+$ T cells. However, it is uncertain whether the inhibition of cathepsins B, L, and S blocks generation of the antigenic peptide on the development of autoimmune diseases. To address this important issue, antigen processing and presentation after specific inhibition of cathepsins were examined in a murine model for SS.

Studies presented here suggest that cathepsin S plays an important rôle in processing of class II-Ii in autoantigen-presenting cells to generate class II molecules competent for binding antigenic peptide, and that inhibition of cathepsin S has important functional consequences in modulating the autoimmune response.

Methods

Mice. Female NFS/N-strain mice carrying the mutant gene *sld* (29) were reared in our specific pathogen-free mouse colony, and given food and water ad libitum. Thymectomy was performed on day 3 after birth (3d-Tx) in NFS/*sld* mice. Nonthymectomied (non-Tx) NFS/*sld* mice and C56BL/6 mice purchased from Charles River Japan Inc. (Atsugi, Japan) were used as controls.

Measurement of endogenous cathepsin activities. Salivary glands, regional lymph nodes, and spleens from 3d-Tx NFS/*sld* SS model, non-Tx NFS/*sld*, and control C57BL/6 mice were used to assay for cathepsin B, L, and S activity. Lysosomes were isolated for the assay by gentle homogenization of samples using a Teflon homogenizer (Microtec Co. Ltd., Funabashi, Japan) pestle in 0.25 M cold sucrose. The suspension was centrifuged at 3,500 *g* for 10 minutes at 4°C. The supernatant was centrifuged at 25,000 *g* for 20 minutes at 4°C. The resulting pellet was resuspended with 50 mM acetate buffer (pH 5.0). The suspension fluid was frozen and thawed three times to disrupt lysosomal membranes. After three cycles of freezing and thawing, the fluid was centrifuged and the supernatant was used as a mitochondria and lysosome fraction. Cathepsin activities were assayed with Z-Arg-Arg-methyl coumarylamide (Peptide Institute, Osaka, Japan) as substrate at pH 5.0 for cathepsin B, with Z-Phe-Arg-methyl coumarylamide for cathepsin L, and using the method described by Inubushi et al. (30) for cathepsin S. The reaction was initiated by addition of substrate (10 μM final concentration) after preincubation with the test compound for 3 minutes at 37°C. The fluorescence of the liberated 7-amino-4-methylcoumarin was measured in a fluorescence spectrophotometer (Hitachi Science Systems Ltd., Hitachinaka, Japan). Emission at 460 nm was measured with excitation at 370 nm.

Cathepsin inhibitors. Specific inhibitors for cathepsin B (CA074), cathepsin L (Clik148), and cathepsin S (Clik60) have been developed with the help of computer-graphic modeling based on the stereo-structure as described previously (25-27). The common fragment, *N*-(L-trans-carbamoyloxyrane-2-carbonyl)-phenylalanine-dimethylamide, is required for specific inhibition of cathepsin L. Seven novel inhibitors of the cathepsin L inhibitor Katunuma (Clik) specifically inhibited cathepsin L at a concentration of 10^{-7} M in vitro, while almost no inhibition of cathepsins B, C, S, and K was observed. Four of the Cliks are stable and showed highly selective inhibition for hepatic cathepsin L in vivo. One of the Clik inhibitors contains an aldehyde group and specifically inhibits cathepsin S at 10^{-7} M in vitro (27).

In vivo treatment with cathepsin inhibitors. We examined the in vivo therapeutic effects of cathepsin S inhibitor (Clik60), cathepsin B inhibitor (CA074), and cathepsin L inhibitor (Clik148) in a murine model for SS. Each inhibitor (Clik60, CA074, and Clik148), dissolved in PBS, was administered intraperitoneally into model mice at doses of 0.1 mg/mouse/day from 4 weeks to 7 weeks (n = 10 for each), and then analyzed at 8 weeks, compared with untreated SS model mice (n = 7).

Histology. All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2), and prepared for histologic examination. The sections were stained with hematoxylin and eosin. Histological grading of the inflammatory lesions was done according to the method proposed by White and Casarett (31). These slides were scored by three independent, pathologists in a blinded manner.

Proliferation assay. Single-cell suspensions of spleen cells or regional lymph node cells (LNCs) from 3d-Tx, non-Tx NFS/*sld*, and C57BL/6 mice were cultured in 96-well flat-bottom microtiter plates (Nalge Nunc Intl. Co., Rochester, New York, USA) in RPMI-1640 containing 10% FCS, penicillin/streptomycin, and β-mercaptoethanol. For proliferation assay, a total of 5×10^5 cells per well were cultured for 72 hours under stimulation of recombinant α-fodrin protein (JS-1, 10 μg/ml) (10), ovalbumin (OVA) (10 μg/ml), and concanabalin A (ConA) (5 μg/ml), and pulsed with 1 μCi/well of [^{3}H]thymidine (NEN Life Science Products Inc., Boston, Massachusetts, USA) during the final 20 hours of the culture. [^{3}H]thymidine incorporation was evaluated using an automated β liquid scintillation counter. We further examined the in vitro preventive effects of cathepsin inhibitors (10^{-7} to 10^{-4} M CA074, Clik148, and Clik60) for antigen-specific proliferative T cell responses. T cell purification was done using CD4 mAb-bounded immunomagnetic beads (Dynal, Oslo, Norway).

