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Experimental Immune-Mediated Pancreatitis in Neonatally Thymectomized Mice Immunized with Carbonic Anhydrase II and Lactoferrin

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SUMMARY: We previously reported that autoantibodies against carbonic anhydrase II and lactoferrin are frequently identified in patients with autoimmune-related pancreatitis. To clarify the role of carbonic anhydrase II and lactoferrin, we created animal models of autoimmune pancreatitis by immunizing neonatally thymectomized mice with carbonic anhydrase II and lactoferrin and also by transferring immunized spleen cells to nude mice. Neonatally thymectomized BALB/c mice were immunized with carbonic anhydrase II or lactoferrin followed by three booster injections ($n = 10$ in each group). We transferred whole, CD4+, or CD8+ spleen cells prepared from immunized neonatally thymectomized mice to nude mice ($n = 5$ in each group). Gene expression of IFN-γ and IL-4 was investigated using semiquantitative reverse transcription-polymerase chain reaction. Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling staining was used to examine apoptosis. In immunized neonatally thymectomized mice, the prevalence of inflammation was significantly higher in the pancreas. Inflammation was present in all mice receiving whole or CD4+ cells. There was no change in any of the mice receiving CD8+ cells or nonimmunized spleen cells. Carbonic anhydrase II or lactoferrin-immunized mice had apoptotic duct cells or acinar cells, respectively. Expression of the IFN-γ gene was up-regulated in each group. Similar findings were observed in the salivary glands and liver. An immunologic mechanism against carbonic anhydrase II or lactoferrin is involved in the pathogenesis of these pancreatitis models, in which the effector cells are Th1-type CD4+ T cells. (*Lab Invest 2002, 82:411–424*).

Since Sarles et al (1961, 1965) reported a type of pancreatitis with hypergammaglobulinemia, several investigators have suggested that an autoimmune mechanism is involved in some patients with pancreatitis. The term "autoimmune pancreatitis" (AIP) was recently proposed (Yoshida et al, 1995) for this type of pancreatitis. Patients with AIP often have other autoimmune diseases such as sialoadenitis (Horiuchi et al, 1998; Ito et al, 1997; Okazaki et al, 2000; Uchida et al, 2000; Wakabayashi et al, 1998), sclerosing cholangitis (Horiuchi et al, 1998; Ito et al, 1997; Okazaki et al, 2000; Wakabayashi et al, 1998), or diabetes mellitus (Ito et al, 1997; Okazaki et al, 2000). Therefore, the terms "autoimmune exocrinopathy" (Strand and Talal, 1979), "a syndrome complex" (Montefusco et al, 1984), or "dry gland syndrome" (Epstein et al, 1982) have been proposed for this disorder, which involves multiple exocrine organs. These observations suggest that there are common target antigens against systemic exocrine organs in this disease, including the pancreas, salivary glands, and bile ducts. Although the exact nature of such antigens remains unclear, some candidates have been proposed. We recently reported that autoantibodies against carbonic anhydrase II (CA-II) and lactoferrin (LF), which are widely distributed in exocrine organs such as the pancreas, salivary glands, and bile ducts, are frequently detected in patients with AIP (Okazaki et al, 2000; Uchida et al, 2000). It remains unknown, however, whether autoimmunity to CA-II and LF has an important role in the development of AIP. Although there are some animal models of autoimmune exocrinopathy (Mustafa et al, 1998; Tsubata et al, 1996; Vallance et al, 1998), the target antigens of those models are unclear. The neonatally thymectomized (nTx) BALB/c mouse is a well-known model of autoimmune gastritis (AIG) (Katakai et al, 1999; Kojima and Prehn, 1981; Nishio et al, 1994; Sakaguchi et al, 1985). In this model, nTx removes CD4+CD25+ regulatory T cells from the periphery (Asano et al, 1996; Itoh et al, 1999), which induces a hyperimmune state. Therefore, these mice might develop other organ-specific autoimmune diseases in addition to AIG. In the present study, we established animal models of immune-mediated pancreatitis associated with multi-organ exocrinopathy by immunizing nTx mice with CA-II or LF and transferring

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the immunized spleen cells to nude mice to determine the mechanisms underlying AIP.

Results

Macroscopic Findings of the Exocrine Organs

The body weight and gross appearance of mice immunized with CA-II or LF, or mice receiving transferred cells, were not different from those of the respective control mice. Macroscopically, ascites or adhesion in the peritoneal cavity was not observed in any group. There was no bleeding or macroscopic atrophy observed in any exocrine organ, including the pancreas, salivary glands, and liver.

The weight of the salivary glands of nTx mice immunized with CA-II or LF was significantly lower than that in the respective control groups ($p < 0.05$; Table 1). The weight of the pancreas in nTx mice immunized with CA-II or LF was also lower than that in the other groups, although there was no significant difference between nTx mice immunized with each respective immunogen, CA-II and LF, and those with BSA (Table 1).

Liver weight was not influenced by immunization with CA-II or LF. Moreover, lymphocytes transferred from mice immunized with CA-II or LF, or from nonimmunized mice did not affect the weight of any exocrine organs in the nude mice (data not shown).

Blood Chemistry and Serologic Findings

Serum amylase levels in the nTx mice immunized with CA-II or LF were significantly higher than those in the respective control groups (Table 1). Other biochemical data that might be suggestive of exocrine organ destruction, such as gamma-glutamyl transpeptidase, alanine aminotransferase, and glucose, were not different among the groups (data not shown).

Serum levels of anti-CA-II antibody were not significantly different among nTx mice, nTx mice immunized with BSA, and nTx mice immunized with CA-II, although they tended to be higher in nTx-mice immunized with CA-II (Table 2). Serum levels of anti-CA-II antibody in nude mice were not affected by transfer of whole spleen cells or CD4+.

Serum levels of anti-LF antibody in nTx mice immunized with LF were significantly elevated compared with the nonimmunized groups with LF. Moreover, serum levels of anti-LF antibodies in nude mice that received whole or CD4+ cells were also significantly higher than in the other groups (Table 2).

Histopathologic Findings

(I) Pancreas. Moderate inflammatory cells were observed mainly around the ducts in the pancreas of nTx mice immunized with CA-II (Fig. 1, b and c). All five nude mice that received whole spleen cells or CD4+ cells prepared from nTx-mice immunized with CA-II had inflammatory cells around the pancreatic duct and edematous changes in the periductal area (Fig. 1, d and e). On the other hand, in nTx mice immunized with LF, there was moderate inflammatory cell infiltration in the pancreatic acini around the duct (Fig. 2a). In the cell transfer experiment, all five nude mice that received whole spleen cells or CD4+ cells prepared from nTx mice immunized with LF had inflammation in the periductal area (Fig. 2, b and c).

On the other hand, there was no inflammation observed in any of the mice that received CD8+ cells from nTx mice immunized with CA-II (Fig. 1f) or LF (Fig. 2d), or whole spleen cells from nonimmunized nTx mice or immunized nTx mice that received BSA (data not shown). In the nTx mice immunized with CA-II or LF, nude mice receiving whole spleen cells and CD4+ spleen cells prepared from nTx-mice immunized with each antigen, the prevalence of inflammation and the inflammation scores of the exocrine pancreas were significantly higher than those in the respective control groups (Tables 3 and 4). The prevalence of insulitis was not influenced by immunization with CA-II or LF or lymphocyte transfer (Tables 3 and 4).

