AICD is triggered in CD4+ T cells by the specific antigenic peptide (e.g., tetanus toxoid or myelin basic protein) presented by the appropriate class II MHC molecules (45), supporting the notion that AICD can be triggered in activated cells through the TCR-mediated recognition of antigen. Autoimmune epitope spreading has been described in patients with systemic lupus erythematosus, multiple sclerosis, and bullous pemphigus (46,47), and it is reported to be B7-1 dependent, playing a major pathologic role in EAE in mice (48). By the time a patient is diagnosed as having an autoimmune disease, significant tissue destruction has already occurred, making it difficult to identify the antigen against which the autoimmune response is directed (47).

It has been shown that membrane FasL is cleaved into a 26-kd soluble form by an MMP (49,50). We previously detected a 26-kd soluble form of FasL and MMP-9 exclusively in JS-1–stimulated splenic T cells in SS model mice (32). In the present study, we detected a significantly increased concentration of MMP-9 in culture supernatant from JS-1– and CII-stimulated splenic T cells activated with anti-CD3 mAb from aged SS model mice. It is possible that autoantigen (JS-1 and/or CII)–stimulated MMP-9 production may play an important role in down-regulation of Fas-mediated AICD, and in bystander T cell activation, resulting in accelerated development of autoimmune lesions.

In conclusion, these results suggest that age-related disturbance of AICD may play a major role in accelerated development of autoimmune lesions. The functional assays of cellular autoimmunity provide convincing evidence for impaired T cell tolerance to a set of closely related self determinants.

REFERENCES

1. Miller RA. The aging immune system: primers and prospectus. Science 1996;273:70–4.
2. Pawelec G, Adibadeh M, Pohla H, Schaudt K. Immunosenescence: ageing of the immune system. Immunol Today 1995;16: 420–2.
3. Kiecolt-Glaser JK, Preacher KJ, MacCallum RC, Atkinson C, Malarkey WB, Glaser R. Chronic stress and age-related increases in the proinflammatory cytokine IL-6. Proc Natl Acad Sci U S A 2003;100:9090–5.
4. Straub RH, Scholmerich J, Cutolo M. The multiple facets of premature aging in rheumatoid arthritis. Arthritis Rheum 2003: 48:2713–21.
5. Bardos T, Zhang J, Mikecz K, David CS, Glant TT. Mice lacking endogenous major histocompatibility complex class II develop arthritis resembling psoriatic arthritis at an advanced age. Arthritis Rheum 2002;46:2465–75.
6. Brunner T, Mogil RJ, LaFace D, Yoo NJ, Mahboubi A, Echeverri F, et al. Cell-autonomous Fas(CD95)/Fas-ligand interaction mediates activation-induced apoptosis in T-cell hybridomas. Nature 1995;373:441–4.
7. Ju ST, Panka DJ, Cui H, Ettinger R, el-Khatib M, Sherr DH, et al. Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. Nature 1995;373:444–8.
8. Hodes RJ. Molecular alterations in the aging immune system. J Exp Med 1995;182:1–3.
9. Nagel JE, Chopra RK, Chrest FJ, McCoy MT, Schneider EL, Holbrook NJ, et al. Decreased proliferation, interleukin 2 synthesis, and interleukin 2 receptor expression are accompanied by decreased mRNA expression in phytohemagglutinin-stimulated cells from elderly donors. J Clin Invest 1988;81:1096–102.
10. Proust JJ, Filburn CR, Harrison SA, Buchholz MA, Nordin AA. Age-related defect in signal transduction during lectin activation of murine T lymphocytes. J Immunol 1988;139:1472–8.
11. Critchfield JM, Racke MK, Zuniga-Pflucker JC, Cannella B, Raine CS, Goverman J, et al. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. Science 1994;263: 1139–43.
12. Chen Y, Inobe JI, Marks R, Gonnella P, Kuchroo VK, Weiner HL. Peripheral deletion of antigen-reactive T cells in oral tolerance. Nature 1995;376:177–80.
13. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456–62.
14. Webb S, Morris C, Sprent J. Extrathymic tolerance of mature T cells: clonal elimination as a consequence of immunity. Cell 1990;63:1249–56.
15. Nagata S, Suda T. Fas and Fas ligand: lpr and gld mutations. Immunol Today 1995;16:39–43.
16. Van Parijs L, Ibraghimov LA, Abbas AK. The roles of costimulation and Fas in T cell apoptosis and peripheral tolerance. Immunity 1996;4:321–8.
17. Drappa J, Brot N, Elkon KB. The Fas protein is expressed at high levels on $CD4^{+}CD8^{+}$ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRLlpr/lpr. Proc Natl Acad Sci U S A 1993;90:10340–4.
18. Fox RI, Stern M, Michelson P. Update in Sjogren's syndrome. Curr Opin Rheumatol 2000;12:391–8.
19. Manoussakis MN, Moutsopoulos HM. Sjogren's syndrome: current concepts. Adv Intern Med 2001;47:191–217.
20. Talal N, Sokoloff L, Barth WF. Extrasalivary lymphoid abnormalities in Sjogren's syndrome. Am J Med 1967;43:50–65.
21. James JA, Gross T, Scofield RH, Harley JB. Immunoglobulin epitope spreading and autoimmune disease after peptide immunization: Sm B/B9-derived PPPGMRPP and PPPGIRGP induce spliceosome autoimmunity. J Exp Med 1995;181:453–61.
22. McRae BL, Vanderlugt CL, dal Canto MC, Miller SD. Functional evidence for epitope spreading in the relapsing pathology of experimental autoimmune encephalomyelitis. J Exp Med 1995; 182:75–85.
23. Yu M, Johnson JM, Tuohy VK. A predictable sequential determinant spreading cascade invariably accompanies progression of experimental autoimmune encephalomyelitis: a basis for peptide-specific therapy after onset of clinical disease. J Exp Med 1996; 183:1777–88.
24. Zhao ML, Fritz RB. The immune response to a subdominant epitope in myelin basic protein exon-2 results in immunity to intra- and intermolecular dominant epitopes. J Neuroimmunol 1995;61: 179–84.
25. Tian J, Atkinson MA, Clare-Salzler M, Herschenfeld A, Forsthuber T, Lehmann PV, et al. Nasal administration of glutamate decarboxylase (GAD65) peptides induces Th2 responses and prevents murine insulin-dependent diabetes. J Exp Med 1996;183: 1561–7.
26. Haneji N, Hamano H, Yanagi K, Hayashi Y. A new animal model for primary Sjogren's syndrome in NFS/sld mutant mice. J Immunol 1994;153:2769–77.

27. Edwards CK III, Zhou T, Zhang J, Baker TJ, De M, Long RE, et al. Inhibition of superantigen-induced proinflammatory cytokine production and inflammatory arthritis in MRL-lpr/lpr mice by a transcriptional inhibitor of TNF-α. J Immunol 1996;157:1758–72.
28. Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, et al. Identification of α-fodrin as a candidate autoantigen in primary Sjogren's syndrome. Science 1997;276:604–7.
29. Feeney AJ, Lawson BR, Kono DH, Theofilopoulos AN. Terminal deoxynucleotidyl transferase deficiency decreases autoimmune disease in MRL-Fas^{lpr} mice. J Immunol 2001;167:3486–93.
30. Fields ML, Sokol CL, Eaton-Bassiri A, Seo S, Madaio MP, Erikson J. Fas/fas ligand deficiency results in altered localization of anti-double-stranded DNA B cells and dendritic cells. J Immunol 2001;167:2370–8.
31. Kageyama Y, Koide Y, Yoshida A, Uchijima M, Arai T, Miyamoto S, et al. Reduced susceptibility to collagen-induced arthritis in mice deficient in IFN-γ receptor. J Immunol 1998;161:1542–8.
32. Ishimaru N, Yanagi K, Ogawa K, Suda T, Saito I, Hayashi Y. Possible role of organ-specific autoantigen for activation-induced cell death (AICD) in murine Sjogren's syndrome. J Immunol 2001;167:6031–7.
33. Alderson MR, Tough TW, Davis-Smith T, Braddy S, Falk B, Schooley KA, et al. Fas ligand mediates activation-induced cell death in human T lymphocytes. J Exp Med 1995;181:71–7.
34. Dhein J, Walczak H, Baumler C, Debatin KM, Krammer PH. Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). Nature 1995;373:438–41.
35. Rieux-Laucat F, le Deist F, Hivroz C, Roberts AG, Debatin KM, Fischer A, et al. Mutations in Fas associated with human lymphoproliferative syndrome and autoimmunity. Science 1995;268: 1347–9.
36. Fisher GH, Rosenberg FJ, Strauss SE, Dale JK, Middleton LA, Lin AY, et al. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. Cell 1995;81:935–46.
37. Buzas EI, Mikecz K, Brennan FR, Glant TT. Mediators of autopathogenic effector cells in proteoglycan-induced arthritic and clinically asymptomatic BALB/c mice. Cell Immunol 1994;158: 292–304.
38. Finnegan A, Mikecz K, Tao P, Glant TT. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. J Immunol 1999;163:5383–90.
39. Lehmann PV, Forsthuber T, Miller A, Sercarz EE. Spreading of T-cell autoimmunity to cryptic determinants of an autoantigen. Nature 1992;358:155–7.
40. Tan LJ, Kennedy MK, dal Canto MC, Miller SD. Successful treatment of paralytic relapses in adoptive experimental autoimmune encephalomyelitis via neuroantigen-specific tolerance. J Immunol 1991;147:1797–802.
41. Kaufman DL, Clare-Salzler M, Tian J, Forsthuber T, Ting GS, Robinson P, et al. Spontaneous loss of T cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature 1993;366:69–71.
42. Tisch R, Yang XD, Singer SM, Liblau RS, Fugger L, McDevitt HO. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 1993;366:72–5.
43. Kabelitz D, Oberg HH, Pohl T, Pechhold K. Antigen-induced death of mature T lymphocytes: analysis by flow cytometry. Immunol Rev 1994;142:157–74.
44. Damle NK, Klussman K, Leytze G, Aruffo A, Linsley PS, Ledbetter JA. Costimulation with integrin ligands intercellular adhesion molecule-1 or vascular cell adhesion molecule-1 augments activation-induced death of antigen-specific CD4$^+$ T lymphocytes. J Immunol 1993;151:2368–79.
45. Pelfrey CM, Tranquill LR, Boehme SA, McFarland HF, Lenardo MJ. Two mechanisms of antigen-specific apoptosis of myelin basic protein (MBP)-specific T lymphocytes derived from multiple sclerosis patients and normal individuals. J Immunol 1995;154: 6191–202.
46. Vanderlugt CL, Neville KL, Nikcevich KM, Eagar TN, Bluestone JA, Miller SD. Pathologic role and temporal appearance of newly emerging autoepitopes in relapsing experimental autoimmune encephalomyelitis. J Immunol 2000;164:670–8.
47. Perriard J, Jaunin F, Favre B, Budinger L, Hertl M, Saurat JH, et al. IgG autoantibodies from bullous pemphigoid (BP) patients bind antigenic sites on both the extracellular and the intracellular domains of the BP antigen 180. J Invest Dermatol 1999;112:141–7.
48. Grenet J, Teitz T, Wei T, Valentine V, Kidd VJ. Structure and chromosome localization of the human CASP8 gene. Gene 1999; 226:225–32.
49. Kayagaki N, Kawasaki A, Ebata T, Ohmoto H, Ikeda S, Inoue S, et al. Metalloproteinase-mediated release of human Fas ligand. J Exp Med 1995;182:1777–83.
50. Gearing AJ, Beckett P, Christodoulou M, Churchill M, Clements J, Davidson AH, et al. Processing of tumor necrosis factor-α precursor by metalloproteinases. Nature 1994;370:555–7.