Flow cytometric analysis. Spleen cells and regional LNCs from 3d-Tx and non-Tx NFS/*sld* mice were analyzed by flow cytometry. Single-cell suspensions were stained with antibodies conjugated to phycoerythrin (anti-CD4; Cedarlane Laboratories Ltd., Hornby, Ontario, Canada) and FITC (anti-CD8 from Cedarlane Laboratories Ltd.; anti-CD44, anti-CD45RB, anti-Mel-14, and anti-I-A^q from Pharmingen, San Diego, California, USA), and analyzed with an EPICS (Beckman Coulter, Miami, Florida, USA). Apoptotic or necrotic cells were detected by flow cytometry with an EPICS (Beckman Coulter) using the Annexin V-FITC Apoptosis Detection Kit (Genzyme Pharmaceuticals Co. Ltd., Cambridge, Massachusetts, USA). Viable cells (1×10^5) were

gated according to size and scatter to eliminate dead cells and debris from analysis.

Measurement of fluid secretion. Detection of tear and saliva volume of the treated and untreated SS animal model of NFS/*sld* mice was done according to a method as described (32). Five mice in each group were analyzed.

Measurement of cytokine production. Cytokine production from spleen cells of 3d-Tx and non-Tx NFS/*sld* mice was tested by two-step sandwich ELISA using a mouse IL-2, IL-4, and IFN-γ kit (Genzyme Pharmaceuticals). In brief, culture supernatants from spleen cells activated with immobilized anti-CD3 mAb (10 μg/ml) (Cedarlane Laboratories Ltd.) for 3 days were added to microtiter plates precoated with anti–IL-2, -IL-4, and -IFN-γ capture antibody and incubated overnight at 4°C. After addition of biotinylated detecting antibody and incubation at room temperature for 45 minutes, avidin-peroxidase was added and incubated at room temperature for 30 minutes. Plates were washed extensively with 1% Tween in PBS between each step. Finally, 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) substrate containing H_2O_2 was added and the colorimetric reaction was read at an absorbance of 450 nm using an automatic microplate reader (BioRad Laboratories Inc., Hercules, California, USA). The concentrations (in pg/ml) of IL-2, IL-4, and IFN-γ were calculated according to the standard curves produced by various concentrations of recombinant cytokines.

Primary culture of mouse salivary gland cells. Mouse salivary gland (MSG) epithelial cells were prepared as previously described (33). Briefly, MSGs were minced into 1-mm^2 pieces, washed with HBSS without Ca^{2+} and Mg^{2+}, and placed in a 60-mm dish containing HBSS with 0.76 μg/ml EDTA, 4.9 μg/ml L-ascorbic acid, and 4.9 μg/ml reduced glutathione. Fragments were washed with DMEM/STI and placed in a mixture of collagenase (type I, 75 U/ml) and hyaluronidase (type IV, 500 U/ml) dissolved in DMEM/F12 containing 10% FBS. The first digest suspension was passed through sterile 100-μm nylon mesh filter and were redigested for 30 minutes by the same digestion procedure, and then the digest suspension was passed through a 100-μm nylon mesh filter. Adherent cells, after culture in MEM containing 10% FBS for 24 hours at 37°C, were isolated as salivary gland epithelial cells. We confirmed that the cells over 95% were positively stained with anti-keratin polyclonal antibody.

Detection of serum autoantibodies against 120-kDa α-fodrin. Serum autoantibody production against 120-kDa α-fodrin was analyzed by immunoblotting and ELISA as described previously (6, 7, 10).

Confocal immunofluorescence analysis. IFN-γ–stimulated and nonstimulated MSG cells were fixed with 1% paraformaldehyde and were incubated with mAb to I-A^q molecule (Pharmingen) and FITC-labeled $AFN_{303-318}$ peptide (described below). In vitro effects of cathepsin inhibitors were examined by the preincubation with each inhibitor (10^{-5} M) for 6–24 hours. The labeled second antibody was Texas red–conjugated goat anti-mouse IgG (Molecular Probes Inc., Eugene, Oregon, USA). For microscopy, a Leica TCS-NT laser scanning microscope (Leica Microsystems Nussloch GmbH, Nussloch, Germany) was used.

Results

Endogenous activities of cathepsins B, L, and S in tissue samples. We first investigated endogenous activities of cathepsin S, cathepsin L, and cathepsin B in tissue samples of the salivary glands, regional lymph nodes, and spleens from SS model mice and controls. As shown in Figure 1, the activities of both cathepsin S and cathepsin L were significantly higher in the salivary glands, lymph nodes, and spleens from model mice than those of controls. No difference in activities of cathepsin B was found in any organ from these mice. Significantly inhibitory effects of cathepsin S inhibitor (Clik60), cathepsin L inhibitor (Clik148), and cathepsin B inhibitor (CA074) were observed in each organ in a dose-dependent manner. Moreover, we confirmed that cathepsin S activity in each organ was clearly inhibited by Clik60, but not by Clik148 and CA074 (data not shown).

