3) MR imaging of the pituitary

A thickened pituitary stalk or mass formation on the stalk was observed in 18 cases, and some of the thickening took place at the level of infundibulum or the proximal end of the stalk. On the other hand, a swelling of the pituitary gland or mass formation in the pituitary was present in 10 cases. Among these 10 cases, 2 showed a pituitary mass alone, and 3 showed both a thickened stalk and pituitary mass occurring simultaneously. The other 5 cases showed a united large mass formation involving both the pituitary and stalk (Fig. 1).

The "bright" signal seen in the posterior portion of the pituitary on T1-weighed imaging was absent in the cases involving central diabetes insipidus and in several cases without clinical diabetes insipidus.

Hypertrophic pachymeningitis was found in 5 patients and orbital lesion including pseudotumor formation was in 2 cases. Para-sinusitis was observed in 3 cases.

4) Laboratory findings

Seven of the 9 patients who were tested for C-reactive protein were found to be positive. Elevated levels of serum immunoglobulin G and serum IgG4 were observed in 7 of 9 cases and in 12 of 13 cases in which they were assessed, respectively. A normal serum level of IgG4 was observed only in patients receiving steroid therapy [29]. Serum levels of IgG4 promptly decreased to reference range after initiation of steroid therapy [21]. Clinical manifestations and laboratory findings did not seem to differ between the cases with and without IgG4 measurement.

In the two cases in which FDG-PET was performed, uptake was observed in both the pituitary gland and other involved lesions [11, 23].

5) Histopathology of pituitary lesion

Pituitary biopsy was performed in only 5 cases via a transsphenoidal approach or transcranial approach. The inflammatory pseudotumor of the pituitary was densely infiltrated with both lymphocyte and plasma cells and fibrous changes were demonstrated in all cases. The plasma cells were stained positive by IgG4 immunostaining (Fig. 2).

6) Associated IgG4-related systemic disease

Various IgG4-related systemic diseases are associated with pituitary and stalk lesion. Among these, retroperitoneal fibrosis was the most prevalent disease (n=10), followed by Mikulicz disease and salivary gland lesions (n=8), pulmonary lesions (m=8), pancreatic lesion (n=6), and lymph node swelling (n=5).

Systemic diseases preceded the pituitary lesions in 11 patients, the two occurred simultaneously in 8 cases, and the pituitary lesion preceded the systemic disease in 2 cases. An isolated pituitary lesion not associated with any systemic IgG-4 related disease was described in one patient [33].

7) Effect of glucocorticoid therapy

Various kinds and doses of glucocorticoid were used for replacing adrenocortical insufficiencies or actively treating the pituitary mass and/or accompanying hypertrophic pachymeningitis, AIP or other lesions. Some of the anterior pituitary insufficiencies were resolved by glucocorticoid even in a lower dose range similar to that prescribed as a replacement for adrenocortical insufficiency. In most cases of diabetes insipidus, glucocorticoid therapy did not lead to remission.

As for the pituitary mass and the stalk thickening, almost all lesions shrank during glucocorticoid therapy. However, several cases showed a relapse of the pituitary mass when the doses of glucocorticoid were decreased. Serum levels of IgG and IgG4 promptly decreased to normal ranges after glucocorticoid therapy.

8) Summary of clinical features

Table 2 provides a summary of the clinical features of the IgG4-related pituitary and stalk lesion. Almost all cases involved middle-aged to elderly men presenting with various degrees of hypopituitarism and diabetes insipidus and demonstrating a thickened pituitary stalk and/or pituitary mass. These structures shrank remarkably in response to glucocorticoid therapy. Some of the anterior pituitary insufficiencies were also resolved by glucocorticoid administration. The presence of IgG4-related sys-

temic diseases and the elevated serum IgG4 levels before glucocorticoid therapy were the main clues to a correct diagnosis of IgG4-related infundibulo-hypophysitis. Several cases were accompanied with pachymeningitis and para-sinusitis, suggesting that both sellar and parasellar structures were involved in chronic inflammation.

3. Relationship to other forms of hypophysitis (Fig.3)

Primary hypophysitis is of unknown etiology and is classified on a histopathological basis as lymphocytic, granulomatous, or xanthogranulomatous hypophysitis [36-38], whereas secondary hypophysitis occurs as a direct result of systemic infectious or inflammatory processes or as a result of local processes such as a ruptured Rathke cleft cyst, craniopharyngioma, adenoma, or germinoma.

Primary hypophysitis may also be categorized into adenohypophysitis, infundibulo-neurohypophysitis, and panhypophysitis based on the tissues involved. Adenohypophysitis typically affects females during the puerperal period and presents a pituitary mass and hypopituitarism, whereas patients with infundibulo-neurophysitis typically show diabetes insipidus with a posterior pituitary mass or thickened pituitary stalk. Both entities are regarded as autoimmune-mediated. On the other hand, panhypophysitis involves both lobes of the pituitary gland, which has a different developmental origin, suggesting that it may not arise solely from an autoimmune mechanism. Inflammation of the anterior or posterior lobe of the pituitary may spread out over the whole pituitary [37]. There are several case reports of panhypophysitis showing an aggressive behavior and invading into the cavernous sinus or hypothalamus and causing cranial nerve paralyses [39]. Whether these types of lymphocytic hypophysitis belong to the same category as other types of hypophysitis is currently unknown.

Chronic parasellar inflammation such as those in hypertrophic pachymeningitis, cavernous sinusitis or Tolosa-Hunt syndrome have been reported to be accompanied by hypopituitarism and/or diabetes insipidus [40]. We have previously reported three such cases but did not measure the subjects' IgG or IgG4 levels [41]. Both sellar and parasellar structures were involved in the chronic inflammation in the cases of IgG4-related hypophysitis with pachymeningitis, para-sinusitis, and/or orbital pseudotumor. The previously reported cases of pituitary lesion associated with multifocal fibrosclerosis were considered to belong the same category as the currently reviewed cases of IgG4-related pituitary diseases.

Cases of isolated pituitary lesions or cases in which onset of diabetes insipidus preceded the other lesions by several years present a challenge for diagnosis. It is recommended that the measurement of serum IgG4 levels be included in the panel during the initial workup for investigating hypopituitarism and/or diabetes insipidus.

4. Pathogenesis

The pathogenesis of IgG4-related systemic disease is currently under intensive investigation [5]. Elevated serum IgG4 and dense infiltration of IgG4-positive plasma cells in various organs suggest that IgG4 plays a major role in the pathogenesis, although the trigger for IgG4 elevation has not been clearly established.

There are increased numbers of activated CD4+ and CD8+ T cells bearing HLA-DR in the pancreas of AIP patients. An inhibitory molecule, cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is expressed on activated memory T cells and CD4+CD25+ regulatory T cells (Tregs), acts as a negative regulator of T cell responses [42]. The soluble isoform of CTLA-4 is reported to be elevated in patients with AIP, enhancing immune responses by blocking the interaction of CD80 on antigen-presenting cells and CTLA-4 on T cells. Tregs are thought to be associated with various autoimmune diseases [43]. Miyoshi *et al.* [44] have observed that the number of circulating naïve Tregs is decreased in the peripheral blood of the patients with AIP, whereas the number of memory Tregs is significantly increased. Prominent infiltration of Tregs has been observed in the liver of patients with sclerosing cholangitis. These finding suggest that regulatory functions of T cells, such as CTLA-4 and Tregs, are involved in the development and pathophysiology of AIP [45].