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Critical role of cathepsin-inhibitors for autoantigen processing and autoimmunity

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Introduction

The cysteinal lysosomal proteases, cathepsin S and cathepsin L have been shown to process invariant chain (Ii), thereby facilitating MHC class II maturation. However, their role in autoantigen processing is not established. Sjögren's 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 (RNP) particles SS-A/Ro and SS-B/La (Fox et al., 1986; Chan et al., 1991; Kruize et al., 1995). Previously, we reported a 120 kD α-fodrin autoantigen on the development of autoimmune exocrinopathy in SS model mice and identified autoantigen-specific T cell responses associated with Th1 cytokine production of interleukin (IL)-2 and interferon (IFN)-γ (Haneji et al., 1994, 1997). Insulin-dependent diabetes mellitus (IDDM) is a T-cell-mediated, organ-specific autoimmune disease that occurs in humans and in animal models such as the non-obese diabetic (NOD) mouse (Katz et al., 1993; Haskins and McDuffie, 1990). The autoimmune diabetes in NOD mice is characterized by lymphocytic infiltration of the islets (insulitis) followed by destruction of islet β-cells. Evidence for the role of $CD4^+$ Th1 cells in IDDM derives from recent studies of the NOD mouse in which the identical glutamic acid decarboxylase (GAD) and proinsulin are key β-cell autoantigens recognized by both T cells and B cells (Kaufman et al., 1993; Liu et al., 2002).

MHC class II molecules encounter and bind antigenic peptides as class II–peptide complexes on the cell surface of antigen-presenting cells (APCs) for recognition by $CD4^+$ T cells (Cresswell 1994; Gremain, 1994; Wolf and Ploegh, 1995). The molecular mechanisms leading to formation of class II–peptide complexes and

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presentation of antigen on the cell surface begin with synthesis of class II $\alpha\beta$ heterodimers in the endoplasmic reticulum. These class II $\alpha\beta$ heterodimers associate early during biosynthesis with a type II membrane protein, the invariant chain (Ii) (Roche and Marks, 1991; Lamb and Cresswell, 1992). 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 (Reyes et al., 1991). Cathepsin L, a potent cysteine-class endoprotease, is specifically inhibited by a fragment of the alternatively spliced Ii form p41 (Bevec et al., 1996). Cathepsin S containing potent endoproteolytic activity is highly expressed in the spleen and professional APCs and other class II-positive cells, and is inducible with by IFN-γ (Shi et al., 1992, 1994). Katunuma and his colleagues have developed specific inhibitors of cathepsin B (CA074), cathepsin L (Clik148), and cathepsin S (Clik60), in vivo as well as in vitro (Towatari et al., 1991; Murata et al., 1991; Katunumua et al., 1999). Matsunaga et al. first reported that CA074 suppresses immune responses (Matsunaga et al., 1993), suggesting that cysteine proteases in 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 block generation of the antigenic peptide on the development of autoimmune diseases. To address this important issue, autoantigen processing and presentation after specific inhibition of cathepsins were examined in murine model for SS and IDDM.

Materials and methods

Mice

Female NFS/N strain carrying the mutant gene *sld* (Hayashi et al., 1988), and NOD mice 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. C56BL/6 mice (Charles River Japan, Inc., Atsugi, Japan), were used as controls.

Cyclophosphamide-induced diabetes

Prediabetic (7–8-week-old) female NOD mice were challenged with i.p. injection of cyclophosphamide (CY) (200 mg/kg) (Sigma-Aldrich) and analyzed 1–4 weeks after CY injection.

Assessment of diabetes

The blood glucose level was monitored weekly with a Glucometer (Kodama, Tokyo, Japan) using 50 µl blood from tail vein. Mice were monitored weekly for development of urine glucose with Keto-Diastix (Bayer-Sankyo Co., Ltd., Tokyo, Japan).

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 (Towatari et al., 1991; Murata et al., 1991; Katunumua et al., 1999).

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 murine model for SS and IDDM in CY-treated NOD mice. Each inhibitor (Clik60, CA074, and Clik148) dissolved in PBS was administered i.p. into model mice at doses of 0.1 mg/mouse/day) and compared with non-treated model mice. All organs were removed from the mice, fixed with 4% phosphate-buffered formaldehyde (pH 7.2) and prepared for histological examination. The sections were stained with hematoxylin and eosin (H&E). Histological grading of the inflammatory lesions in the SS model was done according to the method proposed previously (White and Caserett, 1974).

Proliferation assay

Single cell suspensions of spleen cells or regional lymph node cells (LNCs) were cultured in 96-well flat bottom microtiter plates (Nunc, Roskilde, Denmark). For proliferation assay, a total of 5×10^5 cells/well were cultured for 72 h under stimulation of recombinant α-fodrin protein (JS-1, 10 μg/ml) (10), insulin (10 μg/ml), GAD (10 μg/ml), OVA (10 μg/ml), and ConA (5 μg/ml), and pulsed with 1 μCi/well of [^{3}H]thymidine (NEN Life Science Products, Boston, MA) during final 20 h of the culture. We further examined the in vitro preventive effects of cathepsin inhibitors (10^{-7}–10^{-4} M CA074, Clik148, and Clik60) for antigen-specific proliferative T cell responses. T cell purification was done using CD4mAb-bounded immunomagnetic beads (Dynal, CA).

Measurement of cytokine production

Cytokine production from spleen cells was tested by two-step sandwich ELISA using a mouse IL-2, IL-4, and IFN-γ kit (Genzyme). In brief, culture supernatants from spleen cells activated with immobilized anti-CD3 mAb (10 μg/ml)(Ceder Lane Lab.) for 3 days were added to microtiter plates precoated with anti-IL-2, IL-4, and IFN-γ capture Ab and incubated overnight at 4°C. After addition of biotinylated detecting Ab and incubation at room temperature for 45 min, avidin-peroxidase was added and incubated at room temperature for 30 min. 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, CA). The concentrations of IL-2 (pg/ml), IL-4

(pg/ml), and IFN-γ pg/ml) were calculated according to the standard curves produced by various concentrations of recombinant cytokines.

Detection of serum autoantibodies against 120 kD α-fodrin

Serum autoantibody production against 120 kD α-fodrin was analyzed by immunoblotting as described previously (Haneji et al., 1997).

Measurement of fluid secretion

Detection of tear and saliva volume of the treated, and non-treated SS animal model was done according to a modified method as described (Delporte et al., 1997).