Inhibitory effect of a specific inhibitor of cathepsin S (Clik60) on proliferative T cell response to autoantigen. To address the role of autoantigen-reactive T cells, we examined the proliferative T cell responses in the LNCs and spleen cells from model mice and controls. We found that the LNCs and spleen cells in SS model mice, but not in non-Tx NFS/*sld* and C57BL/6 control mice, at 8 weeks of age showed a significant increase in autoantigen-specific (JS-1–specific) T cell proliferation (Figure 2a; data of the spleen cells not shown). No significant differences were observed in the proliferative response stimulated with OVA (10 μg/ml) and ConA (5 μg/ml) among these mice. We examined the inhibitory effects of cathepsin inhibitors against autoantigen-specific T cell responses in vitro. In regional LNCs, but not in spleens, from model mice, a significantly inhibitory effect of Clik60 was observed in a dose-dependent manner (Figure 2b). In contrast, no inhibitory effects of Clik148 and CA074 were found. Annexin V/propidium iodide (PI) flow cytometric analysis revealed a small proportion of early apoptotic cells (annexin V–positive, PI-negative) and necrotic cells (annexin V–positive, PI-positive), indicating that Clik60 is not toxic and favors T cell survival in the LNCs (Figure 2c). Indeed, the Clik60 inhibitors at 10^{-7} to 10^{-4} M concentrations do not block T cell proliferation to ConA (Figure 2d). These findings indicated that the dose of Clik60, 10^{-5} to 10^{-4} M, was sufficient to inactivate the autoantigen-specific T cell responses in vitro.

In vivo administration of cathepsin inhibitors. We next examined the in vivo therapeutic effects of Clik60, Clik148, and CA074 in a murine model for SS. Interestingly, the treatment with intraperitoneal injection of Clik60 (0.1 mg/mouse/day) was effective in preventing the development of autoimmune lesions in the lacrimal ($P < 0.01$), parotid ($P < 0.05$), and submandibular

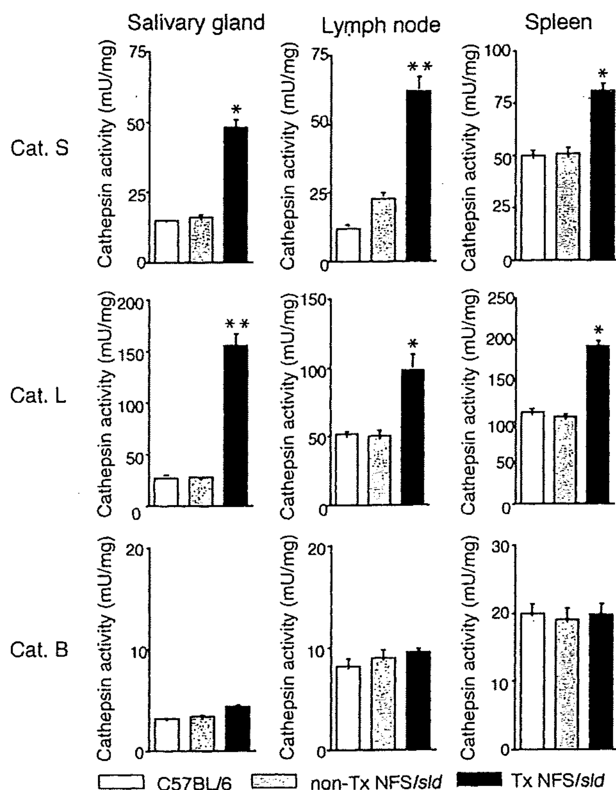

Figure 1
Endogenous cathepsin (Cat.) activities in tissue samples from SS model mice and controls. The activities of both cathepsin S and cathepsin L were significantly higher in the salivary glands, regional lymph nodes, and spleens from model mice than in those from controls (* $P < 0.05$, ** $P < 0.01$, Student's t test). Cathepsin activities were assayed as described in Methods.

($P < 0.05$) glands of the SS model mice, but not in those of groups injected with CA074 and Clik148 (Figure 3a). Representative histological features in the salivary and lacrimal glands are shown in Figure 3b. Moreover, the average saliva and tear volumes of Clik60-treated model mice were significantly higher than those of the untreated SS model mice (Figure 3c). The activation markers (CD44^{high}, CD45RB^{low}, Mel-14^{low}) were clearly downregulated in LNCs gated on CD4 from Clik60-treated model mice (Figure 4a). In addition, autoantigen-specific (JS-1–specific) T cell response was significantly inhibited in LNCs from Clik60-treated mice (Figure 4b). Culture supernatants from anti-CD3 mAb–stimulated splenic T cells obtained from Clik60-treated mice, but not those obtained from non-Tx NFS/*sld* mice, contained high levels of IL-4, but low levels of IL-2 and IFN-γ, by ELISA (Figure 5, a and b). Moreover, serum autoantibody production against 120-kDa α-fodrin was exclusively inhibited in Clik60-treated mice, but not in other groups (Figure 5c). A dilution curve, obtained by ELISA, illustrates that each case is capable of reacting with antigen (Figure 5d). These results strongly suggest that Clik60 plays an important role in preventing autoantigen presentation to generate class II molecules competent for binding antigenic peptide, resulting in inhibition of autoimmunity.