Given the preponderance of the disease amongst elderly males and the dramatic responses to oral steroid therapy, the pathogenesis may not involve an autoimmune mechanism but rather other mechanisms, such as an allergic reaction. Zen *et al.* [46] have reported that the expression of T helper 2 (Th2) cytokines and regulatory cytokines (IL-10 and transforming growth factor-beta) was up-regulated in the affected tissues of patients with IgG4-related pancreatitis and cholangitis. They have suggested that the

predominant Th2 and regulatory immune reactions in this disease reflect an allergic mechanism.

In conjunction to the above mentioned pathogenesis of IgG4-related systemic disease, we should cite two important observations regarding autoimmune hypophysitis. Mirocha *et al.* [47] have observed two separate entities of primary hypophysitis; one entity involves an autoimmune process with Th 17 cell dominance and lack of Tregs, and the other entity involves a process in which Tregs seem to control the immune response, which may not be self- but foreign-targeted. Another important observation is that of drug-induced hypophysitis. Inhibitory antibodies directed against CTLA-4 cause disruption of immune tolerance to antigens on cancer cells and were associated with anti-tumor activity in melanoma and renal cell carcinoma [48]. Anti-CTLA-4 antibody therapy has been associated with autoimmune hypophysitis, and high dose glucocorticoid treatment resulted in markedly improved symptoms and partial recovery of hypopituitarism [49].

5. Summary and conclusion

We have reviewed case reports of possible infundibulo-hypophysitis associated with IgG4-related systemic diseases and described their common clinical features. We consider this disorder not as a variant form of primary autoimmune hypophysitis but as a secondary form of hypophysitis associated with IgG4-related systemic disease. Only 5 cases demonstrated histological proof of inflammatory pseudotumor on pituitary biopsy. Therefore, we should accumulate similar cases of suspected hypophysitis by measuring serum IgG4 levels and try to investigate their histopathology in order to clarify the possible immune- or allergy-mediated pathogenesis.

Acknowledgement

This work was in part supported by Grant-in-Aid for Scientific Research (Research on Measures for Intractable Diseases) from the Ministry of Health, Labor and Welfare in Japan. The authors thank Dr. Satoshi Baba, Department of Pathology, Hamamatsu University School of Medicine, for the valuable discussion of the histopathology.

References

1. Caturegli P, Newschaffer C, Olivi A, *et al.* (2005) Autoimmune hypophysitis. *Endocr Rev* 26(5): 599-614.
2. Rivera JA. (2006) Lymphocytic hypophysitis: disease spectrum and approach to diagnosis and therapy. *Pituitary* 9(1): 35-45.
3. Brazier DJ, Sanders MD. (1983) Multifocal fibrosclerosis associated with suprasellar and macular lesions. *Br J Ophthalmol* 67(5): 292-296.
4. Olmos PR, Falko JM, Rea GL, *et al.* (1993) Fibrosing pseudotumor of the sella and parasellar area producing hypopituitarism and multiple cranial nerve palsies. *Neurosurgery* 32(6): 1015-1021.
5. Kamisawa T, Okamoto A. (2008) IgG4-related sclerosing disease. *World J Gastroenterol* 14(25): 3948-3955.
6. Masaki Y, Dong L, Kurose N, *et al.* (2009) Proposal for a new clinical entity, IgG4-positive multiorgan lymphoproliferative syndrome: analysis of 64 cases of IgG4-related disorders. *Ann Rheum Dis.*, 68(8): 1310-1315.
7. Comings DE, Skubi KB, Van Eyes J, *et al.* (1967) Familial multifocal fibrosclerosis. Findings suggesting that retroperitoneal fibrosis, mediastinal fibrosis, sclerosing cholangitis, Riedel's thyroiditis, and pseudotumor of the orbit may be different manifestations of a single disease. *Ann Intern Med* 66 (5) : 884-892.
8. Kamisawa T, Funata N, Hayashi Y, *et al.* (2003) Close relationship between autoimmune pancreatitis and multifocal fibrosclerosis. *Gut* 52(5): 683-687.
9. Hansen I, Petrossians P, Thiry A, *et al.* (2001) Extensive inflammatory pseudotumor of the pituitary. *J Clin Endocrinol Metab* 86(10): 4603-4610,
10. Murakami K, Muraishi K, Ikeda H, *et al.* (2001) Plasma cell granuloma of the pituitary gland. Case report. *Surg Neurol* 56(4): 247-251.

11. Tanabe T, Tsushima K, Yasuo M, *et al.* (2006) IgG4-associated multifocal systemic fibrosis complicating sclerosing sialadenitis, hypophysitis, and retroperitoneal fibrosis, but lacking pancreatic involvement. *Intern Med* 45(21): 1243-1247.
12. Yamamoto M, Takahashi H, Ohara M, *et al.* (2006) A case of Mikulicz's disease (IgG4-related plasmacytic disease) complicated by autoimmune hypophysitis. *Scand J Rheumatol* 35(5): 410-411.
13. Taniguchi T, Hamasaki A, and Okamoto M. (2006) A case of suspected lymphocytic hypophysitis and organizing pneumonia during maintenance therapy for autoimmune pancreatitis associated with autoimmune thrombocytopenia. *Endocr J* 53(4): 563-566.
14. Wong S, Lam WY, Wong WK, *et al.* (2007) Hypophysitis presented as inflammatory pseudotumor in immunoglobulin G4-related systemic disease. *Hum Pathol* 38(11): 1720-1723.
15. Kishimoto M, Okimura Y, Kimura K, *et al.* (2000) Multifocal fibrosclerosis as a possible cause of panhypopituitarism with central diabetes insipidus. *Endocr J* 47(3): 335-342.
16. Braun J, Schuldes H, Berkefeld J, *et al.* (2001) Panhypopituitarism associated with severe retroperitoneal fibrosis. *Clin Endocrinol (Oxford)* 54(2): 273-276.
17. Sumitani T, Yamamoto H, Saito H, *et al.* (2003) A case of Mikulicz syndrome associated with central diabetes insipidus and hypopituitarism. *Clin Endocrinol (Tokyo)* 51(Suppl. Summer 42): 30-34. (in Japanese)
18. Fukuda W, Kimura M, Akaogi T, *et al.* (2003) Multifocal fibrosclerosis: retroperitoneal fibrosis associated with a suprasellar tumor and pachymeningitis. *Intern Med* 42(10): 1006-1010.
19. Katabami T, Shirai N, Hayashi A, *et al.* (2003) A case of autoimmune hypophysitis associated with Mikulicz syndrome. *Folia Endocrinol Jpn* 79 (Suppl. 2): 5-9. (in Japanese)
20. van der Vliet H and R Perenboom (2004) Multiple pseudotumors in IgG4-associated multifocal systemic fibrosis. *Ann Intern Med* 141: 896-897.
21. Sommerfield AJ, Lockman KA, Bathgate AJ, *et al.* (2008) Multifocal fibrosclerosis: a new case report. *Ann Clin Biochem* 45(Pt 1): 99-101.
22. Miyoshi T, Otsuka F, Tsukamoto N, *et al.* (2008) A case of suspected hypothalamo-hypophyseal dysfunction due to IgG4-related diseases. *Folia Endocrinol Jpn* 84 (Suppl.): 87-89. (in Japanese)
23. Isaka Y, Yoshioka K, Nishio M, *et al.* (2008) A case of IgG4-related multifocal fibrosclerosis complicated by central diabetes insipidus. *Endocr J* 55(4): 723-728.
24. Isaka Y, Yoshioka K, Nishio M, *et al.* (2008) A case of IgG4-related multifocal fibrosclerosis complicated by central diabetes insipidus. *Folia Endocrinol Jpn* 84(Suppl. June): 42-45.(in Japanese)
25. Tsuboi H, Inokuma S, Setoguchi K, *et al.* (2008) Inflammatory pseudotumors in multiple organs associated with elevated serum IgG4 level: recovery by only a small replacement dose of steroid. *Intern Med* 47(12): 1139-1142.
26. Yamamoto T, Takahashi T, Kawahara S, *et al.* (2008) A case of lymphocytic hypophysitis (IgG4-related sclerotic disease) showing difficulty in differentiating sarcoidosis. Abstract in 144th Chubu District Meeting of Japan Radiological Society. (Abstract; in Japanese)
27. Uehara K, Atsumi H, Nakagawa J, *et al.* (2008) A case of IgG4-related hypophysitis presented with partial diabetes insipidus and hypertrophic pachymeningitis. *Folia Endocrinol Jpn* 84(3): 825. (Abstract; in Japanese)
28. Tsukada T, Tachibana O, Yamamoto J, *et al.* (2009) Pachymeningitis and hypophysitis presented as inflammatory pseudotumor in immunoglobulin G4-related systemic disease. Program and Abstracts of the 19th Annual Meeting of the Japan Society for Hypothalamic and Pituitary Tumors. p95. (Abstract; in Japanese)
29. Taji H, Takamura T, Mohri K, *et al.* (2009) A male case of lymphocytic hypophysitis accompanied by IgG4-related disease. *Folia Endocrinol Jpn* 85(Suppl.): 42-44. (in Japanese)
30. Ando K, Hori R, Makita Y, *et al.* (2009) A case of systemic IgG4-related disease manifested as a