Results and discussion

Therapeutic effects of specific inhibitors of Cathepsin S (Clik60) for animal model of SS

To address the role of autoantigen-reactive T cells, we examined the proliferative T cell responses in the cervical LNCs and spleen cells from SS model mice and controls. We found that the LNCs and spleen cells in SS model mice at 8 weeks of age showed a significant increase in autoantigen (JS-1)-specific T cell proliferation (Fig. 1a), but not in C57BL/6 control mice. 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 the spleens, from SS model mice, a significantly inhibitory effect of Clik60 was observed in dose-dependent manner (Fig. 1b). We next examined the in vivo therapeutic effects of Clik60, Clik148, and CA074 in murine model for SS. Treatment with i.p. 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 ($P<0.05$) glands of the SS model mice, but not in groups injected with CA074, and Clik148 (Fig. 2a). Moreover, the average saliva and tear volume of Clik60-treated model mice was significantly higher than that of the non-treated SS model mice (Fig. 2b). In addition, autoantigen (JS-1)-specific T cell response was significantly inhibited in LNCs from Clik60-treated mice. Culture supernatants from anti-CD3mAb-stimulated splenic T cells obtained from Clik60-treated mice contained high levels of IL-4, but low levels of IL-2 and IFN-γ by ELISA (Fig. 3a). Serum autoantibody production against 120 kD α-fodrin autoantigen was exclusively inhibited in Clik60-treated mice, but not in other groups (Fig. 3b). 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 in the salivary and lacrimal glands. A large proportion of class II (I-A^q)-expressing cells was observed

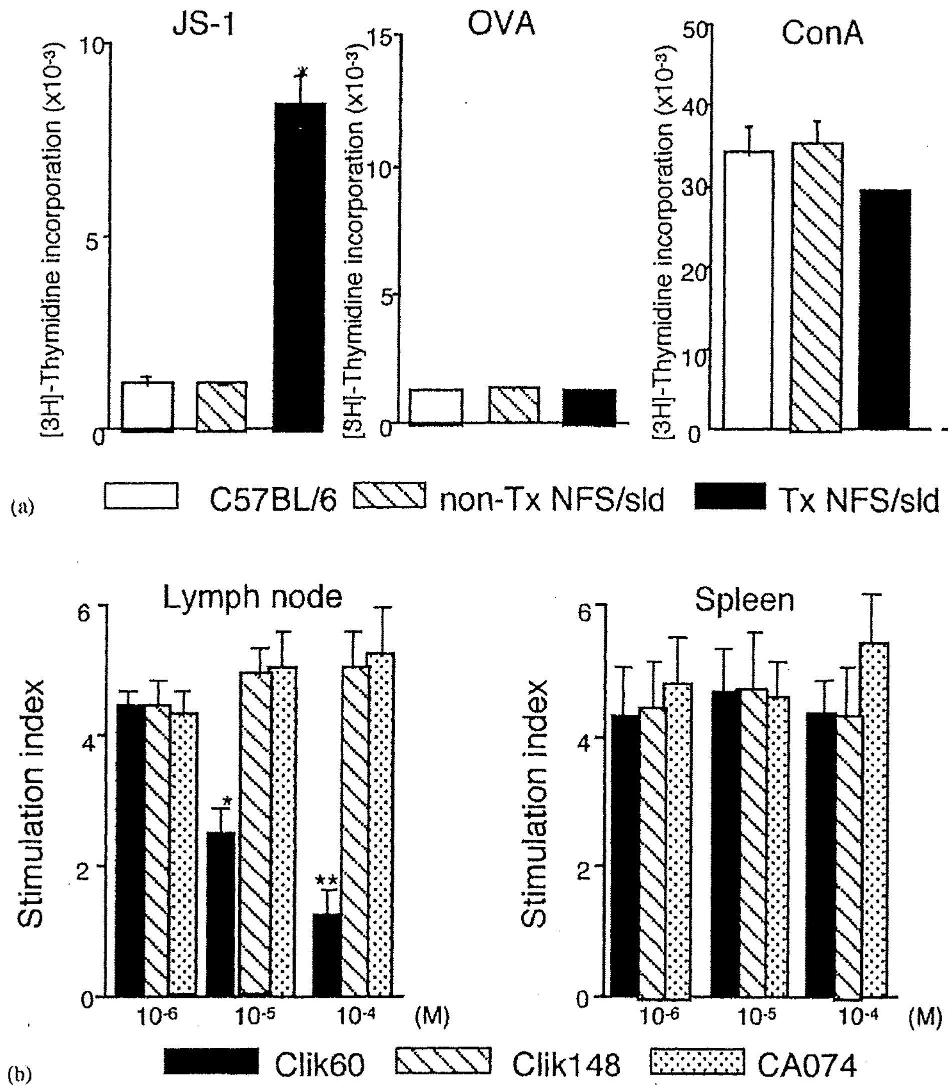

Fig. 1. (a) Detection of proliferative T cell response of LNCs from SS model mice to organ-specific autoantigen (JS-1) (*$P<0.001$, Student's t-test)(a). No differences were found in OVA (10 μg/ml), and ConA (5 μg/ml)-responsiveness. (b) In vitro preventive effect of proliferative T cell response of LNCs, but not spleen cells, to JS-1 by Clik60 in dose-dependent manner. No inhibitory effects of Clik148, and CA074 were found. Data are expressed as stimulation indices (SI) ± standard error of the mean (s.e.m.). 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). Part of this Figure included in reference by Saegusa et al. (2002).

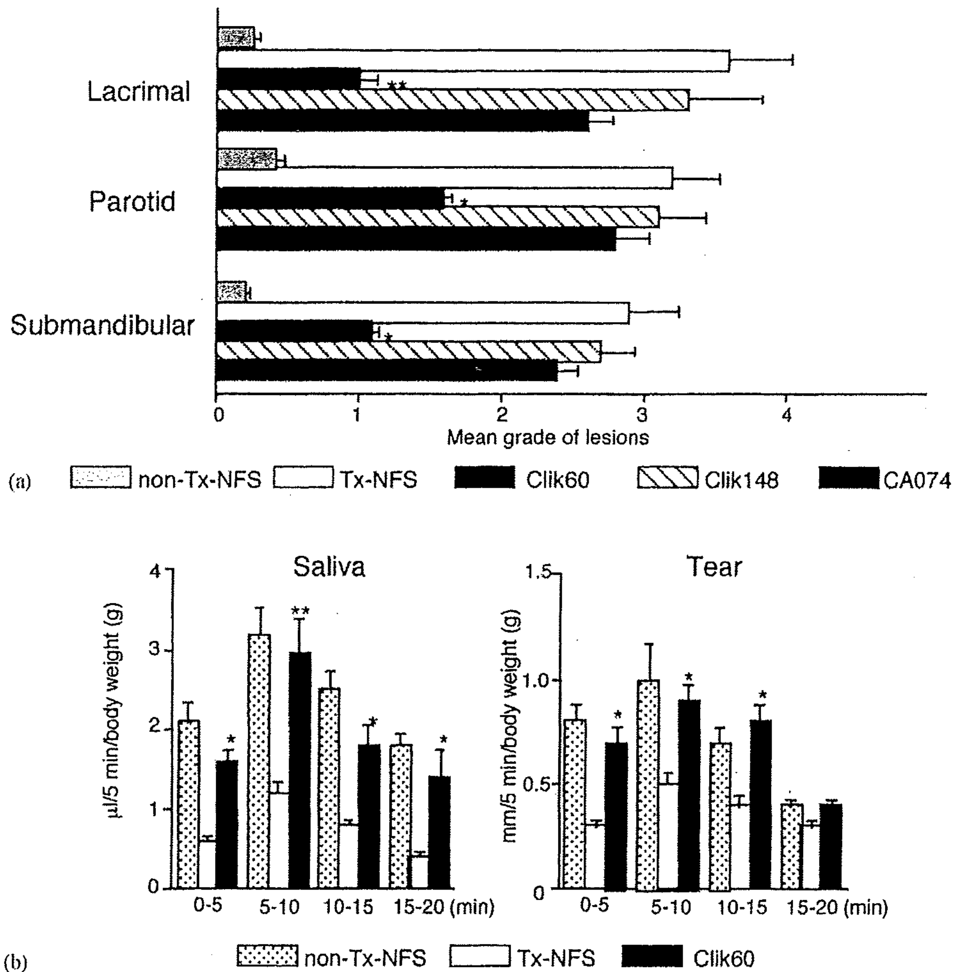

Fig. 2. (a) Effects of in vivo administration of i.p. injection of cathepsin inhibitors (Clik60, Clik148, and CA074). Each inhibitor was administered into SS model mice ($n = 10$, for each) at doses of 0.1 mg/mouse/day from 4 weeks–7 weeks, and then analyzed at 8 weeks, compared with non-treated SS model mice ($n = 7$). The treatment with i.p. injection of Clik60 was effective in preventing the development of autoimmune lesions in the lacrimal, parotid, and submandibular glands of the SS model mice, but not in groups injected with Clik148, and CA074 ($^{*}P<0.01$ and $^{**}P<0.005$, Student's t-test). (b) 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$\pm$SEM in 5 mice examined per each group ($^{*}P<0.05$ and $^{**}P<0.005$, Student's t-test). Part of this Figure included in reference by Saegusa et al. (2002).

on mouse salivary gland (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 (Saegusa et al., 2002). It is possible that the salivary gland