Possible role of MSG cells in autoantigen presentation. It has been well known that nonlymphoid cells that

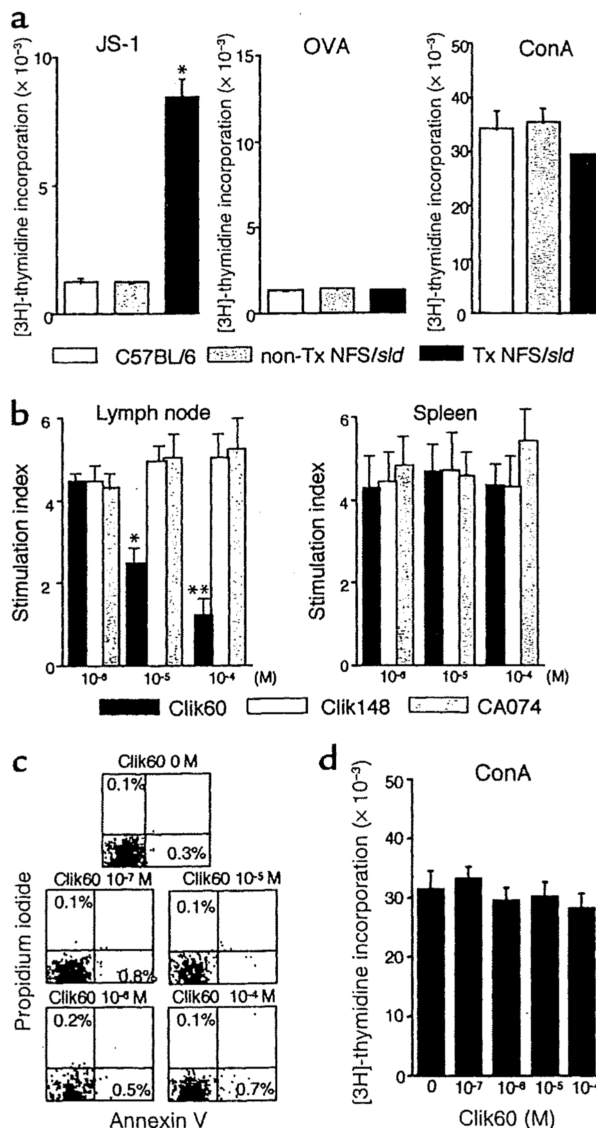

Figure 2
(**a**) Detection of proliferative T cell response of LNCs from SS model mice to organ-specific autoantigen (JS-1) (* $P < 0.001$, Student's t test). No differences were found in OVA (10 μg/ml) and ConA (5 μg/ml) responsiveness. (**b**) In vitro preventive effect of proliferative T cell response of LNCs, but not spleen cells, to JS-1 by Clik60 in a dose-dependent manner. No inhibitory effects of Clik148 and CA074 were found. Data are expressed as stimulation indices ± SEM. Three experiments from each group were performed at 8 weeks of age, and the mean values of index were statistically significant at * $P < 0.05$ and ** $P < 0.01$ (Student's t test). (**c**) Annexin V/propidium iodide (PI) flow cytometric analysis revealed a small proportion of early apoptotic cells (annexin V–positive, PI-negative) and necrotic cells (annexin V–positive, PI-positive). (**d**) The inhibitors at 10^{-7} to 10^{-4} M concentrations of Clik60 do not block T cell proliferation to Con A (5 μg/ml).

express MHC class II molecules provoke autoimmune responses in combination with costimulation (34, 35). To determine whether specific inhibition of cathepsin S activity can functionally alter antigen presentation, primary cultured MSG cells, regional LNCs, and spleen cells from model mice and non-Tx control mice were compared in terms of their capacity to express class II molecule, and to react with organ-specific autoantigen as determined by flow cytometry and proliferation assay. Among these cells, a large proportion of class II-expressing (I-A^q-expressing) cells was observed on MSG cells compared with spleen cells and LNCs from SS model mice (Figure 6a). Moreover, class II molecule can be stably induced by IFN-γ stimulation on MSG cells from non-Tx NFS/*sld* control mice by flow cytometry (Figure 6b). Indeed, purified $CD4^+$ T cells from model mice are capable of responding to IFN-γ-stimulated MSG cells, but not with nonstimulated MSG cells (Figure 6c). Autoantigen-stimulated (JS-1-stimulated) T cell response of LNCs using MSG cells was significantly inhibited by incubation with Clik60, but not incubation with Clik148 and CA074 (Figure 6d). These results suggest that MSG cells may function, at least in part, as autoantigen-presenting cells in the development of murine SS, and that inhibition of cathepsin S prevents both autoantigen presentation in the salivary gland cells and autoantigen-specific T cell proliferation.