(II) Other Organs. In the salivary glands of nTx mice immunized with CA-II or LF, focal inflammation around the ducts was more prevalent than that in the respective control groups (Table 3). In the liver, cholangitis was observed in the nTx mice immunized with BSA and more prominently in the immunized mice compared with the nonimmunized mice. In the cell transfer

Table 1. Weight of Exocrine Organs and Serum Amylase Levels in Mice Immunized with CA-II or LF

	n	Salivary glands (wt%)	Pancreas (wt%)	Amylase (IU/l)
Non-nTx	10	0.889 ± 0.103	0.939 ± 0.121	6796.0 ± 602.8
Non-nTx+BSA	10	0.858 ± 0.201	0.946 ± 0.221	5923.6 ± 613.2
Non-nTx+CA-II	10	0.882 ± 0.154	0.972 ± 0.144	6336.7 ± 411.7
Non-nTx+LF	10	0.843 ± 0.194	0.995 ± 0.192	5806.7 ± 221.2
nTx	10	0.864 ± 0.135 * *	0.950 ± 0.119	6033.0 ± 767.1 * *
nTx+BSA	10	0.897 ± 0.097	0.849 ± 0.151	6636.7 ± 663.3
nTx+CA-II	10	0.556 ± 0.081	0.716 ± 0.183	8242.0 ± 657.7
nTx+LF	10	0.466 ± 0.171	0.714 ± 0.132	8906.0 ± 1207.0

Non-nTx, nonimmunized non-nTx BALB/c mice; Non-nTx+CA-II, non-nTx mice immunized with CA-II; Non-nTx+LF, non-nTx mice immunized with LF; nTx, nonimmunized nTx BALB/c mice; nTx+BSA, nTx mice immunized with BSA; nTx+CA-II, nTx mice immunized with CA-II; nTx+LF, nTx mice immunized with LF; CA-II, carbonic anhydrase II; LF, lactoferrin.

Values are expressed as the percentage of body weight (wt%). * $p < 0.05$ vs control groups.

Table 2. Serum Levels of Anti CA-II or LF Antibody

	n	CA-II	LF
Non-nTx	10	0.280 ± 0.142	0.072 ± 0.018
nTx	10	0.442 ± 0.314	0.066 ± 0.012
nTx + BSA	10	0.494 ± 0.150 *	0.069 ± 0.012 **
nTx + immunogen	10	0.563 ± 0.169	2.675 ± 0.152
Nude	5	0.242 ± 0.027	0.095 ± 0.045
Nude + nTx	5	0.211 ± 0.069	0.099 ± 0.059
Nude + nTx with BSA	5	0.232 ± 0.095	0.117 ± 0.035
Nude + whole with immunogen	5	0.294 ± 0.016	2.363 ± 0.260 **
Nude + CD4 with immunogen	5	0.310 ± 0.136	2.482 ± 0.258
Nude + CD8 with immunogen	5	0.217 ± 0.012	0.387 ± 0.198 **

Non-nTx, nonimmunized non-nTx BALB/c mice; nTx, nonimmunized nTx BALB/c mice; nTx + BSA, nTx mice immunized with BSA; nTx + immunogen, nTx mice immunized with CA-II or LF; Nude, untreated nude mice; Nude + nTx, nude mice that received whole spleen cells prepared from nonimmunized nTx mice; Nude + nTx with BSA, nude mice that received whole spleen cells prepared from nTx mice immunized with BSA; Nude + nTx with immunogen, nude mice that received whole spleen cells from nTx mice immunized with CA-II or LF; Nude + CD4 with immunogen, nude mice that received $CD4^+$ cells from nTx mice immunized with CA-II or LF; Nude + CD8 with immunogen, nude mice that received $CD8^+$ cells from nTx mice immunized with CA-II or LF; CA-II, carbonic anhydrase II; LF, lactoferrin.
Values are absorbency at O.D. 490 nm. * $p < 0.05$ vs non-nTx. ** $p < 0.05$ vs control groups and nude + CD8 with LF.

experiment, all five nude mice, that received whole spleen cells or CD4+ cells prepared from nTx mice immunized with CA-II or LF, had inflammation around the bile duct and the salivary gland ducts (Figs. 3 and 4; Table 4). The prevalence and severity of AIG were not increased in the immunized mice or in the transfer mice (data not shown).

Immunohistochemistry

The majority of lymphocytes infiltrating into the pancreas in nTx mice immunized with CA-II or LF were CD4+ cells (Fig. 5). In contrast, only small numbers of CD8+ and B220+ cells were observed throughout the pancreas. The major infiltrating lymphocytes were also CD4+ T cells in the salivary glands and liver of the transferred nude mice (data not shown).

In Situ End-Labeling of Fragmented DNA

Cells positively stained using the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) method were observed mainly in the ductal cells of the pancreas of the nude mice that received CD4+ spleen cells from nTx mice immunized with CA-II (Fig. 6, a and b). In nude mice that received CD4+ spleen cells from nTx mice immunized with LF, the acinar cells of the pancreas stained positively using the TUNEL method (Fig. 6, c and d). nTx mice immunized with CA-II or LF and nude mice that received whole spleen cells prepared from nTx mice immunized with CA-II or LF had cells with TUNEL staining similar to that of nude mice that received CD4+ spleen cells from nTx mice immunized with CA-II or LF (data not shown). These findings indicate that there was cell-mediated destruction of exocrine organs by CD4+ cells.

Gene Expression of Cytokines by Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Gene expression of IFN-γ was detected using RT-PCR in the salivary glands of nonimmunized nTx mice, nTx mice immunized with CA-II or LF, and nude mice that received whole or CD4+ spleen cells prepared from nTx mice immunized with CA-II or LF. IFN-γ was not detected, however, in nude mice that received CD8+ cells or in non-nTx mice. Expression of the IL-4 gene was not detected in any of the mice (Fig. 7, a and b).

Discussion

Patients with AIP often have other autoimmune diseases, such as sialoadenitis (Horiuchi et al, 1998; Ito et al, 1997; Okazaki et al, 2000; Uchida et al, 2000; Wakabayashi et al, 1998), sclerosing cholangitis (Horiuchi et al, 1998; Ito et al, 1997; Okazaki et al, 2000;Wakabayashi et al, 1998), or diabetes mellitus (Ito et al, 1997; Okazaki et al, 2000), leading to the terms "autoimmune exocrinopathy" (Strand and Talal, 1979), "complex syndrome" (Montefusco et al, 1984), or "dry gland syndrome" (Epstein et al, 1982). The involvement of multiple exocrine organs suggests the existence of common target antigens in the systemic exocrine organs.