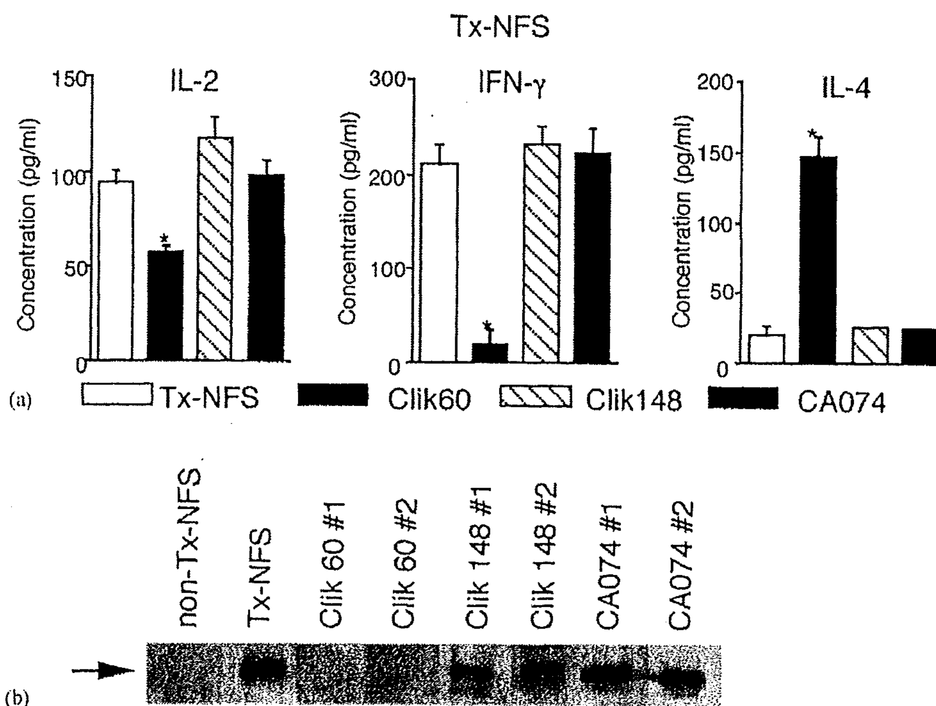

Fig. 3. (a) Down-regulation of the Th1-type cytokine production in the SS model mice treated with Clik60. Culture supernatants from anti-CD3 mAb-stimulated splenic T cells from Clik60-treated model mice (8-week-old) contained a high level of IL-4, but low levels of IL-2 and IFN-γ as measured by ELISA (*$P<0.005$ and **$P<0.001$, Student's t-test). (b) Inhibition of serum autoantibody production to 120 kD α-fodrin was observed in two different recipient mice treated with Clik60 (#1, #2) as shown by immunoblotting. Part of this Figure included in reference by Saegusa et al. (2002).

epithelial cells may function, at least in part, as autoantigen-presenting cells on the development of murine SS, and that inhibition of cathepsin S prevents the 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. 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. The treatment with cathepsin S-inhibitor was effective in preventing the development of autoimmune lesions in the SS model mice. In addition, Clik60-treated mice showed a significant downregulation of autoantigen (JS-1)-specific T cell response and Th1 cytokine expressions. These results indicate that cathepsin S-inhibitor plays an important role for preventing autoantigen presentation that followed by inhibition of autoimme exocrinopathy in SS.

Therapeutic effects of specific inhibitors of Cathepsin L (Clik148) for Autoimmune Diabetes (NOD) mice

We next examined the in vivo therapeutic effects of Clik60, Clik148, and CA074 in NOD mice, a well-known strain of animal model for autoimmune diabetes. Treatment with i.p. injection of Clik148 (0.1 mg/mouse/day) was effective in preventing the glucose production in blood and urine from the cychrophosphamide (CY)-treated NOD mice, but not in groups injected with CA074, and Clik60 (0.1 mg/mouse/day) (Fig. 4). To ensure the role of autoantigen-reactive T cells, we examined the proliferative T cell responses in the peripancreatic LNCs and spleen cells from CY-NOD mice and C57BL/6 control mice. We found that the peripancreatic LNCs and spleen cells in CY-NOD mice showed a significant increase in autoantigen (GAD and insulin)-specific T cell proliferation, but not in control mice. No significant differences were observed in the proliferative response stimulated with OVA (10 μg/ml) and ConA (5 μg/ml) among these mice. Then, we examined the inhibitory effects of cathepsin inhibitors against autoantigen-specific T cell responses in vitro. In peripancreatic LNCs from CY-NOD mice, a significantly inhibitory effect of Clik148 was observed in both anti-GAD and anti-insulin T cell responses, but no effects were found in incubation with Clik60 or CA074 (Fig. 5). Moreover, surface expressions of class II molecule induced by IFN-γ, and TNF-α stimulation in mouse islet β-cell line (Min6) was clearly inhibited by the incubation with Clik148, but not with Clik60 and CA074 determined by flow cytometry (Fig. 6). From these results, it was strongly suggested that Clik148 play a significant role in preventing autoantigen presentation to generate class II molecules competent for binding antigenic peptide, resulting in inhibition of autoimmune diabetes in NOD mice.

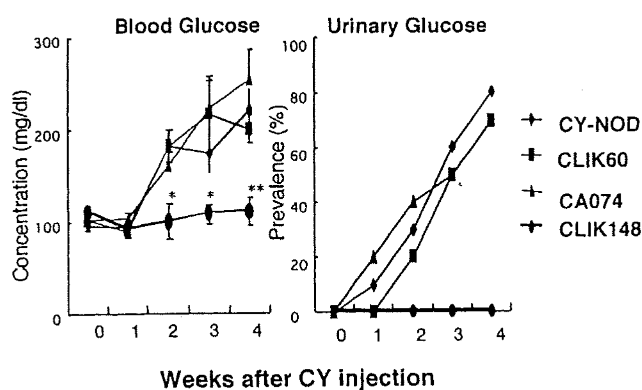

Fig. 4. Treatment with i.p. injection of Clik148 (0.1 mg/mouse/day) was effective in preventing the secretion of blood glucose and urine glucose from the cychrophosphamide (CY)-treated NOD mice (*$P<0.05$, **$P<0.01$, Student's t-test). The blood glucose level was monitored weekly with a glucometer using 50 μl blood from tail vein, and urine glucose was monitored with Keto-Diastix.

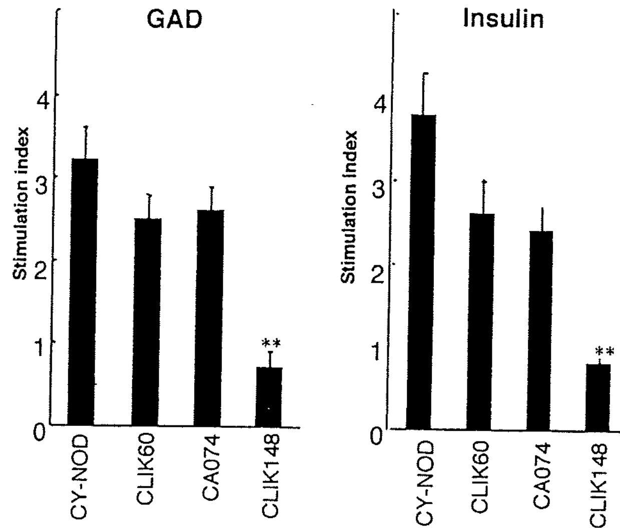

Fig. 5. Autoantigen (GAD and insulin)-stimulated proliferative T cell response of peripancreatic LNCs from NOD mice was significantly inhibited by incubation with Clik148, but not with Clik60, and CA074 (**$P<0.01$, Student's t-test). Data are expressed as stimulation indices (SI)±standard error of the mean (s.e.m.). Three experiments from each group were performed.

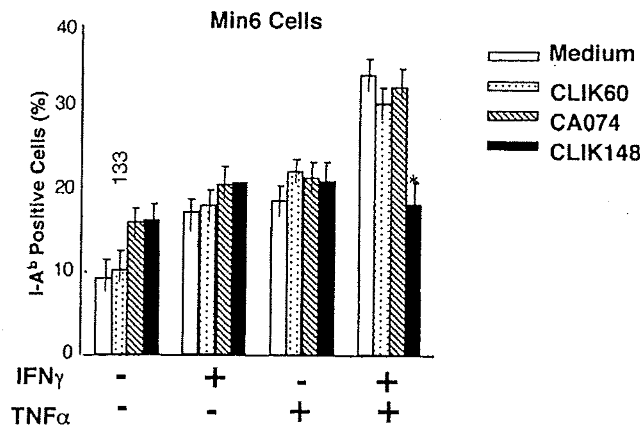

Fig. 6. Expression of MHC class II molecule induced by IFN-γ (100 U/ml), and TNF-α (100 U/ml)-stimulation in mouse islet β-cell line (Min6) was significantly inhibited by the treatment with Clik148, but not with Clik60, and CA074 (**$P<0.01$, Student's t-test). Three experiments from each group were performed.

Differential effect of cathepsin S and L on Antigen presentation

Recent studies have shown that cathepsin S and cathepsin L are important for Ag presentation by discrete populations of cells (Nakagawa et al., 1998, 1999; Shi et al.,

1999). Active cathepsin S was detected in B cells, dendritic cells (DCs), and peritoneal macrophages, where it was shown to be involved in the late stages of Ii degradation (Shi et al., 1999). 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 (Nakagawa et al., 1998). Thus, it would appear that different APCs utilize distinct cathepsins to mediate late stage Ii degradation and 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 (Nakagawa et al., 1999). Because of this cell type-restricted expression, cathepsin L deficiency results in diminished positive selection of CD4$^+$ T cells but does not significantly affect Ag presentation by bone marrow-derived APCs (Nakagawa et al., 1998). In contrast to Ii degradation, little is known about Ag processing, i.e., the proteolytic mechanisms that generate particular T cell epitopes. Early studies found that inhibition of lysosomal acidification interferes with proteolysis and Ag presentation, implicating lysosomal proteases (Watts, 1997). Evaluation of cathepsin L and cathepsin S has focused on their roles in Ii degradation and not in Ag processing (Villadangos and Ploegh, 2000). 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. We confirmed that mouse salivary gland cells and pancreas β islet cells might play an important role for presenting autopeptide in the autoimmune responses, and that inhibition of cathepsin S and cathepsin L prevents the surface expression of peptide/class II complex formation. The differential expression of proteinases by distinct APCs may affect the types of autoantigen peptides that are presented to T cells and thereby the immune responses that are ultimately generated. Our experiments support the hypothesis that cathepsin S, and cathepsin L, 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 proteases cathepsin S and cathepsin L may have important therapeutic potential in modulating class II—restricted autoimmune processes.