hypophysitis 15 yrs after Mikulicz disease, autoimmune hepatitis and interstitial pneumonitis. The 564th Kanto Regional Meeting of Japanese Society of Internal Medicine, (Abstract; in Japanese)

31. Ueda R, Okamura S, Fujita N, *et al.* (2009) A case of retroperitoneal fibrosis complicated with lymphocytic hypophysitis and diabetes insipidus. *Folia Endocrinol Jpn* 85(1): 294. (Abstract; in Japanese)
32. Takeuchi S, Takeuchi A, Takakuwa M, *et al.* (2009) A case of pituitary lesion by IgG4-related disease with multiple lesions. *Folia Endocrinol Jpn.* 85(1): 324. (Abstract; in Japanese)
33. Mizutani A, Okada M, Yokota N, *et al.* (2009) Immunoglobulin G4-related inflammatory pseudotumor as a cause oh panhypopituitarism. Program and Abstracts for the 19th Annual Meeting of the Japan Society for Hypothalamic and Pituitary Tumors. p93. (Abstract; in Japanese)
35. Yoneda K, Matsunami K, Yamaguchi K, *et al.* (2009) A case of isolated ACTH deficiency and IgG4-related disease. The 100th Chugoku District Meeting of Japanese Society of Internal Medicine. (Abstract; in Japanese)
36. Nishimori I, Onishi S, Otsuki M. (2007) Nationwide survey for autoimmune pancreatitis in Japan. *Pancreas (Tokyo)* 22(6): 651-656. (in Japanese)
37. Gutenberg A, Buslei R, Fahlbusch R, *et al.* (2005) Immunopathology of primary hypophysitis: implications for pathogenesis. *Am J Surg Pathol* 29: 329-338.
38. Tashiro T, Sano T, Xu B, *et al.* (2002) Spectrum of different types of hypophysitis : a clinicopathologic study of hypophysitis in 31 cases. *Endocr Pathol* 13: 183-195.
39. Cheung CC, Ezzat S, Smyth HS, *et al.* (2001) The spectrum and significance of primary hypophysitis. *J Clin Endocrinol Metab* 86(3): 1048-1053.
40. Nussnaum CE, Okawara SH, Jacobs LS. (1991) Lymphocytic hypophysitis with involvement of the cavernous sinus and hypothalamus. *Neurosurgery* 28: 440-444.
41. Hama S, Arita K, Kurisu K, *et al.* (1996) Parasellar chronic inflammatory disease presenting Tolosa-Hunt syndrome, hypopituitarism and diabetes insipidus. *Endocr J* 43(5): 503-510.
42. Shimatsu A, Nakamura Y, Murabe H, *et al.* (2000) Three cases of parasellar chronic inflammatory disease manifesting pituitary swelling, diabetes insipidus and Tolosa-Hunt syndrome – relationship to lymphocytic hypophysitis. *Clin Endocrinol (Tokyo)* 48(Suppl, 36): 38-41.(in Japanese)
43. Barry J. (2009) Autoimmunity: CTLA-4: a key protein in autoimmunity. *Nat Rev Rheumatol* 5(5): 244-245.
44. Fehervari Z, Sakaguchi S. (2004) CD4+ Tregs and immune control. *J Clin Invest* 114: 1209-1217.
45. Miyoshi H, Uchida K, Taniguchi T, Yazumi S, Matsushita M, Takaoka M, Okazaki K. (2008) Circulating nai"ve and CD4+CD25high regulatory T cells in patients with autoimmune pancreatitis. *Pancreas* 36(2): 133-140.
46. Okazaki K. (2008) Are regulatory molecules for T cells involved in the development of autoimmune pancreatitis? *Am J Gastroenterol* 103(3): 588-594.
47. Zen Y, Fujii T, Harada K, *et al.* (2007) Th2 and regulatory immune reactions are increased in immunoglobulin G4-related sclerosing pancreatitis and cholangitis. *Hepatology* 45: 1538-1546.
48. Mirocha S, Elagin RB, Salamat S, *et al.* (2008) T regulatory cells distinguish two types of primary hypophysitis, *Clin Exp Immunol* 155: 403-411.
49. Phan GQ, Yang JC, Sherry RM, *et al.* (2003) Cancer regression and autoimmunity induced by CTLA-4 blockade in patients with metastatic melanoma. *Proc Natl Acad Sci USA* 100: 8327-8377.
50. Dillard T, Yedinak CG, Alumkal J, *et al.* (2009) Anti-CTLA-4 antibody therapy associated autoimmune hypophysitis: a serious immune related adverse event across a spectrum of cancer subtypes. *Pituitary* in press (E-pub on line 29 July 2009).