We previously reported that some patients with pancreatitis and Sjögren's syndrome have cellular and humoral immune responses against a 60-kDa protein prepared using the monoclonal antibody SP3-1 (Okazaki et al, 1989) and CA-II (Kino-Ohsaki et al, 1996). We reported that autoantibodies against CA-II and LF are frequently identified in patients with AIP, and the prevalence of these two antibodies is independent (Okazaki et al, 2000; Uchida et al, 2000). These two proteins are similarly distributed in several exocrine organs, including the pancreas, salivary gland, liver (biliary tract), and kidney (renal tubule) (Colomb et al, 1976; Miyauchi, 1984; Okazaki et al, 1989). CA-II, a 38 kDa zinc binding protein, converts carbon dioxide to bicarbonate. In the pancreas, CA-II is located in the duct cells (Kumpulainen and Jalovaara, 1981). Two animal models of sialoadenitis (Nishimori et al, 1995) and cholangitis (Ueno et al, 1998) using different mouse strains immunized with CA-II were previously

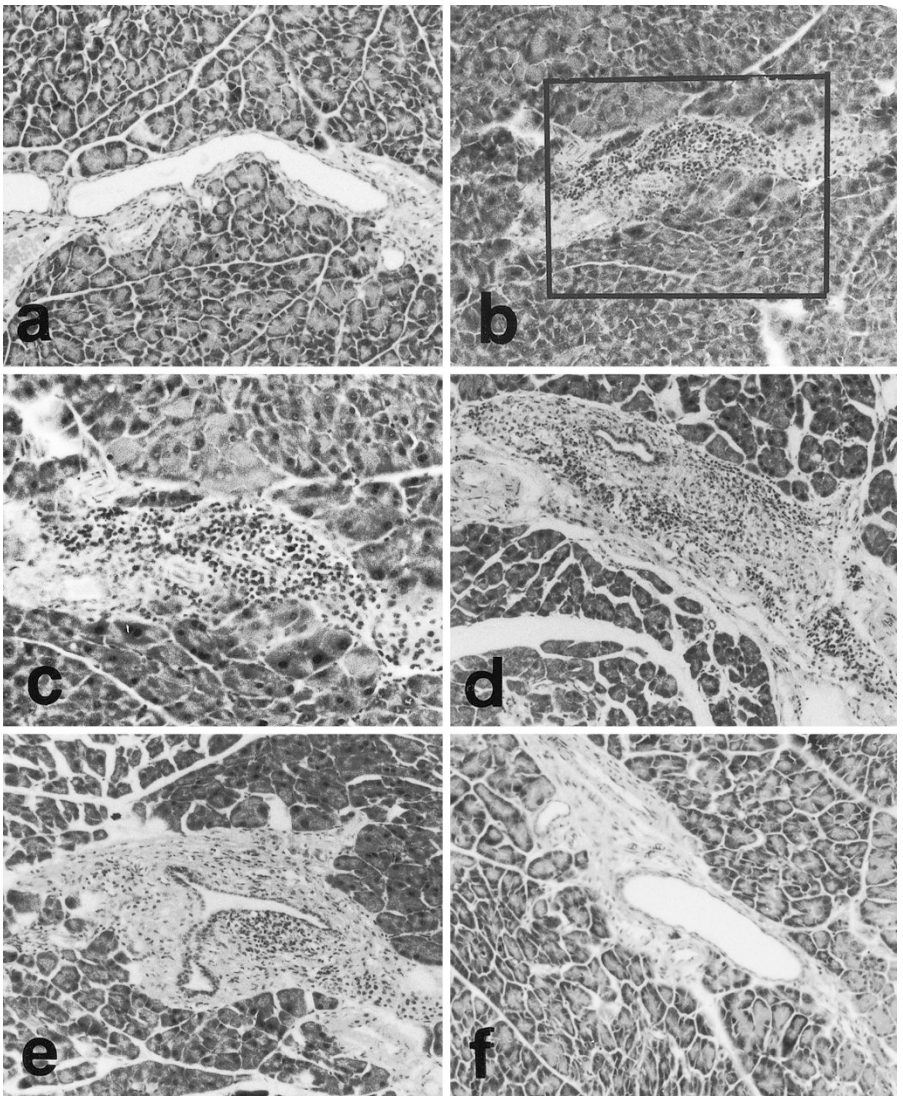

Figure 1.

Histopathology of the pancreas in the carbonic anhydrase II (CA-II) experiment. Hematoxylin and eosin-stained tissue sections from nonimmunized neonatal thymectomy (nTx) mice show no lymphocyte infiltration (a). In the nTx mice immunized with CA-II (b and c), moderate lymphocyte infiltration and destruction of the small duct were observed. Lymphocyte infiltration with edematous changes was observed in the nude mice that received whole (d) or CD4+ (e) spleen cells from nTx mice immunized with CA-II. No inflammation was observed in any of the mice that received CD8+ cells (f). Original magnification, ×200 (c) and ×100 (a and b; d–f).

reported. Subcutaneous immunization with CA-II induced sialoadenitis in PL/J mice (H-2^u) and SJL/JCr (H-2^s) but not in other mouse strains (H-2^p, H-2^q, and H-2^r)(Nishimori et al, 1995). On the other hand, intraperitoneal immunization of BALB/c mice (H-2^d) and DBA/1 mice (H-2^q) with CA-II induced cholangitis but not sialoadenitis (Ueno et al, 1998). These two models suggest that CA-II-induced sialoadenitis or cholangitis develops independently as a consequence of different strain susceptibility, major histocompatibility complex (MHC) haplotype, or immunization route. Although the effector cells for sialoadenitis in PL/J mice immunized with CA-II are unknown, the cholangitis model in BALB/c mice by Ueno et al (1998) suggests that CD4+ cells are the effector cells.

LF, a single chain 78 kDa glycoprotein, has bacteriostatic activity (Ellison et al, 1988) as well as iron transporting capacity (Ainscough et al, 1979). It is rich in granulocytes and secretory fluid from the exocrine organs such as the mammary, lachrymal, and salivary glands (Miyauchi and Watanabe, 1987; Reitamo et al, 1980). LF is also detected in the normal bile duct (Saito and Nakanuma, 1992) and pancreatic acini (Colomb et al, 1976), although in very small amounts. On the other hand, immunohistochemical studies indicate that LF is strongly positive in the biliary epithelial cells in hepatolithiasis (Saito and Nakanuma, 1992), and the concentration of LF in pancreatic juice is increased in patients with chronic pancreatitis (Fedail et al, 1978; Hayakawa et al, 1983). Although the role of LF in cholangitis and pancreatitis is unknown, LF might be involved in the pathophysiology of inflammation in these organs. Moreover, it was recently reported that orally administered LF activates intestinal mucosal