Inhibition of cathepsin S prevents class II–peptide association. We confirmed that activity of cathepsin S in MSG cells was clearly inhibited by Clik60 (10^{-5} M) (Figure 7a). Surface expression of class II molecule induced by IFN-γ stimulation in MSG cells from non-Tx NFS/*sld* mice was clearly inhibited by incubation with Clik60, but not incubation with Clik148 and CA074, as determined by flow cytometry (Figure 7b). MHC class II molecules are synthesized with their peptide-binding site blocked by Ii, and they acquire the capacity to bind antigens only after Ii has been degraded in the compartments. Cathepsin S is known to play a pivotal role in proteolytic degradation of the Ii as a prerequisite for peptide binding to class II molecules (36). Recently, we have identified the pathogenic T cell epitope peptide ($AFN_{303-318}$) on α-fodrin autoantigen involved in autoimmune responses in this model (unpublished data). We observed colocalization of FITC-labeled $AFN_{303-318}$ peptide and class II (I-A^q) molecule in the IFN-γ-stimulated MSG cells (Figure 7c), suggesting that MSG cells may play a role in presenting autopeptide in the autoimmune responses. Moreover, confocal immunofluorescence analysis confirmed that inhibition of cathepsin S prevents the surface expression of peptide-class II complex formation, while inhibition of cathepsin L and cathepsin B did not effect expression of peptide-bound class II molecules (Figure 7c).

Discussion

Therapeutic strategies for modulating the immune response in autoimmune disorders have centered on altering generation of the effector molecules that mediate the inflammatory reactions, e.g., corticosteroids, cyclosporin, and inhibitors of cytokines (37, 38). Our experiments demonstrate that selective inhibition of cysteine proteases is an additional potential strategy for modulating the autoantigen-derived immune response in class II–restricted disease processes. To our knowledge, this manuscript presents the first evidence that selective inhibition of cysteine protease cathepsin S in vivo has important functional consequences for MHC class II-dependent immune responses in autoimmune disease.

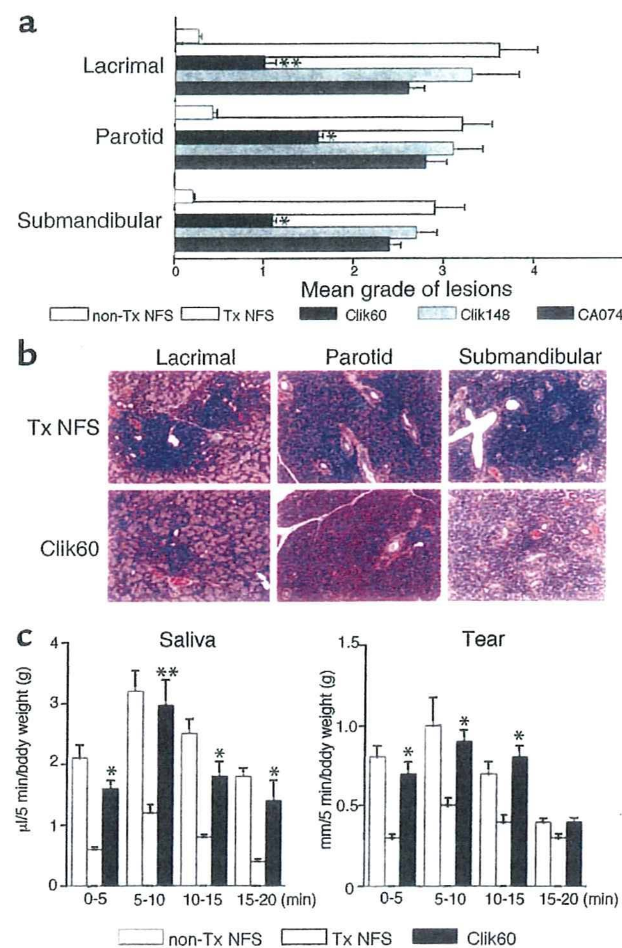

Figure 3

(**a**) Effects of in vivo administration of intraperitoneal injection of cathepsin inhibitors (Clik60, Clik148, and CA074). Each inhibitor was administered into SS model mice (n = 10 for each) at doses of 0.1 mg/mouse/day from 4 weeks to 7 weeks, and the results were analyzed at 8 weeks compared with those in untreated SS model mice (n = 7). Treatment with intraperitoneal injection of Clik60, but not treatment with Clik 148 and CA074, was effective in preventing the development of autoimmune lesions in the lacrimal, parotid, and submandibular glands of the SS model mice (*P < 0.01, **P < 0.005, Student's t test). (**b**) Representative histologic features in the salivary and lacrimal glands showing preventive effects in Clik60-treated mice at 8 weeks of age. (**c**) The average saliva and tear volume of the SS model mice treated with Clik60 was significantly higher than that of control mice. Results are expressed as mean ± SEM in five mice examined per group (*P < 0.05, **P < 0.005, Student's t test).