Summary

The cysteinal lysosomal proteases, cathepsin S and cathepsin L have been shown to process invariant chain, thereby facilitating MHC class II maturation. However, their role in antigen processing is not established. Studies examined the functional significance of cathepsin inhibition on antigen processing and autoimmune diseases in murine models for SS and non-obese type-I diabetes model (NOD). Specific inhibitor of cathepsin S (Clik60) in vitro markedly impaired presentation of an organ-specific autoantigen, 120 kD α-fodrin, by interfering with MHC class II–peptide binding. Antigen-specific T cell responses were significantly inhibited by incubation with Clik60 in dose-dependent manner. Ttreatment with Clik60 in vivo profoundly blocked lymphocytic infiltrations in the salivary and lacrimal glands and

abrogated a rise in serum autoantibody production. Moreover, treatment with i.p. injection of specific inhibitor of cathepsin L (Clik148) was effective in preventing the glucose production in blood and urine from NOD mice, but not in groups injected with CA074 and Clik60. Clik148 markedly impaired presentation of autoantigens including insulin and GAD in NOD mice. Thus, inhibition of cathepsin activity in vivo alters autoantigen presentation and development of autoimmunity. 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 autoimmune diseases including Sjögren's syndrome and autoimmune diabetes.

References

Bevec T, Stoka V, Pungercic G, Dolenc I, Turk V. Major histocompatibility complex class II-associated p41 invariant chain fragment is a strong inhibitor of lysosomal cathepsin L. J Exp Med 1996;183: 1331–8.

Chan EK, Hamel JC, Buyon JP, Tan ET. Molecular definition and sequence motifs of the 52-kD component of human SS-A/Ro autoantigen. J Clin Invest 1991;87:68–76.

Cresswell P. Antigen presentation. Getting peptides onto MHC class II molecules. Curr Biol 1994;4:541–3.

Delporte BC, Delporte C, O'Connell BC, He X, Lancaster HE, O'Connell AC, Agre P, Baum BJ. Increased fluid secretion after adenoviral-mediated transfer of the aquaporin-1 cDNA to irradiated rat salivary glands. Proc Nat Acad Sci 1997;94:3268–73.

Fox RI, Robinson CA, Curd JG, Kozin F, Howell FV. Sjögren's syndrome. Proposed criteria for classification. Arthritis Rheum 1986;29:577–85.

Germain RN. MHC-dependent antigen processing and peptide presentation: providing ligands for T lymphocyte activation. Cell 1994;76:287–99.

Haneji N, Hamano H, Yanagi K, Hayashi Y. A new animal model for primary Sjögren's syndrome in NFS/*sld* mutant mice. J Immunol 1994;153:2769–77.

Haneji N, Nakamura T, Takio K, Yanagi K, Higashiyama H, Saito I, Noji S, Sugino H, Hayashi Y. Identification of α-fodrin as a candidate autoantigen in primary Sjögren's syndrome. Science 1997;276:604–7.

Haskins K, McDuffie M. Acceleration of diabetes in young NOD mice with a CD4 islet-specific T cell clone. Science 1990;249:1433–6.

Hayashi Y, Kojima A, Hata M, Hirokawa K. A new mutation involving the sublingual gland in NFS/N mice: partially arrested mucous cell differentiation. Am J Pathol 1988;132:187–91.

Katunumua N, Murata E, Kakegawa H, Matsui A, Tsuzuki H, Tsuge H, Turk D, Turk V, Fukushima M, Tada Y, Asao T. Structure based development of novel specific inhibitors for cathepsin L and cathepsin S in vivo and in vitro. FEBS Lett 1999;458:6–10.

Katz JD, Wang B, Haskins K, Benoist C, Mathis D. Following a diabetogenic T cell from genesis through pathogenesis. Cell 1993;74:1089–100.

Kaufman DL, Clare-Salzier M, Tian J, Forsthuber T, Ting GSP, Robinson P, Atkinson MA, Sercarz EE, Tobin AJ, Lehmann OV. Spontaneous loss of T-cell tolerance to glutamic acid decarboxylase in murine insulin-dependent diabetes. Nature 1993;366:69–72.

Kruize AA, Smeenk RJT, Kater L. Diagnostic criteria and immunopathogenesis of Sjögren's syndrome: implications for therapy. Immunol Today 1995;16:557–9.

Lamb C, Cresswell P. Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. J Immunol 1992;148:3478–82.

Liu E, Moriyama H, Abiru N, Miano D, Yu L, Taylor RM, Finkelman FD, Eisenbarth GS. Anti-peptide autoantibodies and fetal anaphylaxis in NOD mice in response to insulin self-peptide B:9-23 and B:13-23. J Clin Invest 2002;110:1021–7.

Matsunaga Y, Saibara T, Kido H, Katunuma N. Participation of cathepsin B in processing of antigen presentation to MHC class II. FEBS Lett 1993;324:325–30.

Murata M, Miyashita S, Yokoo C, Tamai M, Hanada K, Hatayama K, Towatari T, Nikawa T, Katunuma N. Novel epoxysuccinyl peptides: selective inhibitors of cathepsin B, in vitro. FEBS Lett 1991;280:307–10.

Nakagawa T, Roth W, Wong P, Nelson A, Farr A, Deussing J, Villadangos JA, Ploegh H, Peters C, Rudensky AY. Cathepsin L: critical role in Ii degradation and CD4T cell selection in the thymus. Science 1998;280:450–3.

Nakagawa T, Brissette WH, Lira PD, Griffiths RJ, Petrushova N, Stock J, McNeish JD, Eastman SE, Howard ED, Clarke SR, Rosloniec EF, Elliott EA, Rudensky AY. Impaired invariant chain degradation and antigen presentation and diminished collagen-induced arthritis in cathepsin S null mice. Immunity 1999;10:207–17.

Reyes VE, Lu S, Humphreys RE. Cathepsin B cleavage of Ii from MHC α- and β-chains. J Immunol 1991;146:3877–80.

Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. Nature 1991;354:392–4.

Saegusa K, Ishimaru N, Yanagi K, Arakaki R, Ogawa K, Saito I, Katunuma N, Hayashi Y. Cathepsin S inhibitor prevents autoantigen presentation and autoimmunity. J Clin Invest 2002;110:361–9.

Shi GP, Munger JS, Meara JP, Rich DH, Chapman HA. Molecular cloning and expression of human alveolar macrophage cathepsin S, an elastinolytic cysteine protease. J Biol Chem 1992;267:7258–62.

Shi GP, Webb AC, Foster KE, Knoll JHM, Lemere CA, Munger JS, Chapman HA. Human cathepsin S: chromosomal localization, gene structure, and tissue distribution. J Biol Chem 1994;269:11530–6.

Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, Riese R, Ploegh HL, Chapman HA. Cathepsin S required for normal MHC class II peptide loading and germinal center development. Immunity 1999;10:197–206.

Towatari T, Nikawa T, Murata M, Yokoo C, Tamai M, Hanada K, Katunuma N. Novel epoxysuccinyl peptides: a selective inhibitor of cathepsin B, in vivo. FEBS Lett 1991;280:311–5.

Villadangos JA, Ploegh HL. Proteolysis in MHC class II antigen presentation: who's in charge? Immunity 2000;12:233–9.

Watts C. Capture and processing of exogenous antigens for presentation on MHC molecules. Annu Rev Immunol 1997;15:821–50.

White SC, Caserett GW. Induction of experimental autoallergic sialadenitis. J Immunol 1974;112:178–85.

Wolf PR, Ploegh HL. How MHC class II molecules acquire peptide cargo: biosynthesis and trafficking through the endocytic pathway. Annu Rev Cell Dev Biol 1995;11:267–306.

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Effective Treatment of a Mouse Model of Sjögren's Syndrome With Eyedrop Administration of Anti-CD4 Monoclonal Antibody

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Objective. **To determine whether eyedrop administration of an anti-CD4 monoclonal antibody (mAb) is effective in the treatment of Sjögren's syndrome (SS) using a mouse model of the disease.**

Methods. **The anti-CD4 mAb was administered daily into the eyes of mice with SS from ages 4 to 8 weeks or ages 10 to 12 weeks. During treatment, tear volume was monitored and after final treatment, histologic features of the lacrimal and salivary glands, the phenotypes and function of T cells, and serum titers of anti-α-fodrin antibody were examined.**

Results. **Eyedrop administration of anti-CD4 mAb before the onset of SS prevented the autoimmune pathology seen in the lacrimal glands but not that in the salivary glands. Furthermore, eyedrop administration of anti-CD4 mAb after the development of SS inhibited mononuclear cell infiltration and the destruction of parenchyma only in the lacrimal glands. Eyedrop administration of anti-CD4 mAb suppressed the local activation of CD4+ T cells rather than deleting CD4+ T cells, which reduced the expansion of pathologic CD4+ T cells against α-fodrin.**

Conclusion. **These results demonstrate the remarkable efficacy of anti-CD4 mAb eyedrops in the treatment of SS eye symptoms, which illustrates a new antibody-based therapeutic strategy for patients with eye problems caused by SS as well as other diseases.**

The immune system has acquired regulatory systems that preclude the reactivity of mature T cells against self antigens presented by major histocompatibility complex (MHC), while maintaining an ability to respond to non-self antigens presented by self MHC (1,2). The deletion of T cells that have T cell receptors with a high affinity for self antigens in the thymus is an important mechanism for self-tolerance induction and many other systems, including apoptosis, anergy of mature T cells and regulatory T cells, and control T cell tolerance (3–5). The dysregulation of T cell tolerance induction/maintenance is considered to be responsible for many types of autoimmune diseases, and a variety of mechanisms for causing autoimmune diseases have been proposed (5–9). However, the precise mechanisms of human autoimmune diseases remain unclear, and this prevents the establishment of specific therapeutic strategies for these conditions.