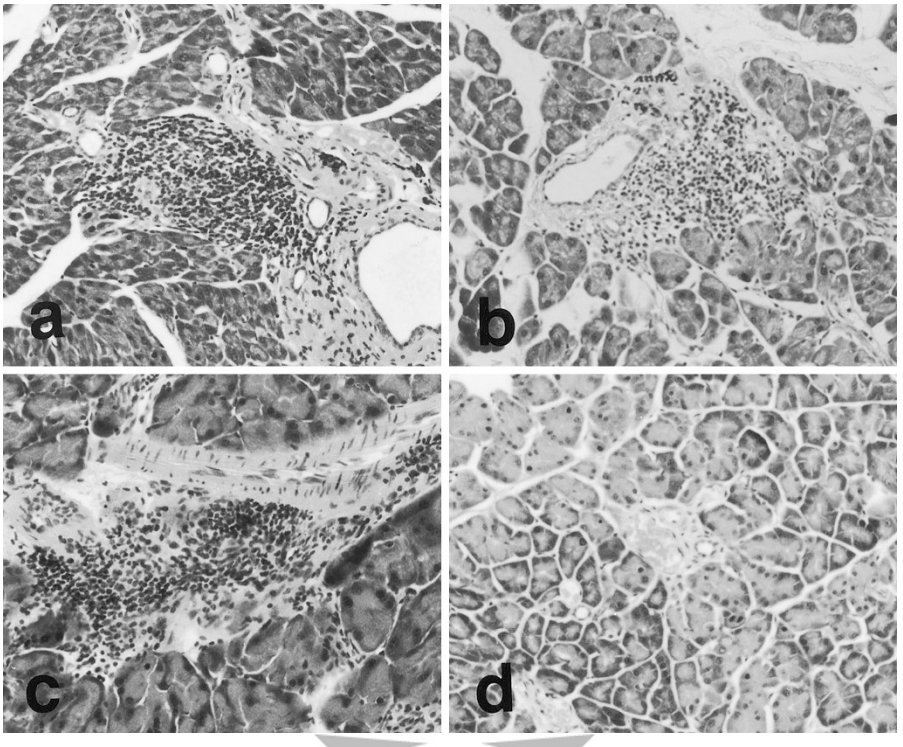

Figure 2.
Histopathology of the pancreas in the lactoferrin (LF) experiment. In the pancreas of nTx mice immunized with LF, there was moderate inflammatory cell infiltration in pancreatic acini around the duct (a). In the cell transfer experiment, all five nude mice that received whole spleen cells or CD4+ cells prepared from nTx mice immunized with LF had inflammation in the periductal area (b and c). In contrast, there was no inflammation observed in any of the nude mice that received CD8+ cells (d). Original magnification, ×200 (a–d).

immunity by increasing CD4+ and CD8+ T cells, which suggests that LF also has potent immunomodulating or immunogenic activities (Brock, 1995). Indeed, LF is one target antigen recognized by perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) and anti-LF antibodies are often detected in patients with primary sclerosing cholangitis and ulcerative colitis as well as AIP (Peen et al, 1996). We previously reported an antibody to LF in autoimmune liver diseases, particularly in patients with autoimmune cholangitis (Ohana et al, 1998). By itself, however, the presence of autoantibodies against CA-II or LF does not necessarily mean that these proteins are involved in the pathogenesis.

In the present study, we successfully developed immune-mediated pancreatitis using nTx BALB/c mice immunized with CA-II and LF, although it was not necessarily due to autoimmunity. Apart from antigen-specific immunity, mild cholangitis could be induced by control immunization with BSA or Freund's complete adjuvant (FCA) alone (data not shown). As inflammation in nTx-mice immunized with CA-II or LF was more severe compared with controls, antigen-specific immunity might be involved in immune-mediated cholangitis as well as in sialoadenitis and pancreatitis. The adoptively transferred BALB/c nu/nu mice spontaneously developed persistent inflammation in the same organs as the immunized nTx mice. nTx BALB/c mice spontaneously develop various organ-specific autoimmune diseases such as gastritis, thyroiditis, oophoritis, orchitis, and insulitis. Autoimmune gastritis is induced in 60% of nTx mice, whereas thyroiditis, sialoadenitis, or insulitis occur at a very low incidence. In the present models, the prevalence of pancreatitis, sialoadenitis, and cholangitis was drastically increased, whereas other lesions, such as AIG (data not shown) or insulitis, were not increased. Although we did not evaluate other strains in this study, several animal models of autoimmune disease using nTx mice showed that organ specific inflammation depends on strains such as sialoadenitis in NFS/sld mice (Hayashi et al, 1996), hepatitis in A/J mice (Kamiyasu et al, 1997; Masanaga et al, 1998), and gastritis in BALB/c mice. To clarify the strain specificity in our models, further study using other strains is necessary.

In nTx BALB/c mice with AIG, the effector cells were reported to be CD4+ Th1 cells (Asano et al, 1996; Itoh et al, 1999; Katakai et al, 1999; Kojima and Prehn, 1981; Nishio et al, 1994; Sakaguchi et al, 1985). In general, the pathophysiology of autoimmune diseases usually depends on the interaction of self-antigen-specific T cells and regulatory T cells. Moreover, nTx depletes the CD4+ and CD25+ regulatory T cells in the periphery (Asano et al, 1996; Itoh et al, 1999), which results in a hyperimmune state. Whole or CD4+ spleen cells prepared from nTx mice immunized with CA-II or LF induced persistent inflammation of the same organs in the syngenic nude mice as in those in the nTx mice immunized with the respective proteins, whereas CD8+ cells were never pathogenic. In the present models, we confirmed antigen-specific immu-

Table 3. Histologic Assessment of Various Organs in Mice Immunized with CA-II or LF

		Salivary glands		Pancreas				Liver	
				Exocrine pancreas		Endocrine pancreas			
Immunogen	Mouse	Number[a]	Score[b]	Number[a]	Score[b]	Number[a]	Score[b]	Number[a]	Score[b]
None	Non-nTx	0/10	0	0/10	0	1/10	0	0/10	0
	nTx	1/10	0.100 ± 0.316	1/10	0.100 ± 0.316	1/10	0.100 ± 0.316	2/10	0.200 ± 0.422
BSA	Non-nTx	0/10	0	0/10	0	0/10	0	10/10	1.200 ± 0.447
	nTx	2/10	0.200 ± 0.422	1/10	0.200 ± 0.422	1/10	0.100 ± 0.316	10/10	1.200 ± 0.447
CA-II	Non-nTx	0/10	0	0/10	0	0/10	0	10/10	1.200 ± 0.447
	nTx	8/10	1.600 ± 1.075	5/10	0.700 ± 0.823	1/10	0.100 ± 0.316	10/10	2.000 ± 0.775
LF	Non-nTx	0/10	0	0/10	0	0/10	0	10/10	1.200 ± 0.447
	nTx	8/10	1.600 ± 1.075	6/10	0.800 ± 0.919	1/10	0.100 ± 0.316	10/10	1.800 ± 0.632

Non-nTx, non-nTx BALB/c mice; nTx, nTx BALB/c mice; CA-II, carbonic anhydrase II; LF, lactoferrin.

[a] Number of mice with inflammation/total number of mice is shown.

[b] Score indicates means ± SD of inflammation score.

* $p < 0.05$ vs control groups.

Table 4. Histologic Assessment of Various Organs in BALB/c nu/nu Mice that Received Splenocytes from Immunized nTx-mice

Nude mice		Salivary glands		Pancreas				Liver	
				Exocrine pancreas		Endocrine pancreas			
Immunogen	Transferred cells[a]	Number[b]	Score[c]	Number[b]	Score[c]	Number[b]	Score[c]	Number[b]	Score[c]
None	None	0/5	0	0/5	0	0/0	0	0/5	0
	Whole	0/5	0	0/5	0	1/5	0.200 ± 0.447	0/5	0
BSA	Whole	0/5	0	0/5	0	1/5	0.200 ± 0.447	0/5	0
CA-II	Whole	5/5	1.800 ± 0.837	5/5	1.200 ± 0.447	1/5	0.200 ± 0.447	5/5	1.800 ± 0.837
	CD4	5/5	1.200 ± 0.477	5/5	1.200 ± 0.477	1/5	0.200 ± 0.447	5/5	1.800 ± 0.837
	CD8	0/5	0	0/5	0	0/5	0	0/5	0
LF	Whole	5/5	1.400 ± 0.548	5/5	1.200 ± 0.447	1/5	0.200 ± 0.447	5/5	1.800 ± 0.837
	CD4	5/5	1.200 ± 0.477	5/5	1.400 ± 0.548	1/5	0.200 ± 0.447	5/5	1.800 ± 0.837
	CD8	0/5	0	0/5	0	0/5	0	0/5	0

Whole, nude mice that received whole spleen cells from nonimmunized nTx mice or nTx mice immunized with BSA, CA-II, or LF; CD4, nude mice that received CD4-positive cells from nTx mice immunized with CA-II or LF; CD8, nude mice that received CD8-positive cells from nTx mice immunized with CA-II or LF; CA-II, carbonic anhydrase II; LF, lactoferrin.