Table 1. Reported cases of pituitary and stalk lesion associated with IgG4-related systemic diseases (since 2000)

Case	Age/ Sex	Pituitary function DI Hypopituitarism	MRI	IgG/IgG4 (mg/dL)	IgG4-related lesions	Ref.	Report
1	53/M	DI Hypopituitarism	Stalk	—/—	Dura Orbita Parasinus Lung	15	Kishimoto (2000)
2	43/M	DI Hypopituitarism	Stalk	—/—	Retroperitoneum	16	Braun (2001)
3	66/M	DI Hypopituitarism	Stalk	6060/—	Mikulicz Pelvis Lung Parasinus	17	Sumitani (2003)
4	42/M	Hypopituitarism	Stalk	1400/—	Dura Retroperitoneum	18	Fukuda (2003)
5	65/M	DI Hypopituitarism	Stalk Pituitary mass	3277/—	Mikulicz Dura	19	Katabami (2003)
6	66/F	Hypopituitarism	Pituitary mass	—/485(20-250)	Pancreas Retroperitoneum Salivary Lung	20	van der Vliet (2004)
7	71/M	Hypopituitarism	Stalk Pituitary mass	3015/405	Salivary Retroperitoneum Lymph node	11	Tanabe (2006)
8	70/M	Hypopituitarism	Stalk	—/2220	Mikulicz	12	Yamamoto (2006)
9	75/M	Hypopituitarism	Stalk - Pituitary mass	6040/—	Pancreas Eye Lung	13	Taniguchi (2006)
10*	62/M	Hypopituitarism	Pituitary mass	1330/720 (0-291)	Pancreas Gallbladder	14	Wong (2007)
11	61/M	DI Hypopituitarism	Stalk	—/—	Peritoneum Cholangitis	21	Sommerfield (2008)
12	73/M	Hypothalamic dysfunction	Stalk	1581/22 (4.8-105)	Retroperitoneum Pancreas	22	Miyoshi (2008)
13	55/M	DI Hypopituitarism	Stalk	2701/1860	Parasinus Retroperitoneum	23, 24	Isaka (2008)
14	62/M	DI Hypopituitarism	Stalk	1990/292 (<135)	Pancreas Lung Lymph node Retroperitoneum	25	Tsuboi (2008)
15*	68/M	—	Stalk - Pituitary mass	—/elevated	Kidney Lymph node	26	Yamamoto (2008)
16*	77/M	DI Hypopituitarism	Stalk	2370/229	Dura	27,28	Uehara(2008) Tsukada(2009)
17*	59/M	DI Hypopituitarism	Stalk - Pituitary mass	1515*/111*	Pancreas Orbita Eye Lymph node Retroperitoneum Lung Kidney Thyroid	29	Taji (2009)
18	70/M	DI Hypopituitarism	Stalk - Pituitary mass	—/949	Liver Mikulicz Lung	30	Ando (2009)
19	58/M	DI	Stalk	—/466*	Retroperitoneum Dura	31	Ueda (2009)
20	77/M	DI Hypopituitarism	Stalk Pituitary mass	—/1950	Pancreas Liver Lymph node Salivary	32	Takeuchi (2009)
21*	72/M	Hypopituitarism	Stalk - Pituitary mass	—/—	— (Pituitary alone)	33	Mizutani (2009)
22	64/M	Hypopituitarism	—	—/—	Retroperitoneum Lung Mikulicz	34	Yoneda (2009)

case*: case with pituitary biopsy
DI: diabetes insipidus
Stalk: stalk thickening or mass on the stalk; Stalk - Pituitary mass: united large mass formation in the pituitary and stalk
—: not described
IgG/IgG4*: under steroid therapy, ( ): reference ranges

Table 2. Clinical characteristics of pituitary and stalk lesions associated with IgG4-related systemic diseases.

1. Preponderance of the disease amongst elderly males
2. Presented with various degrees of hypopituitarism and diabetes insipidus
3. MRI demonstrated a thickened pituitary stalk and/or pituitary mass
4. Thickened stalk and pituitary mass shrank in response to glucocorticoid therapy
5. Some of the anterior pituitary insufficiencies were resolved by glucocorticoid therapy
6. Presence of IgG4-related systemic diseases
7. Elevated serum IgG4 levels before glucocorticoid therapy
8. Some cases accompanied with pachymeningitis or para-sinusitis, suggesting that both sellar and parasellar structures were involved in the chronic inflammation

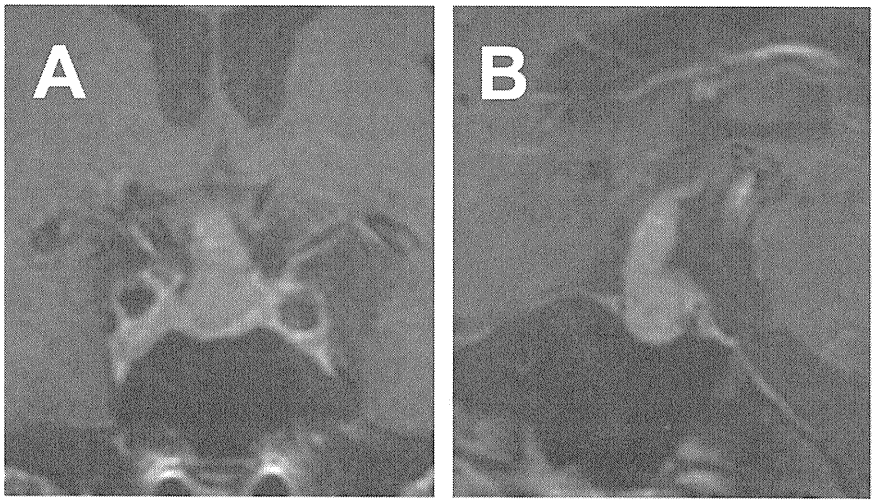

Fig. 1. MR imaging of the pituitary in a patient with IgG4-related infundibulo-hypophysitis [33]. T1-weighted gadolinium enhanced imaging (A: coronal section, B: saggital sections)

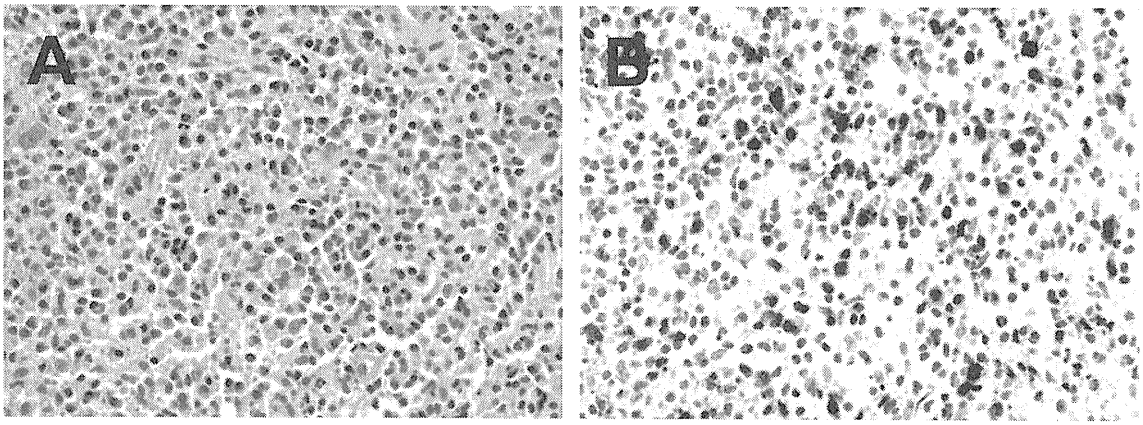

Fig. 2. Histopathology of the pituitary gland in a patient with IgG4-related infundibulo-hypophysitis [33]. A: HE staining, B: IgG4 immunostaining.