Sjögren's syndrome (SS) is an autoimmune disease characterized by the destruction of lacrimal and salivary glands, resulting in dry eyes and dry mouth as the major symptoms (10). Because SS patients have high titers of autoantibodies, including anti-SSA/Ro and SSB/La, abnormal T and B cell activation has been considered to cause SS (10,11). We have established and investigated an animal model of SS in NFS/*sld*-mutant mice thymectomized 3 days after birth and found that the 120-kd α-fodrin protein is a critical autoantigen in the development of SS in this mouse model (12–14). Furthermore, we and other investigators (12,15) have found that patients with SS have high titers of serum

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anti–α-fodrin antibody, suggesting that α-fodrin is a critical autoantigen for the onset or progression of human SS. Although the mechanism of autoimmune disorders in the lacrimal and salivary glands in this mouse model is still unclear, autoreactive CD4+ T cells are responsible for the destruction of the lacrimal and salivary glands in this model (16–18) as well as in human SS (19,20).

Patients with SS are treated with oral or intravenous immunosuppressive drugs, including steroids and cyclosporin A (CSA), which suppress T cell proliferation (10). Such drugs are effective in certain SS patients, but sometimes induce severe side effects (10). The characteristic symptom of SS is dry eyes (10,21), so it is better to treat this symptom with eyedrop (ED) administration of drugs rather than systemically. In this regard, topical CSA has been successfully used as a treatment for the eye symptoms of SS (22), although double-blind clinical studies of SS patients are necessary for the final determination of its efficacy.

To establish a topical therapeutic strategy for the eye symptoms of SS patients, we evaluated ED administration of anti-CD4 monoclonal antibody (mAb) in a mouse model of SS. The anti-CD4 mAb specifically affects CD4+ T cell activation, the dysregulation of which is responsible for the development of SS symptoms in mice and humans (16–18). Although an antibody is a high molecular weight glycoprotein, we found that ED administration of anti-CD4 prevented the onset as well as the progression of autoimmune responses in the lacrimal glands in mice with SS by inhibiting CD4+ T cell activation, possibly through infiltration of the antibody into the lacrimal glands. These findings suggest that it might be possible to treat the eye symptoms of humans with SS as well as other diseases with ED administration of antibodies that recognize critical molecules that cause each disease.

MATERIALS AND METHODS

Mice and experimental protocol. Female NFS/*sld* mice carrying the mutant gene *sld* (23) were bred in our specific pathogen–free mouse colony and were provided with food and water ad libitum. A thymectomy was performed on NFS/*sld* mice 3 days after birth (3d-Tx mice), as previously described (12,24). For the eyedrops, 2 μl of anti-CD4 mAb (GK1.5) dissolved in phosphate buffered saline was prepared in our laboratory (1 mg/ml) and applied with a micropipette to both eyes once a day from 4 to 8 weeks of age or from 10 to 12 weeks of age. As a control, rat IgG eyedrops (Inter-Cell Technologies, Hopewell, NJ) were used.

Histologic examination. The mice were killed at 8 or 12 weeks of age. All organs were removed, fixed in 4% phosphate buffered formaldehyde (pH 7.2), and prepared for histologic examination. Sections were stained with hematoxylin and eosin (H&E). Histologic grading of inflammatory lesions was performed according to the method proposed by White and Casarett (25), as follows: 1 = 1–5 foci composed of >20 mononuclear cells per focus, 2 = >5 such foci, but without

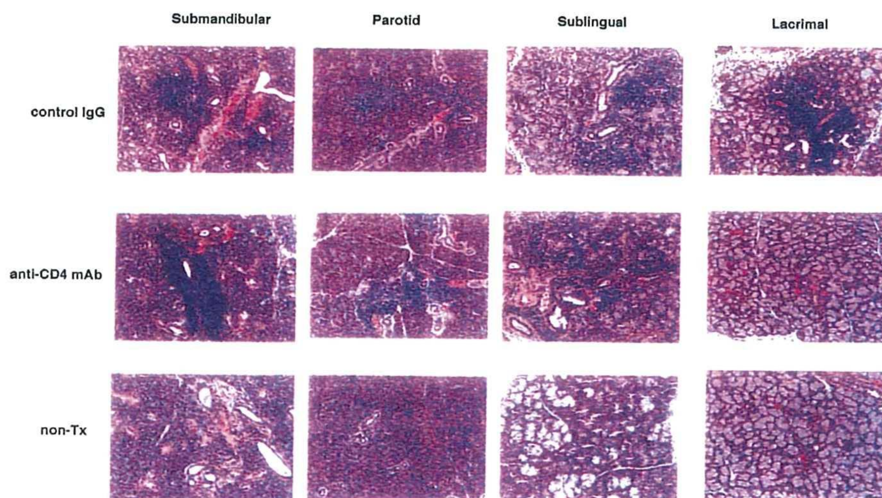

Figure 1. Suppression of autoimmune responses in lacrimal glands by treatment with eyedrop (ED) administration of CD4. Mice thymectomized 3 days after birth were treated with ED administration of control IgG or anti-CD4 monoclonal antibody (mAb) from ages 4 to 8 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 4 weeks after initial treatment and stained with hematoxylin and eosin. As a control, nonthymectomized (non-Tx) NSF/*sld* mice were used. Results are representative of 4 independent experiments.

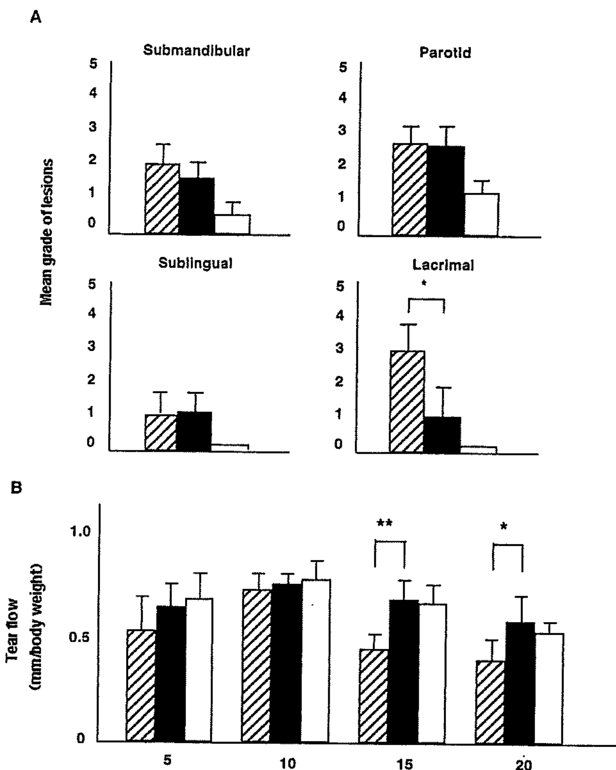

Figure 2. Prevention of the onset of autoimmune responses by ED administration of CD4 in lacrimal glands only. **A,** The 3-day-old thymectomized (3d-Tx) mice were treated with eyedrop (ED) administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 4 to 8 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 4 weeks after initial treatment and stained with hematoxylin and eosin. As a control, nonthymectomized (non-Tx) NSF/*sld* mice were used (open bars). The histologic scores of each gland were evaluated as described in Materials and Methods. Values are the mean and SEM of 7 mice. * = $P < 0.01$. **B,** The 3d-Tx mice were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 4 to 8 weeks. As a control, non-Tx NSF/*sld* mice were used (open bars). The tear volume of each mouse was evaluated as described in Materials and Methods. Values are the mean and SEM of 9 mice. * = $P < 0.05$; ** = $P < 0.01$.

significant parenchymal destruction, 3 = degeneration of parenchymal tissue, 4 = extensive infiltration of the glands with mononuclear cells and extensive parenchymal destruction, and 5 = severe destructive foci with focal fibrosis, ductal dilation, and/or fatty infiltration in addition to the grade 4 lesions. Histologic evaluation of the lacrimal and salivary glands was performed in a blinded manner, and 1 tissue section from each lacrimal and salivary gland was examined.

Flow cytometric analysis. Single-cell suspensions from the lymph nodes or spleen were stained with a combination of phycoerythrin (PE)–conjugated anti-CD4 mAb and fluorescein isothiocyanate–conjugated anti-CD8 mAb or PE-conjugated anti-CD4 mAb and cytochrome-conjugated anti-CD44 mAb and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cells were gated according to size and scatter to eliminate dead cells and debris from analysis. All antibodies were from BD Transduction Laboratories (San Jose, CA).