[a] Transferred cells prepared from nTx mice with or without immunization.

[b] Number of mice with inflammation/total number of mice is shown.

[c] Score indicates means ± SD of inflammation score. § $p < 0.05$ vs control groups and CD8.

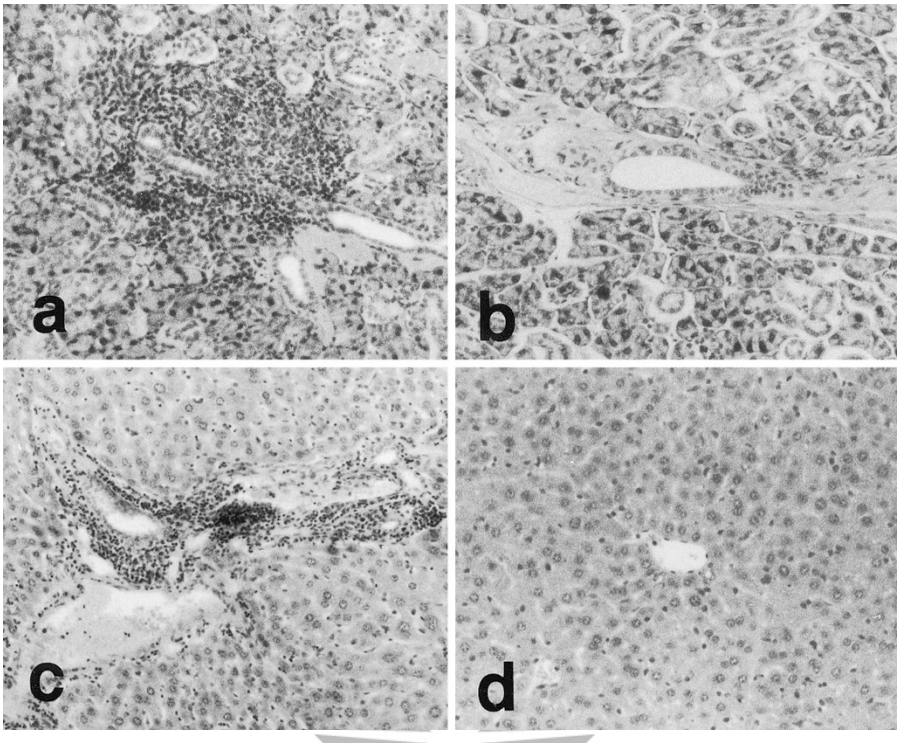

Figure 3.
Hematoxylin and eosin-stained section of salivary glands and liver tissue in the nude mice that received spleen cells sensitized with CA-II. In the salivary glands (a and b) and liver (c and d), inflammation around the draining duct was observed in nude mice that received CD4+ spleen cells prepared from nTx mice immunized with CA-II (a and c). On the other hand, there was no inflammation in the nude mice that received CD8+ cells from nTx mice immunized with CA-II (b and d). Original magnification, ×200.

nity by increased cellular and humoral immune responses against CA-II or LF. These findings suggest that MHC-class II restricted CD4+ T cells react to CA-II or LF, which allows them to escape from negative selection in the thymus and depletion of regulatory T cells such as CD4+CD25+ T cells in the periphery, and have important roles in the development of immune-mediated pancreatitis and exocrinopathy in nTx BALB/c mice. Moreover, in inflamed tissues, gene expression of IFN-γ, but not IL-4, was detected in both models, which suggests that a Th1-type of immune response predominates over the Th2-type. Th1-type CD4+ cells might be involved in patients with AIP (Okazaki et al, 2000) and Sjögren's syndrome (Fox et al, 1994). Thus, the microenvironment of the Th1/Th2 immune balance in nTx mice with exocrinopathy is similar to that in patients with AIP. Taken together, these results suggest autoimmune-mediated exocrinopathy against CA-II and LF as common target antigens.

Although the antigens involved are unknown, aly−/− mice and MRL/lpr mice spontaneously develop pancreatitis in which the effector cells are also Th1-type CD4+ T cells (Mustafa et al, 1998; Tsubata et al, 1996). Recent observations also suggest a functional role of T lymphocytes, such as cytotoxicity (Hunger et al, 1997) or neuroimmune interactions in chronic pancreatitis (Keith et al, 1985). In mice with cerulein-induced experimental pancreatitis, CD4+ T cells are required for complete development of pancreatic lesions (Demols et al, 2000). Apart from autoimmunity, these CD4+ T cells probably induce the activation of macrophages and further proinflammatory reactions during the early stage of acute pancreatitis, as well as direct cytotoxicity effects through Fas ligand expression (Demols et al, 2000).

In the model of Sjögren's syndrome using nTx NFS/sld mice, Th1-type CD4+ T cells also play an important role in the development of sialoadenitis. In this model and human Sjögren's syndrome, α-fodrin seem to be involved as an autoantigen (Haneji et al, 1998; Hayashi et al, 1996). The homology of the amino acid sequences among α-fodrin, CA-II, and LF is less than 10%, which suggests that these three proteins are completely different and are not likely to be cross-reactive. After induction of organ specific inflammation by an autoantigen, immune responses against other antigens in the inflamed tissues are also induced (Peakman and Vergani, 1997). Therefore, we cannot rule out a possibility of secondary involvement of autoimmune responses against a-fodrin in our models. Further study including measurement of anti α-fodrin antibody is needed.

There remains a possibility that CD8+ lymphocytes are also the effector cells, because CD8+ T cells might be the effector cells in MHC class-II-deficient mice, which spontaneously develop pancreatitis (Vallance et al, 1998). Indeed, HLA-DR- and CD8+ cells also increased in the peripheral blood and infiltrated into pancreatic tissue in patients with AIP, although CD4+ cells predominated over CD8+ cells (Okazaki et al, 2000). TGF-β is an important regulating factor in

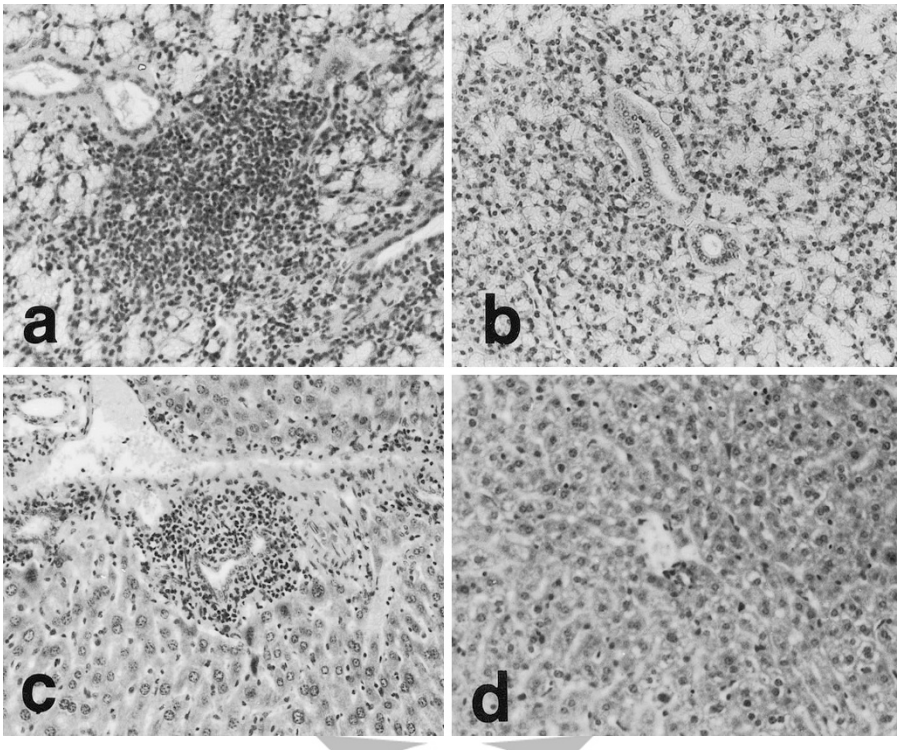

Figure 4.