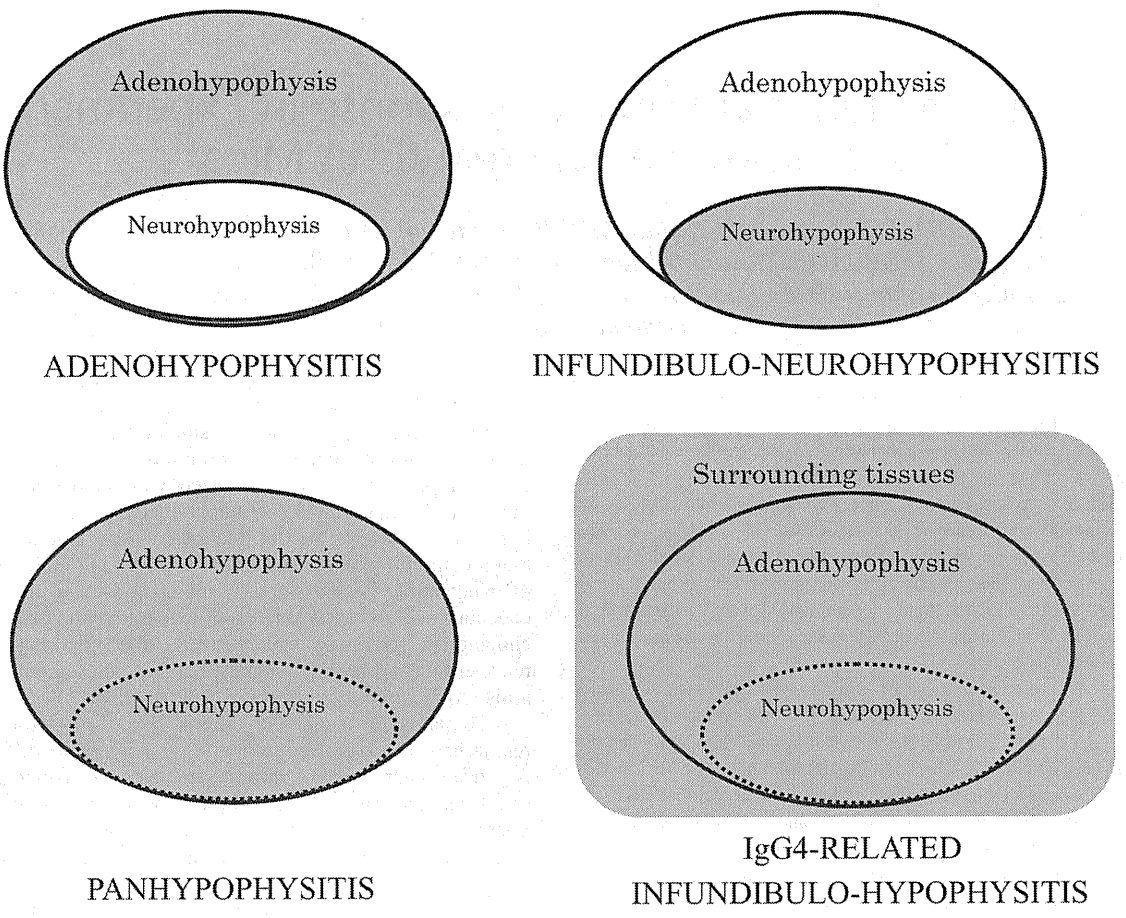

Fig. 3. Involvement of pituitary gland with hypophysitis (conceptual figures).
Shaded area represents the involved tissues.

Analysis of Humoral Immune Response in Experimental Autoimmune Pancreatitis in Mice

Masanori Asada, MD, PhD, Akiyoshi Nishio, MD, PhD,† Takuji Akamatsu, MD,‡ Junya Tanaka, MD,‡ Kazuyuki Saga, MD,‡ Masahiro Kido, MD, PhD,‡ Norihiko Watanabe, MD, PhD,‡ Kazushige Uchida, MD, PhD,† Toshiro Fukui, MD, PhD,† Kazuichi Okazaki, MD, PhD,† and Tsutomu Chiba, MD, PhD‡*

Objectives: To study the autoimmune response in MRL/Mp mice, which spontaneously develop pancreatitis in the exocrine pancreatic tissue.

Methods: Six-week-old female mice were injected intraperitoneally with polyinosinic polycytidylic acid at a dose of 5 mg/kg of body weight twice a week for up to 12 weeks. The mice were serially killed, and the severity of their pancreatitis was graded with a histological scoring system. Immunohistological examinations were performed, and the serum levels of autoantibodies were measured by enzyme-linked immunosorbent assay.

Results: The administration of polyinosinic polycytidylic acid accelerated the development of pancreatitis, with abundant infiltration of $B220^+$ B cells and $CD138^+$ plasmacytes. Various autoantibodies directed against autoantigens, including carbonic anhydrase II and lactoferrin, were detected but none against glutamic acid decarboxylase. Of these, autoantibodies directed against the pancreatic secretory trypsin inhibitor (PSTI; 91.7%) were more prevalent than those against carbonic anhydrase II (33.3%) or lactoferrin (45.8%). Determination of the epitope of the anti-PSTI antibody showed that most immunoreactivity was directed at the site on PSTI that is active in the suppression of trypsin activity.

Conclusions: The autoimmune response to PSTI protein may induce a failure of PSTI activity, resulting in the activation of trypsinogen and the subsequent disease progression.

Key Words: autoimmune pancreatitis, autoantibody, pancreatic secretory trypsin inhibitor

(*Pancreas* 2010;39: 224–231)

Chronic pancreatitis is characterized by chronic inflammation and progressive fibrosis of the pancreas, which leads to irreversible pancreatic dysfunction and, finally, to pancreatic insufficiency. Although the cause of chronic pancreatitis is frequently associated with excessive alcohol use and gall stones, approximately 30% to 40% of cases are idiopathic.[1] Since Sarles et al[2] reported a case of chronic pancreatitis with hyperglobulinemia, an increasing number of similar cases have been documented, with or without other autoimmune diseases. Many such cases of pancreatitis are associated with increased immunoglobulin G (IgG), IgG4, or autoantibody production and are highly responsive to steroid treatment. These findings suggest that an autoimmune mechanism is involved in the development of pancreatitis. Recent studies have demonstrated the frequent cooccurrence of extrapancreatic lesions, such as sclerosing cholangitis, sclerosing sialoadentitis, interstitial nephritis, or retroperitoneal fibrosis,[3] suggesting that autoimmune pancreatitis (AIP) is a discrete form of pancreatitis.[4,5]

A series of diagnostic criteria have been proposed by researchers in several countries in an attempt to differentiate AIP from other forms of chronic pancreatitis or pancreatic cancer. These criteria are based on a combination of findings from imaging, laboratory testing, and histological analysis.[6–8] A typical radiological image shows narrowing of the main pancreatic duct and enlargement of the pancreas. Laboratory data show abnormally elevated levels of serum γ-globulin, IgG, or IgG4, or the presence of autoantibodies. Histopathological analysis of the pancreas demonstrates marked fibrosis and prominent infiltration by lymphocytes and plasma cells. Recently, widespread awareness of the disease and the proposed diagnostic criteria has resulted in an increasing number of patients with AIP reported throughout the world.

However, little is known about the precise pathogenesis of AIP, although reports have shown that the disease is associated with the progressive infiltration of lymphocytes and plasmacytes, predominantly localized to ductal structures, in addition to varying degrees of parenchymal and acinar destruction.[9] The natural course of the disease is also as yet unknown. The disease may remain largely asymptomatic for prolonged periods, and symptoms develop in patients with advanced stages of the disease. Therefore, the early immune response underlying the pathogenesis of AIP is difficult to study in patients with the disease. A serological hallmark of AIP is considered to be elevated levels of IgG and IgG4 and the presence of autoantibodies against antigens such as carbonic anhydrase II (CA-II), lactoferrin (LF), pancreatic secretory trypsin inhibitor (PSTI), and nuclear antigens,[10–14] although these autoantibodies are not found in all patients with AIP and their role in its pathogenesis is not fully understood.