Measurement of fluid secretion. Analysis of the tear and saliva volume of 15 treated and 15 untreated 3d-Tx NFS/*sld* mice and 12 non–3d-Tx NFS/*sld* mice was performed according to a previously described method (26). A total of 42 mice were examined.

Proliferation assay. Total spleen cells or cervical lymph node cells (5×10^5/well) in RPMI 1640 containing 10% fetal calf serum, penicillin/streptomycin, and 2-mercaptoethanol were stimulated with recombinant α-fodrin protein (JS-1) (12) or 2.0 μg/ml concanavalin A (Con A; Sigma, St. Louis, MO) in 96-well, flat-bottomed plates for 72 hours. Then, ^{3}H-thymidine (1 μCi/well; NEN Life Science Products, Boston, MA) was pulsed into the cell mixture during the final 20 hours of culture. Incorporation of ^{3}H-thymidine was evaluated by an automated β–liquid scintillation counter (MicroPlus; Wallace, Turku, Finland).

Enzyme-linked immunosorbent assay (ELISA). The 96-well plates were coated with JS-1 (12). After washing the protein, diluted serum from mice with SS was added. Biotinylated anti-mouse IgG (Vector, Burlingame, CA) was added as the second antibody. The JS-1–specific antibodies were measured by an automatic ELISA reader (Flow, McLean, VA).

Statistical analysis. The results of histologic, tear secretion, and flow cytometric analyses as well as proliferation assay and ELISA were evaluated by Student's *t*-test.

RESULTS

Prevention of the onset of autoimmune pathology in lacrimal glands by ED administration of CD4. The 3d-Tx NFS/*sld* mice began to develop autoimmune lesions in the lacrimal and salivary glands at 4 weeks of age or later, while no inflammatory lesions were observed in non–3d-Tx NFS/*sld* mice (12). To evaluate whether ED administration of CD4 was effective for preventing the onset of SS autoimmune pathology, anti-CD4 mAb was applied daily to both eyes of 3d-Tx mice from 4 to 8 weeks of age. Then the histologic features of the lacrimal, parotid, submandibular, and sublingual glands of anti-CD4 mAb– or control IgG-treated mice were evaluated by H&E staining (Figures 1 and 2). The lacrimal, parotid, submandibular, and sublingual glands of control IgG-treated 3d-Tx mice showed massive infiltration of mononuclear cells around ducts, as well as destruction of parenchyma, 4 weeks after initial treatment (Figure 1).

In contrast, the infiltration of mononuclear cells as well as the destruction of parenchyma was inhibited in the lacrimal glands, but not the parotid, submandibular, and sublingual glands, of 3d-Tx mice treated with ED administration of CD4 (Figure 1). The non–3d-Tx NSF/*sld* mice did not develop any autoimmune responses

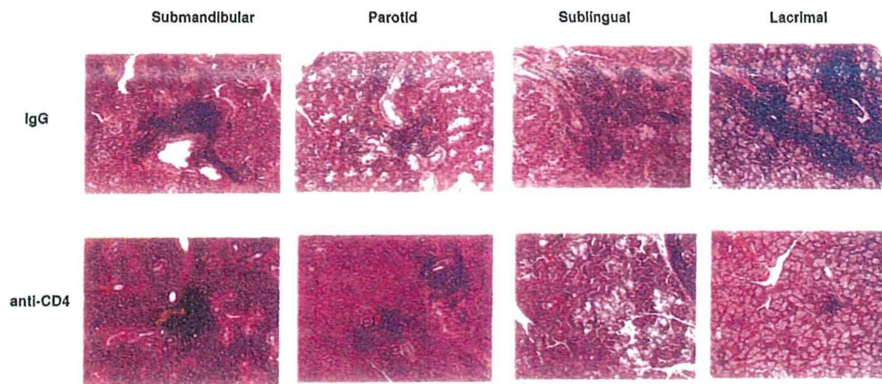

Figure 3. Suppression of established autoimmune responses in lacrimal glands by ED administration of CD4. The 3d-Tx mice were treated with ED administration of control IgG or anti-CD4 mAb from ages 10 to 12 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 2 weeks after initial treatment and stained with hematoxylin and eosin. Results are representative of 3 independent experiments. See Figure 2 for definitions.

(Figure 1). Those histologic findings were scored 4 weeks after initial treatment, as described in Materials and Methods (Figure 2A). The 3d-Tx NFS/*sld* mice treated with ED administration of CD4 had significantly inhibited autoimmune responses only in the lacrimal glands ($P < 0.01$) (Figure 2A), although there were still very small foci of infiltrating mononuclear cells in the lacrimal glands. Furthermore, the average tear volume of mice treated with ED administration of CD4 significantly recovered to the level of control IgG-treated mice (Figure 2B). These results demonstrate that ED administration of CD4 specifically suppressed the onset of autoimmune responses in the lacrimal glands, but not the salivary glands.

Suppression of already developed autoimmune pathology by ED administration of CD4. It is necessary to consider the clinical use of a therapeutic strategy and establish one that can suppress autoimmune pathology that has already developed. Thus, in order to examine the effect of ED administration of CD4 on the autoimmune lesions of mice that had already developed SS, we initiated treatment at 10 weeks of age and continued it for 2 weeks. Then, histologic sections of the lacrimal, parotid, submandibular, and sublingual glands were examined by H&E staining 2 weeks after initial treatment. ED administration of CD4 suppressed the cell infiltration and parenchyma destruction of the lacrimal glands, but not of the parotid, submandibular, or sublingual glands (Figure 3), similar to the therapeutic effects of ED administration of CD4 before the onset of autoimmune diseases (Figures 1 and 2). The histologic scores clearly demonstrated that the therapeutic effect is limited only to the lacrimal glands (Figure 4A), indicating that ED administration of CD4 also has the potential to suppress progression of already developed autoimmune diseases. In contrast, the tear volume of mice treated with ED administration of CD4 was not significantly increased compared with that of mice treated with control IgG (Figure 4B).

CD4+ T cell number augmented and activation inhibited by ED administration of CD4. The anti-CD4 mAb used can cause the deletion of CD4+ T cells when injected into mice and can block the interaction between CD4 and class II MHC (27,28). Thus, we next examined whether the therapeutic effect of ED administration of CD4 on 3d-Tx NFS/*sld* mice was attributable to the deletion of CD4+ T cells or the inhibition of CD4+ T cell activation.

Cervical lymph node cells and spleen cells were purified from 3d-Tx NFS/*sld* mice treated with ED administration of CD4 from 4 to 8 weeks of age or 10 to 12 weeks of age. In mice treated from 4 to 8 weeks of age, the relative number of CD4+ T cells from lymph nodes was increased compared with the number of CD8+ T cells and compared with the control IgG-treated group (Figure 5A). Since the total cell number did not change with ED administration of CD4 (Figure 5A), only the total CD4+ T cells and not the CD8+ T cells increased. The relative and total numbers of both CD4+ and CD8+ T cells from lymph nodes were increased in mice treated with ED administration of CD4 from 10 to 12 weeks of age (Figure 5B). In contrast, the number of CD4+ and CD8+ T cells from the spleen did not change with ED administration of CD4 in either group (data not shown).

We next examined CD44 expression on CD4+ T

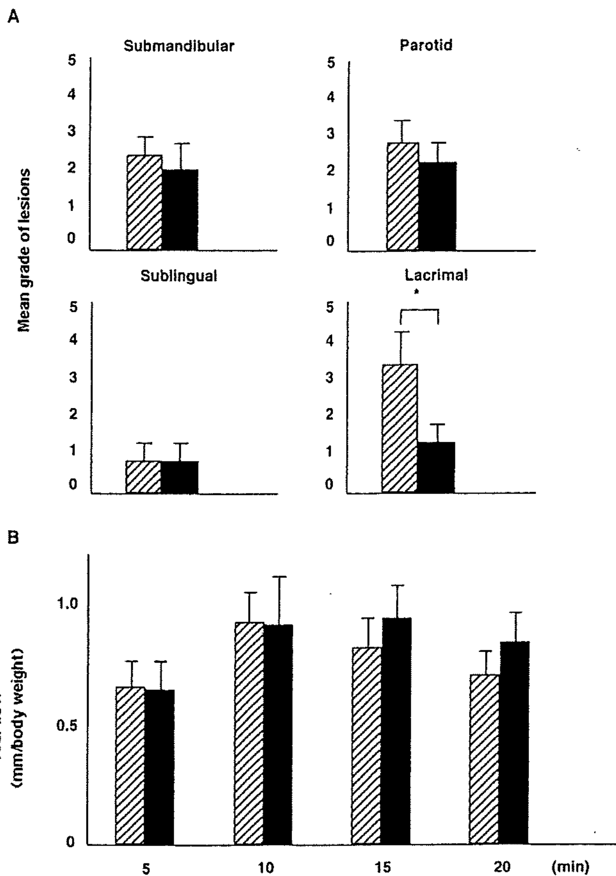

Figure 4. Prevention of the progression of autoimmune responses by ED administration of CD4 in lacrimal glands only. **A,** The 3d-Tx mice were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 10 to 12 weeks. The lacrimal, parotid, submandibular, or sublingual glands were removed 2 weeks after initial treatment and stained with hematoxylin and eosin. The histologic scores of each gland were evaluated as described in Materials and Methods. Values are the mean and SEM of 7 mice. * = $P < 0.05$. **B,** The 3d-Tx mice were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 10 to 12 weeks. The tear volume of each mouse was evaluated as described in Materials and Methods. Values are the mean and SEM of 5 mice. See Figure 2 for definitions.

cells because CD44 is known to be highly expressed on activated T cells and memory T cells (29). Lymph node CD4+ T cells from mice treated with ED administration of CD4 from ages 4 to 8 weeks and ages 10 to 12 weeks expressed lower levels of CD44 compared with those from the control IgG-treated group (Figures 5A and B). In contrast, the expression level of CD44 on splenic CD4+ T cells from 3d-Tx mice treated with ED administration of CD4 was similar to that from control IgG-treated mice (data not shown). Taken together, these results demonstrate that ED administration of CD4 inhibited the activation of CD4+ T cells infiltrating into the lacrimal glands, but did not delete the CD4+ T cells.