Hematoxylin and eosin-stained section of salivary glands and liver tissue in nude mice that received spleen cells sensitized with LF. In nude mice that received CD4+ spleen cells prepared from nTx mice immunized with LF, there was inflammation around the draining duct of the salivary glands (a) and bile duct (c) inflammation. On the other hand, there was no inflammation in the nude mice that received CD8+ cells from nTx mice immunized with LF in salivary gland (b) and liver (d). Original magnification, ×200.

the maintenance of immune homeostasis (Marth et al, 1996). Loss of TGF-β signaling in TGF-β dominant negative mutant mice might contribute to AIP (Hahm et al, 2000). TGF-β gene expression in our mouse model was not different compared with control mice (data not shown).

In conclusion, an autoimmune mechanism against CA-II or LF via Th1-type CD4+ cells might be involved in the pathogenesis of AIP and associated exocrinopathy. Further studies, including identification of the antigenic motif in the amino acid sequence and of the genetic background that confers susceptibility, are necessary to clarify the precise mechanism.

Materials and Methods

Mice and Immunization

Male and female BALB/c CrSlc and BALB/c nu/nu CrSlc mice (Japan SLC, Shizuoka, Japan) were bred in the Facility of Experimental Animals at the Faculty of Medicine of Kyoto University, Kyoto, Japan, under specific pathogen-free conditions. nTx was performed in BALB/c CrSlc mice on the third day after birth under ether anesthesia, as described previously (Asano et al, 1996; Itoh et al, 1999; Katakai et al, 1999; Kojima and Prehn, 1981; Nishio et al, 1994; Sakaguchi et al, 1985). Bovine CA-II or LF (Sigma Chemical, St. Louis, Missouri) was used as an antigen for the immunization. In a preliminary study using Western immunoblotting, we confirmed that an extract of murine pancreas reacts with sera from animals immunized with bovine CA-II and LF (data not shown). nTx mice were injected with an emulsion of 0.1 mg of CA-II or LF in 0.1 ml PBS with 0.1 ml FCA (Chemicon International Inc., Temecula, California) at three to four sites subcutaneously at 6 weeks of age and were killed after three booster injections at 2-week intervals. Control mice were immunized with 0.1 mg of BSA (Sigma Chemical) in 0.1 ml of PBS and FCA. The mice were divided into eight groups (n = 10 in each group): normal BALB/c mice not immunized (non-nTx); normal mice immunized with BSA (non-nTx + BSA); nonimmunized nTx mice (nTx); nTx mice immunized with BSA (nTx + BSA); normal mice immunized with CA-II (non-nTx + CA-II); nTx mice immunized with CA-II (nTx + CA-II); normal mice immunized with LF (non-nTx + LF); and nTx mice immunized with LF (nTx + LF). The experimental procedures in this study were approved by the Animal Protection Committee of Kyoto University.

Adoptive Transfer

To clarify which cells are the effector cells for the development of exocrinopathy whole, CD4+, and CD8+ spleen cells (1×10^6) prepared from nTx mice immunized with CA-II or LF were transferred to 6-week-old BALB/c nu/nu (nude) mice. In control mice, spleen cells prepared from nTx mice immunized with BSA were transferred. Spleen cells prepared from nTx mice immunized with CA-II or LF were incubated

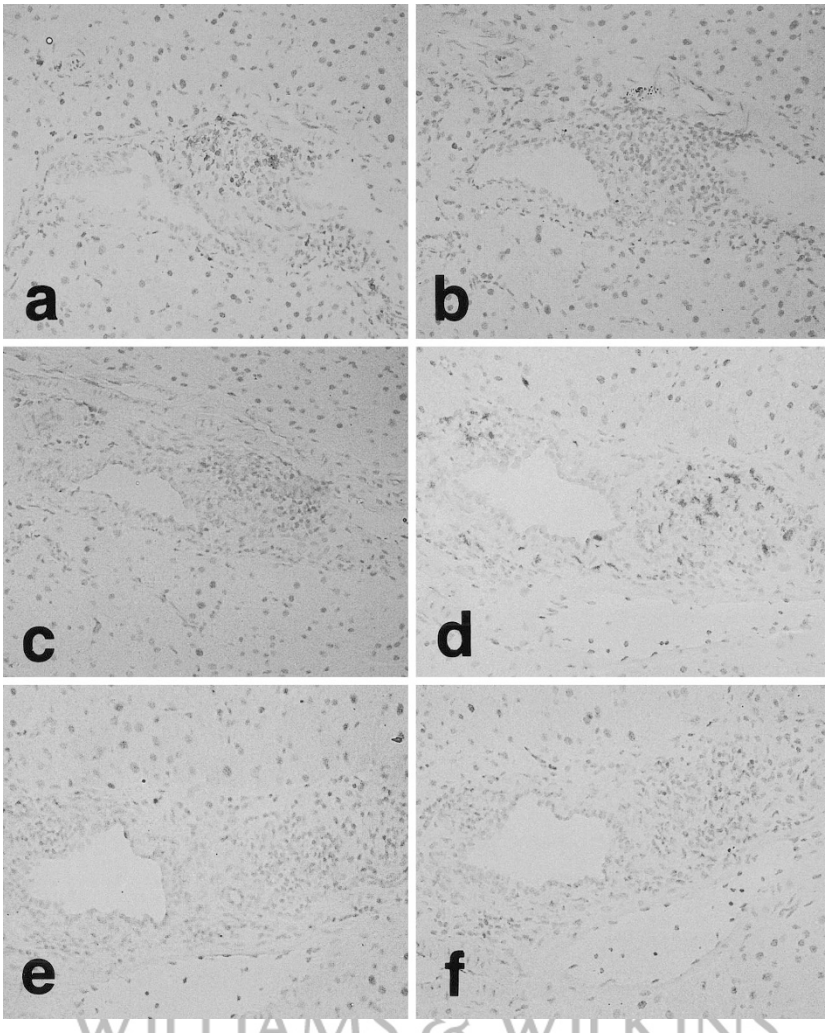

Figure 5.
Immunohistochemistry of the pancreas from nTx mice immunized with CA-II (a–c) or LF (d–f). CD4+ cells predominantly infiltrated around the small duct (a and d). CD8+ and B220+ cells were rarely observed in the serial sections (b and c; e and f). Original magnification, ×200.