To investigate the autoimmune mechanism involved in the development of human AIP, several animal models have been studied that develop AIP-like pancreatic lesions spontaneously or after immunization with exogenous antigens.[15–21] Among these, MRL/Mp mice spontaneously develop pancreatitis via an autoimmune mechanism at 34 weeks of age or older.[15] The administration of polyinosinic polycytidylic acid (poly I:C), a synthetic double-stranded RNA, accelerates the development of the disease, with an incidence of 100% at 18 weeks without any

From the *Digestive Disease Center, Tazuke Kofukai Medical Research Institute, Kitano Hospital, Osaka; †Third Department of Internal Medicine, Kansai Medical University, Moriguchi; and ‡Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan.

Received for publication March 18, 2009; accepted August 4, 2009.

Reprints: Akiyoshi Nishio, MD, PhD, Third Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono-cho, Moriguchi 570-8507, Japan (e-mail: nishioa@takii.kmu.ac.jp).

This study was supported in part by grants-in-aid for scientific research (C19590762 and C20590810) from the Japan Society for the Promotion of Science, by a research fund from the Pancreas Research Foundation, and by a Health and Labor Science Research grant for Intractable Diseases from the Japanese Ministry of Health, Labor and Welfare.

other severe autoimmune diseases.[19] Although the pancreatitis in MRL/Mp mice has been shown to be cell mediated, the humoral autoimmune response has not been fully investigated. In this study, we investigated the autoimmune response in MRL/Mp mice treated with poly I:C, with a specific focus on AIP-related autoantibody production.

MATERIALS AND METHODS

Mice

Female MRL/Mp mice were purchased from the Jackson Laboratory (Bar Harbor, Me). Wild-type female C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All mice were bred at the animal facility of Kyoto University under specific-pathogen–free conditions.

Induction of Pancreatitis

The 6-week-old female MRL/Mp mice were injected intraperitoneally with poly I:C (Sigma Chemical Co, St Louis, Mo) at a dose of 5 mg/kg of body weight twice a week for up to 12 weeks. The control mice were injected with phosphate-buffered saline (PBS). All experiments were conducted with the approval of the Ethics Committee for the Use of Experimental Animals of Kyoto University.

Histological Examination

The mice were killed at the age of 12 or 18 weeks. Blood was collected, and sera were stored at −20°C until use. Several tissues, including the pancreas, the liver, the salivary gland, and the kidney, were removed for histopathological examination. The tissues were fixed in 10% phosphate-buffered formaldehyde (pH 7.2) and embedded in paraffin. The sections were stained with hematoxylin and eosin and examined histopathologically under a light microscope. The severity of the pancreatitis was scored on a 0 to 4 scale based on the histopathological scoring system described by Kanno et al[15]: 0, pancreas without mononuclear cell infiltration; 1, mononuclear cell aggregation and/or infiltration within the interstitium, with no parenchymal destruction; 2, focal parenchymal destruction with mononuclear cell infiltration; 3, diffuse parenchymal destruction but some intact parenchymal residue retained; and 4, almost all pancreatic tissue, except the pancreatic islets, destroyed or replaced with

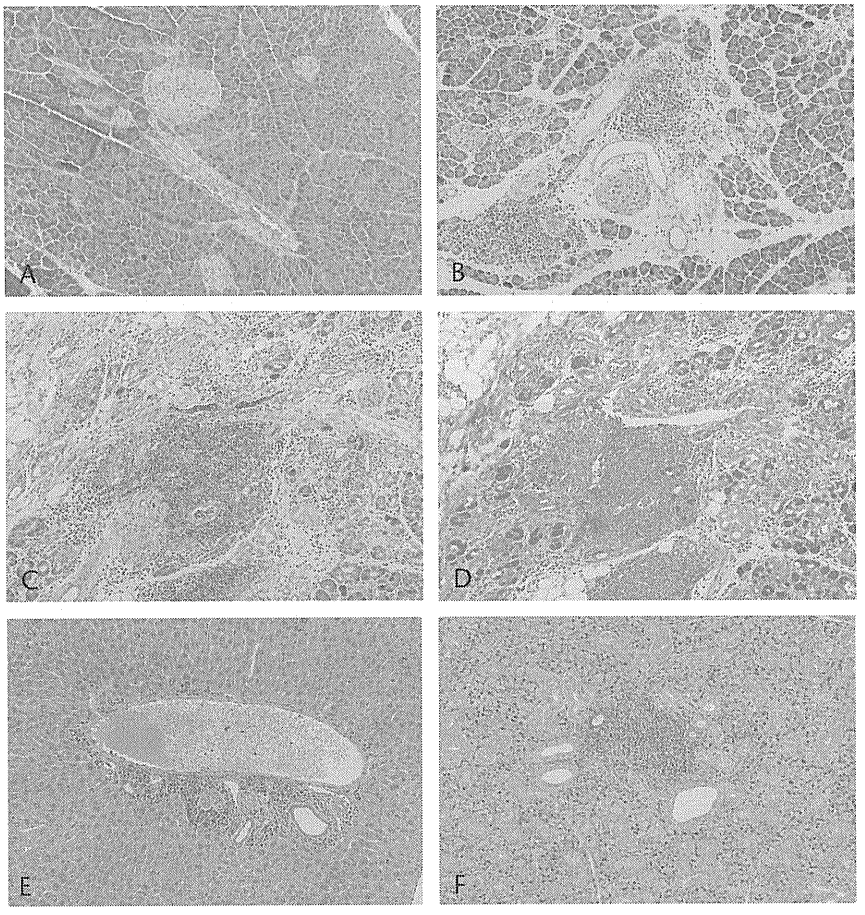

FIGURE 1. Histopathological examination of the pancreas, the liver, and the submandibular salivary gland. Representative pancreatic sections stained with hematoxylin and eosin or Azan: 12-week treatment with PBS (A), 6-week treatment with poly I:C (B), 12-week treatment with poly I:C (C), and 12-week treatment with poly I:C (Azan staining) (D). Liver (E) and submandibular salivary gland sections (F) after treatment with poly I:C for 12 weeks. After the mice were injected with poly I:C for 6 weeks, interstitial edema and moderate inflammatory cell infiltration of the pancreas were observed. After treatment for 12 weeks, marked inflammatory cell infiltration with severe destruction of the acini, irregular fibrosis, and fatty changes of the pancreas was seen. Mononuclear cell infiltration was observed around some of the portal areas of the liver. Periductular infiltration by mononuclear cells was scattered in the submandibular salivary gland (original magnification ×100).