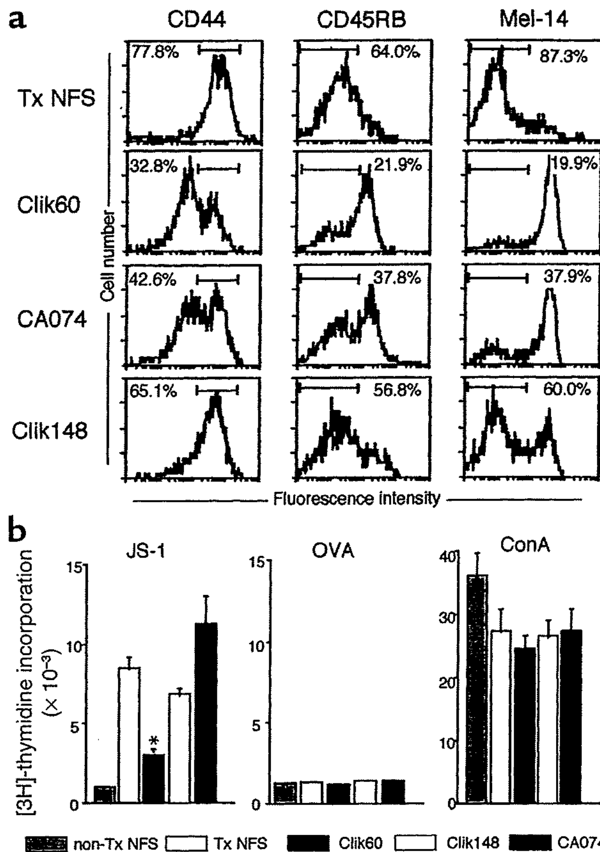

Figure 4
(**a**) Upregulation of the activation markers (CD44^{high}, CD45RB^{low}, Mel-14^{low}) in LNCs gated on CD4 from Clik60-treated mice, compared with control mice. (**b**) SS model mice treated with Clik60 showed a significant decrease of autoantigen-specific (JS-1-specific) T cell proliferation in LNCs, compared with controls ($P < 0.01$, Student's t test). Data are expressed as cpm per culture in triplicate.

We reported previously a cleavage product of 120-kDa α-fodrin as an important autoantigen in the pathogenesis of SS in both an animal model and humans (10). The observation that thyrocytes express MHC class II molecules in Graves thyroiditis led Bottazzo and coworkers (39) to theorize that nonlymphoid cells that express MHC class II molecules provoke autoimmune responses by presenting autoantigens. Salivary gland epithelial cells from SS patients have been found to express MHC class II molecules in a large proportion of biopsy samples, and the number of positive cells generally increases with the number of lymphocytes infiltrating the gland (40–42). In the present experiments, a large proportion of class II–expressing (I-A^q-expressing) cells was observed on MSG epithelial cells from SS model mice, and MHC class II molecule can be stably induced by IFN-γ stimulation on MSG cells from syngeneic control mice. Moreover, autoantigen-stimulated (JS-1-stimulated) proliferative T cell response using MSG cells was clearly inhibited by the incubation with cathepsin S inhibitor (Clik60). In light of these results, it is possible that the salivary gland epithelial cells may function, at least in part, as autoantigen-presenting cells in the development of murine SS, and that inhibition of cathepsin S prevents autoantigen presentation and subsequent peptide binding by class II molecules. MHC class II molecules bind a diverse array of peptides derived from the endocytic pathway and present them to CD4$^+$ T cells. APCs have a pool of active class II mol-