with phycoerythrin (PE)- or FITC-conjugated anti-mouse CD4 or CD8 (Coulter, Miami, Florida) at 4° C for 30 minutes. CD4+ or CD8+ cells were isolated by sorting FITC- or PE-labeled spleen cells with FACS Vantage (Becton & Dickinson, Lincoln Park, New Jersey). We confirmed that these cells significantly responded to CA-II or LF using a ^{3}H-thymidine proliferation assay (data not shown). In the transfer experiment, the mice were divided into nine groups ($n = 5$ in each group): nontransferred BALB/c nu/nu mice (nude); nude mice receiving whole spleen cells from nTx mice without immunization (nude + nTx); nude mice receiving whole spleen cells from nTx mice immunized with BSA (nude + nTx with BSA); nude mice receiving whole spleen cells from nTx mice immunized with CA-II (nude + nTx with CA-II); nude mice receiving CD4 + spleen cells from nTx mice immunized with CA-II (nude + CD4 with CA-II); nude mice receiving CD8+ cells from immunized nTx mice (nude + CD8 with CA-II); nude mice that received whole spleen cells from nTx mice immunized with LF (nude + whole with LF); nude mice that received CD4+ spleen cells from nTx mice immunized with LF (nude + CD4 with LF); and nude mice that received CD8+ cells from nTx mice immunized with LF (nude + CD8 with LF). Histology and serology were studied 3 months after transfer.

Blood Chemistry

Serum biochemistry, including amylase, gamma-glutamyl transpeptidase, alanine aminotransferase, and glucose, were measured using a biochemical auto-analyzer for clinical use (Hitachi 7170; Hitachi, Ltd., Ibaragi, Japan). To evaluate humoral immunity to CA-II or LF, antibodies against CA-II or LF were measured with a solid phase ELISA. CA-II or LF (50 μl of a 20 ng/ml solution) was coated onto 96-well microtiter plates (Corning Costar Corporation, Cambridge, Massachusetts). Normal BALB/c mouse serum served as controls. After overnight incubation at 4° C, serially diluted mouse serum (0.1 ml) was plated followed by incubation at room temperature for 1 hour. After washing the plate, horseradish peroxidase-

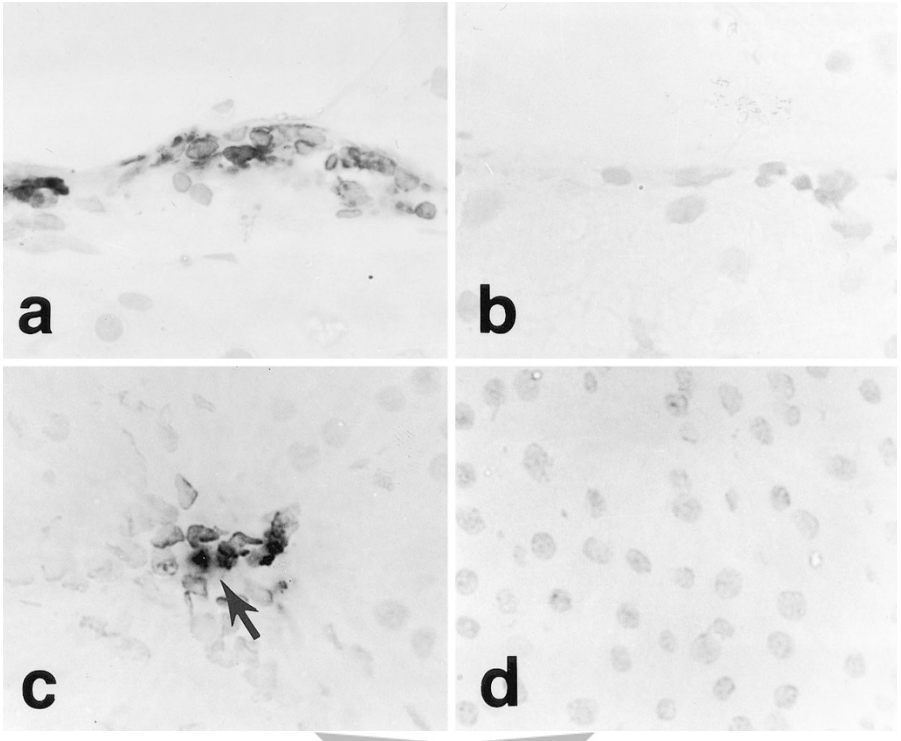

Figure 6.
Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining of the pancreas. Positive cells were observed in the pancreatic duct cells of the nude mice that received CD4 spleen cells from nTx mice immunized with CA-II (a). There were no positive cells in the negative control (b). c, Nude mice that received CD4+ spleen cells from the nTx mice immunized with LF. Acinar cells were positively stained *(arrow)*. Infiltrating lymphocytes around these acinar cells were also positively stained. d, Control mice. There were similar results in nTx mice immunized with CA-II or LF and nude mice that received whole spleen cells prepared from nTx mice immunized with CA-II or LF (data not shown). Original magnification, ×800.

labeled goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, Pennsylvania), diluted to a predetermined concentration, was added to each well. After 1 hour of incubation followed by rigorous washing, each well was reacted with a substrate (o-para-aminobenzidine) solution for 15 minutes. The reaction was terminated with 25 μl of 2M H_2SO_4 and the absorbency at 490 nm was determined using an enzyme-linked immunosorbent assay reader (Elx808; Bio-Tek Instruments, Highland Park, Vermont).

Histology

Pancreas, salivary glands, and liver were fixed with 4% phosphate-buffered formaldehyde (pH 7.2) and prepared for histologic examination. The sections were stained with hematoxylin and eosin and then evaluated independently by two pathologists who were blind to the origin of the specimens. To evaluate inflammatory activity, the degree of inflammation was scored into four categories according to those reported for an animal model of lupus nephritis and cholangitis: grade 0 = no inflammation; grade 1 = mild; grade 2 = moderate; or grade 3 = severe). Each of these scores was then assigned a number (0–3, respectively) for statistical studies, similar to those reported for the animal model of lupus nephritis and cholangitis (Ueno et al, 1998; Watson et al, 1992).

Immunohistochemistry

Immunohistochemical staining was performed on freshly frozen sections using the avidin-biotin immunoperoxidase method. Briefly, freshly frozen sections were fixed in acetone for 10 minutes, rinsed in PBS (pH 7.2), and incubated with the appropriate blocking agent for 20 minutes. Biotin-conjugated rat anti-mouse B220, CD4, and CD8 (BD Pharmingen, San Diego, California) monoclonal antibodies were applied to the sections for 2 hours. The sections were washed with cold PBS for 30 minutes and incubated with avidin-biotin peroxidase complex (ABC) (Vector Laboratories, Inc., Burlingame, California) for 30 minutes. After washing with PBS, the sections were reacted with a fresh mixture of 0.05% 3,3'-diaminobenzidine and 0.005% H_2O_2 in Tris-buffer (0.05 M, pH 7.6) for 5 minutes and washed with distilled water. Controls were incubated with normal rat serum (Cappel Laboratories) instead of the monoclonal antibodies. These control samples produced negative results.