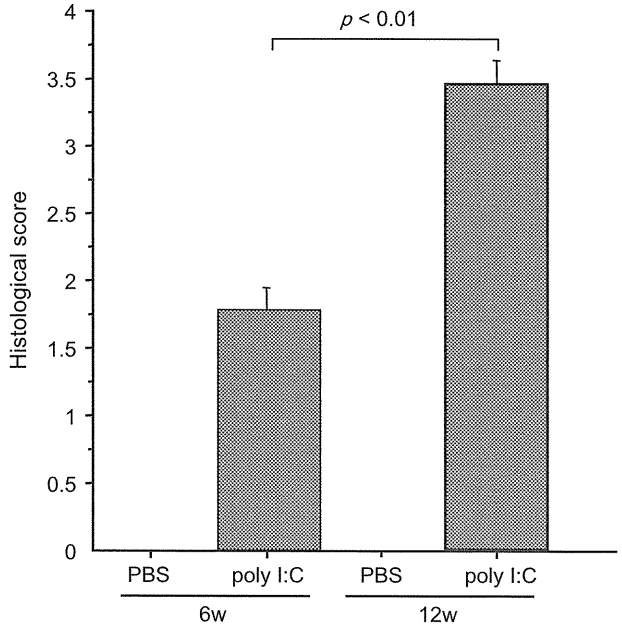

FIGURE 2. Histological scoring of pancreatitis. The severity of pancreatitis was scored on a 0 to 4 scale based on a histopathological scoring system. The histological scores for pancreatitis increased according to the duration of the treatment (6 weeks, 1.8 ± 0.4, 12 weeks, 3.5 ± 0.7). The results are expressed as mean ± SD.

fibrosis or adipose tissue. The maximum score was used as the grade of pancreatitis in each mouse. To estimate the incidence of pancreatitis, mice with pancreatic lesions that were scored 2 or higher were defined as positive. The fibrosis of the sections was also examined histologically with Azan staining.

Immunohistochemistry

Immunohistochemical staining was performed on 5-μm-thick, formalin-fixed, paraffin-embedded tissue sections. The sections were deparaffinized in xylene, rehydrated in graded alcohol, and washed in PBS. Antigen retrieval was accomplished by microwave irradiation. After the serial sections had been blocked in 1.5% normal rabbit serum for 15 minutes at room temperature, they were incubated for 1 hour at room temperature with 1 of the following primary antibodies at a dilution of 1:50: rat anti–mouse B220 monoclonal antibody (mAb), rat anti-mouse CD4 mAb, rat anti–mouse CD8 mAb, or rat anti–mouse CD138 mAb (all from BD Bioscience, San Jose, Calif). After brief rinsing, the sections were treated with biotinylated rabbit anti–rat IgG secondary antibody (Serotec Inc, Kidlington, Oxford, United Kingdom) for 30 minutes at room temperature, rinsed, and incubated with peroxidase-conjugated avidin-biotin complex (ABC Elite kit; Vector Laboratories, Burlingame, Calif) for 30 minutes at room temperature. The peroxidase activity was visualized by the application of a fresh mixture of 3,3′-diaminobenzidine and 0.005% H_2O_2 in Tris-buffered saline (0.05 mol/L, pH 7.6). The sections were then counterstained with hematoxylin, dehydrated, cleared, and mounted.

Measurement of Autoantibodies

To evaluate the humoral immunity against PSTI, CA-II, and LF, the levels of specific autoantibodies in the sera were quantified with enzyme-linked immunosorbent assays (ELISAs) according to a previous report,[12] with minor modifications. Microtiter plates (Maxi Sorp; Nalge Nunc International, Roskilde, Denmark) were coated with 50 μL of a 20-μg/mL solution of bovine PSTI, CA-II, or LF (Sigma Chemical Co) and incubated overnight at 4°C. The plates were then incubated with 200 μL of 10% skim milk in PBS containing 0.05% Tween 20 (PBST) to block nonspecific binding and rinsed 4 times with PBST. The murine sera, at a dilution of 1:40, were tested in duplicate for 2 hours at room temperature. The optimal dilution

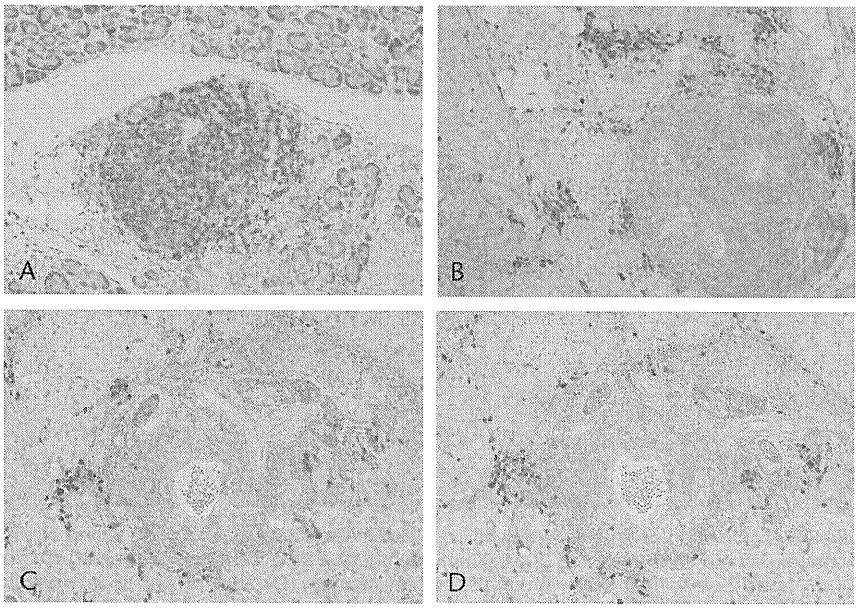

FIGURE 3. Immunohistochemical analysis of the pancreas. Representative pancreatic sections from mice treated with poly I:C for 6 weeks: anti-B220 (A), anti-CD138 (B), anti-CD4 (C), and anti-CD8 staining (D). $B220^+$ mononuclear cells infiltrated into the pancreatic parenchyma and formed lymphoid follicles at the periductal region of the pancreatic ducts. $CD138^+$ cells infiltrated around the follicles and interstitium, in addition to $CD4^+$ and $CD8^+$ T cells (original magnification ×200).

of the sera (1:40) was determined in preliminary experiments in which 4-fold serial dilutions of the sera (1:40 to 1:640) were tested for the detection of autoantibodies by ELISA. After the samples were washed with PBST, the bound antibodies were reacted specifically with diluted (1:4000) goat anti–mouse IgG antibody conjugated with horseradish peroxidase (Serotec Inc, Raleigh, NC) for 2 hours at room temperature. After 4 washes with PBST, the plates were incubated with 50 μL of 0.4-mg/mL *o*-phenylene diamine in 0.1-mol/L citrate phosphate buffer (pH 5.0) with H_2O_2 for 15 minutes at room temperature. The reaction was terminated by the addition of 50 μL of 2 N H_2SO_4, and the absorbance was determined at an optical density (OD) of 490 nm. Positive results were defined as OD values greater than the mean plus 3 SDs (mean + 3 SDs) of the values obtained for the sera of untreated C57BL/6 mice. Anti–glutamic acid decarboxylase (GAD) antibody was measured by a commercial laboratory (SRL Inc, Osaka, Japan).