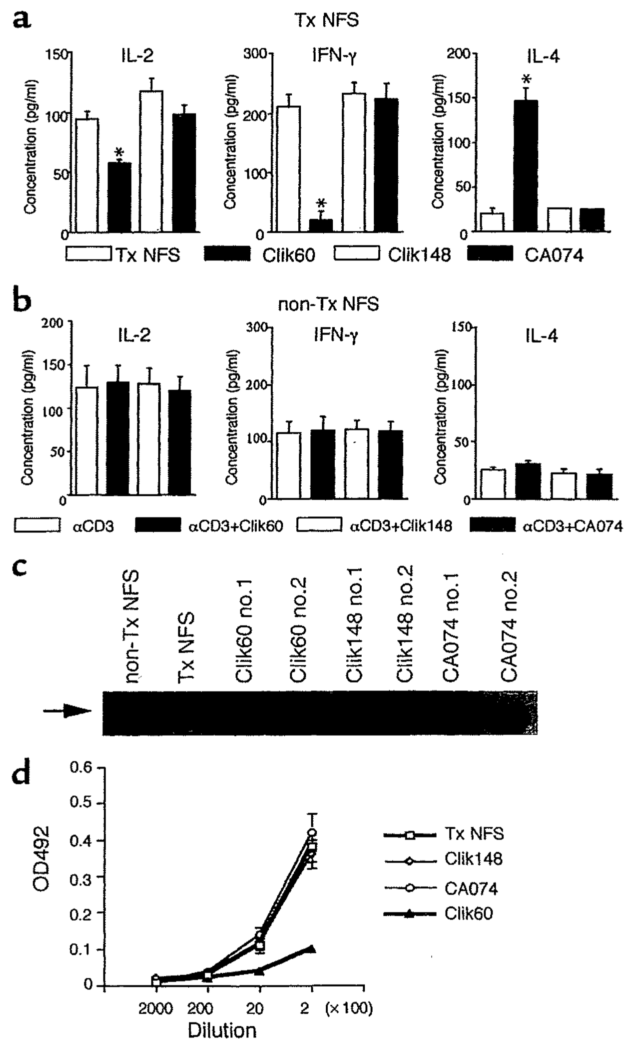

Figure 5
(**a**) Downregulation of Th1 cytokine production in SS model mice treated with Clik60. Culture supernatants from anti-CD3 mAb-stimulated splenic T cells from Clik60-treated model mice (8 weeks old) contained a high level of IL-4, but low levels of IL-2 and IFN-γ, as measured by ELISA (*$P < 0.005$, **$P < 0.001$, Student's t test). (**b**) No significant cytokine production in culture supernatants from anti-CD3 mAb-stimulated splenic T cells from non-Tx NFS/*sld* mice (8 weeks old). (**c**) Production of serum autoantibodies to 120-kDa α-fodrin was diminshed in two mice (no. 1 and no. 2) treated with Clik60, as shown by immunoblotting. (**d**) A dilution curve illustrates that each case is capable of reacting with antigen. The same dilution of sera was examined in all cases by ELISA. OD492, optical density of 492 nm.

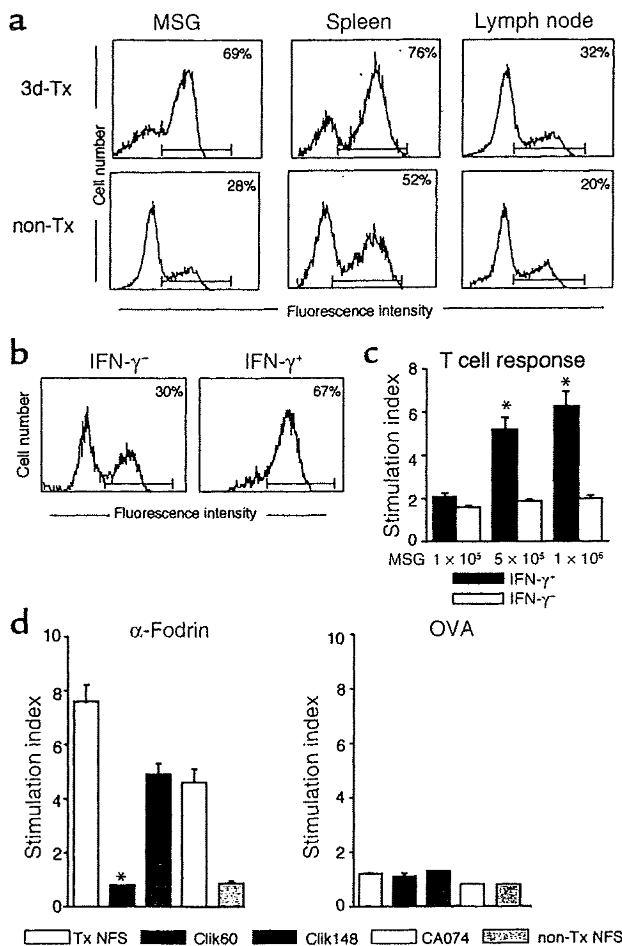

Figure 6
(**a**) A large proportion of class II-expressing (I-A^q-expressing) cells observed on MSG cells from SS model mice compared with those on spleen and LNCs, as determined by flow cytometry. (**b**) MHC class II molecule induced by IFN-γ (100 U/ml) stimulation in MSG cells from non-Tx NFS/*sld* control mice by flow cytometry. (**c**) Significant proliferative responses of purified CD4⁺ T cells (2×10^5) from model mice with IFN-γ-stimulated MSG cells (5×10^5 and 1×10^6, $*P < 0.01$, Student's *t* test), but not from those with non-stimulated MSG cells. (**d**) Autoantigen-stimulated (JS-1-stimulated) proliferative T cell response of LNCs using MSG cells from SS model mice was significantly inhibited by the incubation with Clik60, but not the incubation with Clik148 and CA074 ($*P < 0.01$, Student's *t* test). Data are expressed as stimulation indices ± SEM. Three experiments from each group were performed.

ecules on their surface that can quickly load peptides from the extracellular milieu for T cell presentation (43). The kinetics of this T cell response may correlate with the kinetics of peptide binding to the surface class II MHC molecules (44–46).