In Situ End-Labeling of Fragmented DNA

Fragmented DNA was studied using an in situ end-labeling technique with the commercially available TUNEL staining kit (ApopTag; Oncor, Gaithersburg, Maryland) as described previously (Anderson et al, 1997). Briefly, fresh frozen sections were fixed with

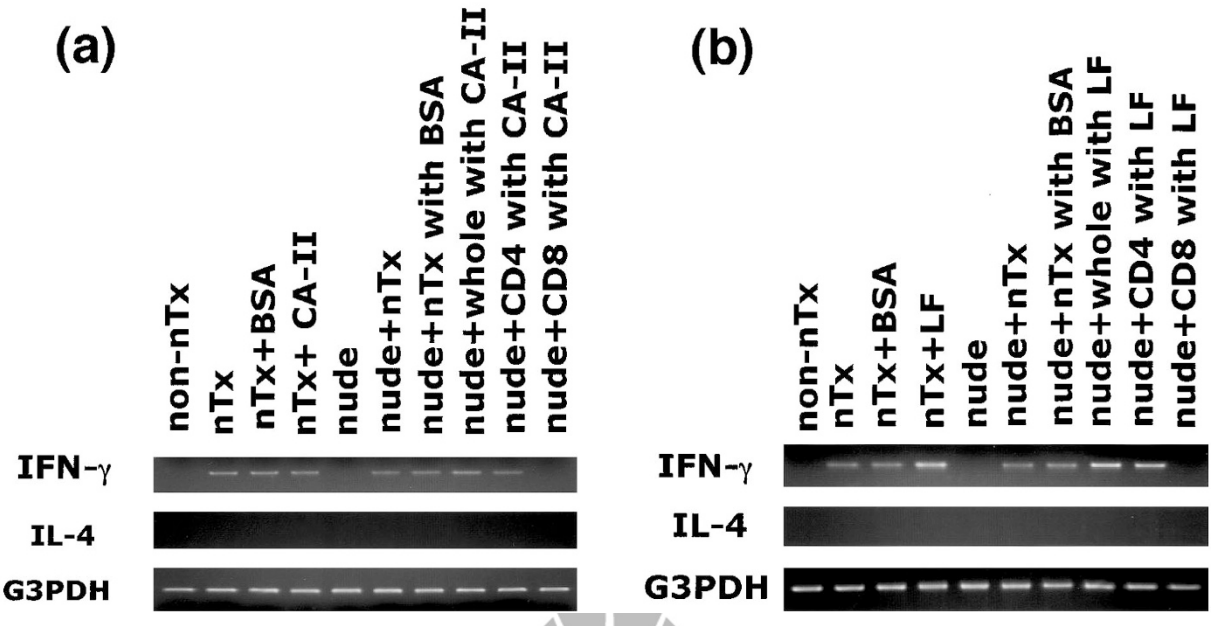

Figure 7.

Gene expression of cytokines in the salivary glands determined using reverse transcription-polymerase chain reaction (RT-PCR). a, The mRNA of IFN-γ was expressed in the salivary glands of nTx mice, but was not significantly changed by immunization. Nude mice that received whole or CD4+ spleen cells from nTx mice immunized with CA-II also expressed the IFN-γ message in the salivary glands. b, The mRNA of IFN-γ was expressed in the salivary glands of nTx mice glands, and immunization with LF up-regulated IFN-γ gene expression. Nude mice that received whole or CD4+ spleen cells from nTx-mice immunized with LF had an up-regulated IFN-γ message compared with nude mice that received whole spleen cells from nonimmunized nTx mice or nTx mice immunized with BSA, whereas nude mice that received CD8+ spleen cells did not express the IFN-γ gene. IL-4 mRNA was not expressed in any mice. Nonimmunized non-nTx, nonimmunized non-nTx BALB/c mice; nonimmunized nTx, nonimmunized nTx BALB/c mice; nTx + BSA, nTx mice immunized with BSA; nTx + CA-II, nTx mice immunized with CA-II; nude, untreated nude mice; nude + nTx, nude mice that received whole spleen cells prepared from nonimmunized nTx mice; nude + nTx with BSA, nude mice that received whole spleen cells prepared from nTx mice immunized with BSA; nude + nTx with CA-II, nude mice that received whole spleen cells from nTx mice immunized with CA-II; nude + CD4 with CA-II, nude mice that received CD4+ cells from nTx mice immunized with CA-II; nude + CD8 with CA-II, nude mice that received CD8+ cells from nTx mice immunized with CA-II; nTx + LF, nTx mice immunized with LF; nude + whole with LF, nude mice that received whole spleen cells from nTx mice immunized with LF; nude + CD4 with LF, nude mice that received CD4+ cells from nTx mice immunized with LF; nude + CD8+ with LF, nude mice that received CD8+ cells from nTx mice immunized with LF.

10% buffered formalin for 10 minutes at room temperature and washed three times with PBS. Digoxigenin-labeled dUTP was incorporated at the 3'-OH ends of the fragmented DNA by terminal deoxynucleotidyl transferase, anti-digoxigenin antibody conjugated with peroxidase was applied, and the peroxidase activity was revealed by treatment with 3-amino-9-ethylcarbazol. Nuclei were lightly counterstained with methylgreen.

Reverse Transcription-Polymerase Chain Reaction

To analyze cytokine gene expression by RT-PCR, total RNA was extracted with the RNA extraction solution Isogen (Nippon Gene, Tokyo, Japan). RNA prepared from the pancreas was partially degraded. Therefore, we performed RT-PCR using the salivary glands, which had lesions similar to those of the pancreas. RNA was pooled from three murine salivary glands in each group. Total RNA was reverse-transcribed into DNA using the Super Script Preamplification System (Gibco BRL, Life Technologies, Inc., Rockville, Maryland). Total RNA in the reaction mixture was heated at 42° C for 50 minutes and at 70° C for another 15 minutes and then chilled on ice. PCR was performed with the following mouse-specific primers (Clontech, Palo Alto, California): IL-4: 5,'CCAGCTAGTTGTCATCCTGCTCTCCTTTCTCG and 3', CGTGGTACTTACTCAGGTTCAGGTGTAGTGAC; IFN-γ: 5', TGCATCTTGGCTTTGCAGCTCTTCCTCATGGC and 3', CGGTTCA AACTCCAGTTGTTGGTGTCCAGGT; Glyceraldehyde-3-phosphate dehydrogenase (G3PDH): 5', GTGGGCCGCTCTAGGCACCAA and 3', CTCTTTGATGTCACGCACGATTTC.

The reaction mixture consisted of 20 μl of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 mM each deoxy-nucleotide triphosphate (Perkin-Elmer, Branchburg, New Jersey), 50 pM of each specific primer, 1.0 U of *Taq* DNA polymerase (Ampli *Taq* Gold; Perkin-Elmer), and 1 μg of the cDNA preparation. Amplification was performed with an automated thermal cycler (GeneAmp PCR System 9600R; Perkin-Elmer) for 35 cycles, each of which consisted of 20 seconds at 95° C for denaturing, 1 minute at 55° C for annealing, and 1 minute at 72° C for extension. The final cycle included a 10-minute extension step at 72° C to ensure full extension of the product. Each PCR product (10 μl) was electrophoresed on a 2.0% agarose gel containing ethidium bromide, and the bands were examined using an image auto-analyzing system (Fotodyne, FOTOanalyst and Archive ECLIPSE; Advanced American Biotechnology, Fullerton, California).

Statistical Analysis

All data are expressed as the mean ± SD. Statistical analysis of the data was performed using a two-tailed Wilcoxon's *t* test. A p value of < 0.05 was considered statistically significant.

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