Reduction of JS-1–specific T cell response by ED administration of CD4. We previously reported that CD4+ T cells from 3d-Tx mice responded to the α-fodrin JS-1 peptide (13). Thus, we examined whether ED administration of CD4 affects the JS-1–specific proliferative response of lymph node cells from 3d-Tx mice. Cervical lymph node cells were purified after ED administration of CD4 from 4 to 8 weeks of age or 10 to 12 weeks of age and were stimulated with JS-1 or Con A. As previously reported, lymph node T cells from control IgG-treated mice at 8 to 12 weeks of age vigorously proliferated in response to JS-1 (Figures 6A and B). In contrast, the JS-1–specific T cell responses were lower in mice treated with ED administration of CD4 both from 4 to 8 weeks of age and 10 to 12 weeks of age than in

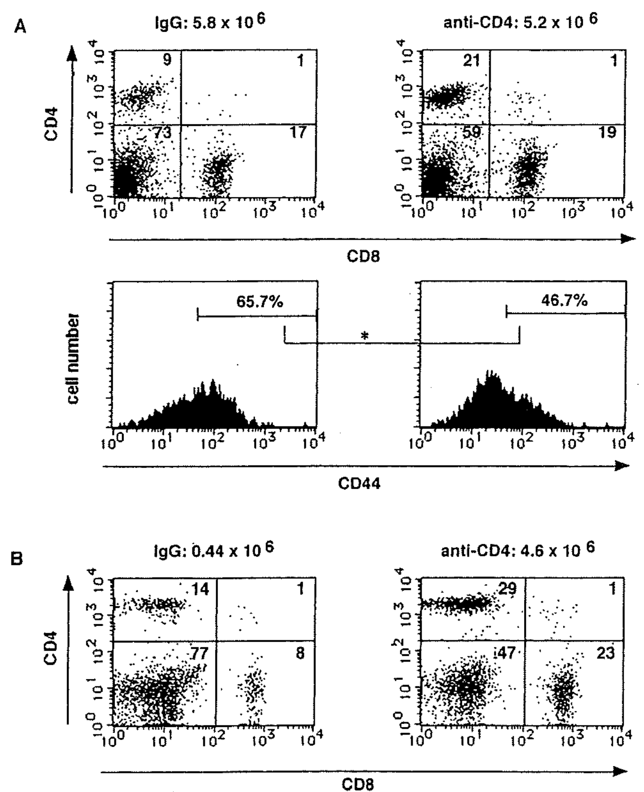

Figure 5. Flow cytometric analysis of lymph node cells. Cervical lymph node cells from 3d-Tx mice that were treated with ED administration of control IgG or anti-CD4 mAb from ages 4 to 8 weeks (**A**) or ages 10 to 12 weeks (**B**) were stained with phycoerythrin (PE)–conjugated anti-CD4 mAb and fluorescein isothiocyanate–conjugated anti-CD8 mAb or PE-conjugated anti-CD44 mAb and cytochrome-conjugated anti-CD44 mAb and analyzed by flow cytometry. The total cell number is indicated across the top. Results are representative of at least 3 independent experiments. * = $P < 0.05$ by Student's unpaired t-test. See Figure 2 for other definitions.

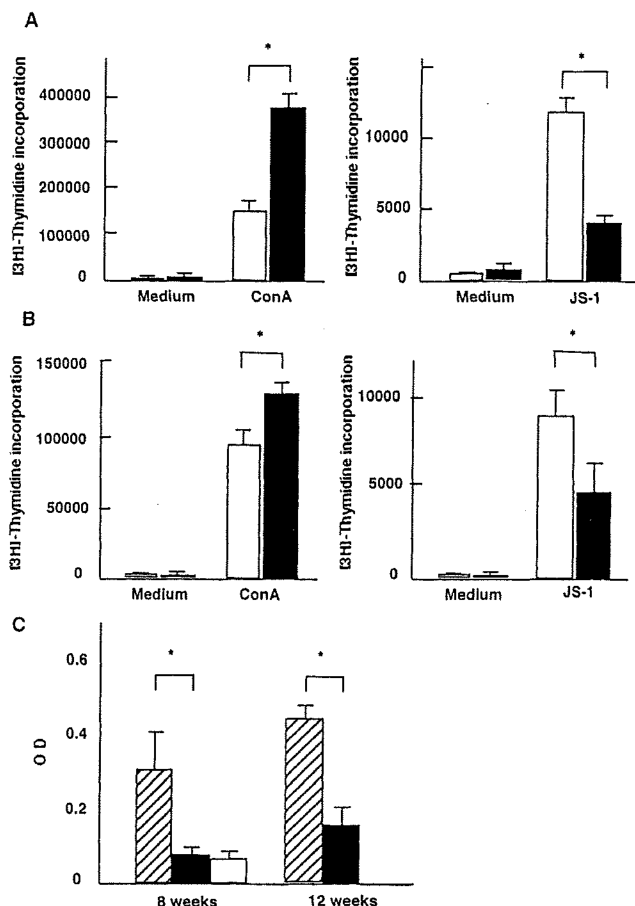

Figure 6. Proliferative response of JS-1–specific T cells and serum titer of anti–JS-1 antibody. The cervical lymph node cells from 3d-Tx mice that were treated with ED administration of control IgG (**left**) or anti-CD4 mAb (**right**) from **A**, ages 4 to 8 weeks or **B**, ages 10 to 12 weeks were stimulated with JS-1 peptide or concanavalin A (Con A) for 72 hours, and incorporation of ^{3}H-thymidine into cells during the final 12 hours was evaluated. Values are the mean and SEM of 7 mice. * = $P < 0.01$. C, Levels of serum autoantibody against 120-kd α-fodrin (JS-1) in 3d-Tx mice that were treated with ED administration of control IgG (hatched bars) or anti-CD4 mAb (solid bars) from ages 4 to 8 weeks or ages 10 to 12 weeks were measured by enzyme-linked immunosorbent assay. Non-Tx mice were used as controls (open bars). Values are the mean and SEM of 7 mice. OD = optical density; * = $P < 0.05$. See Figure 2 for other definitions.

control IgG-treated mice (Figures 6A and B). The inhibitory effects of anti-CD4 mAb appeared to be stronger when administered from 8 to 12 weeks of age (Figures 6A and B). The response to Con A was higher in groups treated with ED administration of CD4 (Figures 6A and B), arguing against the possibility that ED administration of CD4 suppressed total T cell responses. These results demonstrate the reduced expansion of JS-1–specific T cells in mice treated with ED administration of CD4, which is consistent with the low CD44 expression on CD4+ T cells from mice treated with ED administration of CD4.

Reduced titer of serum antibody against α-fodrin. The 3d-Tx mice have high titers of serum autoantibody against recombinant α-fodrin protein (JS-1) (12). Thus, we examined whether the local treatment with ED administration of CD4 affected serum levels of autoantibody against α-fodrin in the mice with SS. The titer of serum antibody against α-fodrin was evaluated after treatment of 3d-Tx mice with ED administration of CD4 from 4 to 8 weeks of age or 10 to 12 weeks of age. As shown in Figure 6, the titer of autoantibody against α-fodrin was lower in mice treated from ages 4 to 8 weeks and ages 10 to 12 weeks compared with control IgG-treated groups (Figure 6C). The decreased serum titer of autoantibody against α-fodrin suggests that ED administration of CD4 affected autoimmune pathology only in the lacrimal glands but was able to suppress systemic production of α-fodrin–specific autoantibody.

DISCUSSION

Patients with SS have generally been treated with systemic administration of immunosuppressive drugs (10,21). Despite the effectiveness of such drugs, it is better to establish a local therapeutic strategy for eye and mouth symptoms of SS because the systemic use of immunosuppressive drugs induces severe side effects (21). In this study, we evaluated whether ED administration of CD4 effectively inhibits autoimmune pathology in the lacrimal glands of a mouse model of SS (12). We have previously reported that anti-CD86 mAb treatment improved the autoimmune pathology in both the lacrimal and the salivary glands of mice with SS (30). We used an antibody because the specific binding ability of a mAb against the target molecule allows the establishment of a molecule-specific therapeutic strategy with fewer side effects. Although an antibody is a large molecular weight glycoprotein, we demonstrated that ED administration of CD4 effectively inhibited both the onset and the progression of autoimmune responses only in the lacrimal glands of mice with SS. Although we did not simply compare the efficacy of topical administration of anti-CD4 mAb with that of systemic administration, this successful therapeutic effect of anti-CD4 mAb would provide the possibility of establishing a new form of antibody-based therapy for patients with eye symptoms caused by SS as well as other types of diseases.

ED administration of CD4 increased the total number of CD4+ T cells in cervical lymph nodes after a