Recent studies using cathepsin-deficient mice have shown that cathepsin D and cathepsin B are unnecessary for MHC class II presentation (47, 48), whereas cathepsin S and cathepsin L are important for antigen presentation by discrete populations of cells (49–52). Active cathepsin S was detected in B cells, dendritic cells, and peritoneal macrophages, where it was shown to be involved in the late stages of Ii degradation (50–52). By contrast, cathepsin L was detected only in macrophages and cortical thymic epithelial cells, where a defect resulted in severely impaired CD4⁺ T cell selection (49). In addition to cathepsin S and cathepsin L, cathepsin F has been recently implicated in Ii degradation and antigen presentation in alveolar macrophages (53). Thus, it would appear that different APCs utilize distinct cathepsins to mediate late-stage Ii degradation and to regulate MHC class II presentation. Moreover, cathepsin S-deficient mice showed diminished susceptibility to collagen-induced arthritis, suggesting a potential therapeutic target for regulation of immune responsiveness (50). In this study, the treatment with cathepsin S inhibitor was effective in preventing the development of autoimmune lesions in the salivary and lacrimal glands of the SS model mice. Almost entire remissions of SS were induced in the salivary and

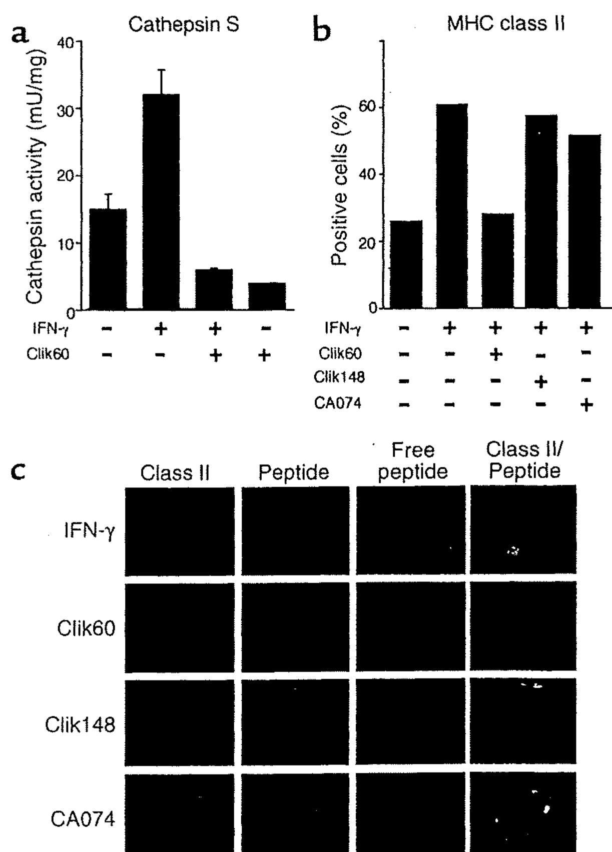

Figure 7
(**a**) Detection of cathepsin S activity in MSG cells and its inhibition by Clik60 (10^{-5} M). (**b**) Surface expression of class II molecule induced by IFN-γ stimulation on MSG cells was clearly inhibited by the incubation with Clik60, but not the incubation with CA074 and Clik148, as determined by flow cytometry. (**c**) Colocalization of FITC-labeled $AFN_{303-318}$ peptide and class II (I-A^q) molecule in the IFN-γ-stimulated MSG cells. Prevention of the surface expression of peptide-class II complex formation was confirmed by the inhibition of Clik60, but not by inhibition of Clik148 and CA074.

lacrimal glands by the Clik60 treatment. In addition, Clik60-treated mice showed a significant downregulation of autoantigen-specific (JS-1-specific) T cell response and Th1 cytokine expressions. Although we could not exclude the possibility that the Clik60 treatment in vivo inhibits only cathepsin S and that other enzymes could be involved, these results indicate that cathepsin S inhibitor plays an important role in preventing autoantigen presentation which is followed by inhibition of autoimmunity.

Ubiquitous expression and involvement in the terminal degradation of proteins that intersect the endocytic pathway were previously perceived to be the hallmarks of these proteinases. However, recent evidence has demonstrated that several cathepsins are expressed in a tissue-specific fashion and that partial proteolysis of specific biological targets is a key function of cathepsins in antigen processing. Analysis of cathepsin L-deficient mice revealed a profound defect in Ii degradation in thymic cortical epithelial cells but not in bone marrow-derived APCs. We have recently identified the pathogenic T cell epitope peptide ($AFN_{303-318}$) on α-fodrin autoantigen involved in organ-specific autoimmune responses (unpublished data). We confirmed here that MSG cells may play an important role for presenting autopeptide in the autoimmune responses, and that inhibition of cathepsin S prevents the surface expression of peptide–class II complex formation, while inhibition of cathepsin L and cathepsin B did not. The differential expression of proteinases by distinct APCs may affect the types of peptides that are presented to T cells and thereby the immune responses that are ultimately generated.

Taken together, our experiments support the hypothesis that cathepsin S, previously shown to be important in Ii processing in vitro, regulates MHC class II function and subsequent autoimmune responses in vivo. Thus, selective inhibition of cysteine protease cathepsin S may have important therapeutic potential in modulating class II-restricted autoimmune processes.

Acknowledgments

This work was supported in part by Grants-in-Aid for Scientific Research (12307040, 12557022) from the Ministry of Education, Science and Culture of Japan. K. Saegusa is a Research Fellow of the Japan Society for the Promotion of Science.

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