Measurement of Serum IgG Subclasses

To clarify which IgG subclass was predominant in mice with AIP, we measured the levels of the IgG1, IgG2a, IgG2b, and IgG3 subclasses using the Mouse IgG1, IgG2a, IgG2b, and IgG3 ELISA Quantitation kits (Bethyl Laboratories Inc, Montgomery, Tex), according to the manufacturer's instructions. Briefly, microtiter plates (Maxi Sorp) were coated with 100 μL of capture antibody for 1 hour at room temperature. After the plates were blocked with blocking solution, the sera (serially diluted to fall within the concentration range of the standards) were added to the plates, and the plates were incubated for 1 hour at room temperature. After the plates were washed, the bound IgG was reacted specifically for 1 hour at room temperature with the diluted detection antibody conjugated with horseradish peroxidase. After the plates were washed with PBST, they were incubated with 100 μL of tetramethylbenzidine/H_2O_2 (R&D Systems, Inc, Minneapolis, Minn) for 15 minutes at room temperature. The reaction was terminated by the addition of 100 μL of 2 N H_2SO_4, and the absorbance was determined at an OD of 450 nm. The levels of the IgG subclasses were calculated from the respective standard curves.

Immunoglobulin G Subclass and Epitope Mapping of Anti-PSTI Antibody

To determine the IgG subclass of the serum anti-PSTI antibodies, an ELISA was performed using bovine PSTI as the antigen. The murine sera were tested in duplicate for 1 hour at room temperature at a dilution of 1:40. The bound anti-PSTI antibody was reacted with optimally diluted (1:5000 to 1:100,000) goat anti–mouse IgG1, IgG2a, IgG2b, or IgG3 antibody conjugated with horseradish peroxidase (Bethyl Laboratories Inc). The plates were incubated with 100 μL of tetramethylbenzidine/H_2O_2 (R&D Systems, Inc) for 15 minutes at room temperature. The reaction was terminated by the addition of 100 μL of 2 N H_2SO_4, and the absorbance was determined at an OD of 450 nm as described previously. To define the epitopic region for the serum anti-PSTI antibodies, we synthesized overlapping peptides that covered the entire amino acid sequence of the serine protease inhibitor, Kazal type 3 (Spink3), a mouse homologue of bovine PSTI, consisting of the 56 amino acids following the secretion signal sequence composed of the first 24 amino acids. The first peptide included amino acids 1 to 25, KVTGKEASCHDAVAGCPRIYDPVCG; the second peptide included amino acids 17 to 41, PRIYDPV CGTDGITYANECVLCFEN; and the third peptide included amino acids 32 to 56, ANECVLCFENRKRIEPVLIRKGGPC. The peptides with the previously mentioned sequences were synthesized to order (immunological purity and no conjugation) by Invitrogen Japan KK (Tokyo, Japan). The serum levels of the autoantibodies directed against these synthetic peptides were measured by ELISA.

Statistical Analysis

The data were analyzed using the Student *t* test when 2 groups were compared. When multiple groups were compared, the data were examined by 1-way analysis of variance followed by Fisher protected least significant difference. A 2-tailed $P < 0.5$ was deemed to indicate statistical significance.

RESULTS

Histological Examination

The administration of poly I:C accelerated the development of pancreatitis in MRL/Mp mice, but that of PBS did not (Fig. 1A). After injection of poly I:C for 6 weeks, atrophic changes in the parenchyma, interstitial edema, and moderate inflammatory cell infiltration were observed (Fig. 1B). After injection for 12 weeks, marked inflammatory cell infiltration was observed, with severe destruction of the acini, irregular

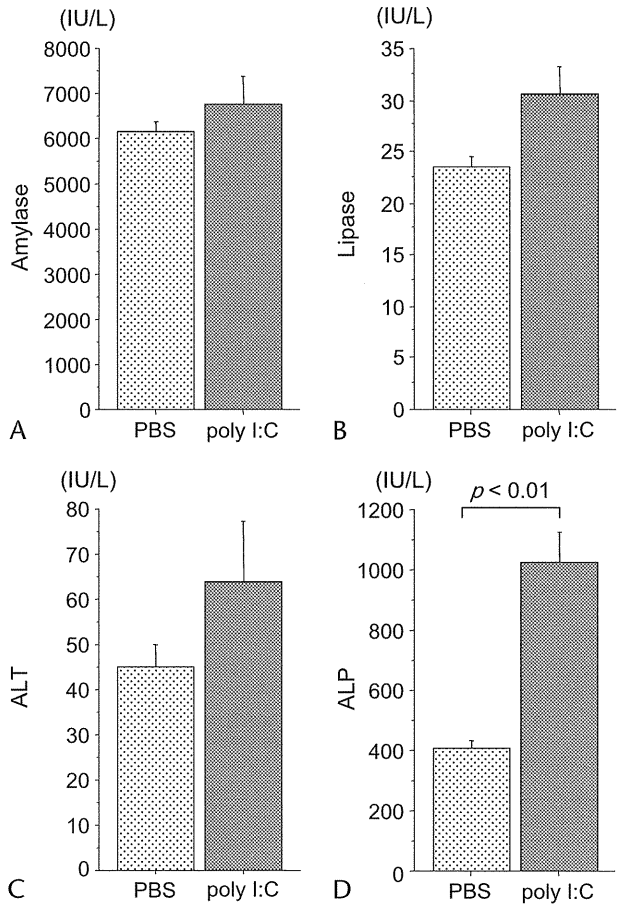

FIGURE 4. Serum pancreaticobiliary enzymes of the control mice and mice treated with poly I:C for 12 weeks: amylase (A), lipase (B), alanine aminotransferase (ALT) (C), and alkaline phosphatase (ALP) (D). There was no significant increase in the serum amylase, the lipase, or the ALT levels in the mice treated with poly I:C relative to those treated with PBS. The serum levels of alkaline phosphatase were significantly higher in the poly I:C–treated mice. The results are expressed as mean ± SD.

fibrosis, and fatty changes (Figs. 1C, D). Some of the acinar cells also showed vacuolar changes in their cytosol (cellular vacuolization). The histological scores for pancreatitis increased with the duration of the treatment (6 weeks, 1.8 ± 0.4, 12 weeks, 3.5 ± 0.7; Fig. 2). However, the endocrine glands showed few changes, and the tissues were well preserved. In the liver, mononuclear cell infiltration was observed around some of the portal areas, indicating the coexistence of cholangitis (Fig. 1E). Mild periductul infiltration by mononuclear cells was also observed in the submandibular salivary gland (Fig. 1F).

Immunohistochemistry

Immunohistochemistry showed that most of the infiltrates in the pancreatic parenchyma were $B220^+$ mononuclear cells, which infiltrated the pancreatic parenchyma and formed lymphoid follicles in the periductal regions of the pancreatic ducts (Fig. 3A). $CD138^+$ plasmacytes infiltrated around the follicles and interstitium (Fig. 3B). $CD4^+$ and $CD8^+$ T cells were mainly observed around the follicles (Figs. 3C, D).

Blood Chemistry

There were no significant increases in serum amylase, lipase, or alanine aminotransferase levels in mice treated with poly I:C relative to those in mice treated with PBS (Fig. 4). The serum levels of alkaline phosphatase in the poly I:C–treated mice were significantly higher than those in the control mice ($P < 0.01$), which is consistent with the histopathological finding of cholangitis in the livers of mice treated with poly I:C.

Measurement of Serum IgG Subclasses

The IgG subclass levels in the murine sera were measured by ELISA. The levels of the IgG subclasses in the control mice (n = 6) and mice treated with poly I:C (n = 6) were as follows (mg/dL): IgG1, 133.5 ± 25.8 and 363.5 ± 44.6, respectively; IgG2a, 255.2 ± 125.4 and 560.2 ± 211.6, respectively; IgG2b, 24.1 ± 13.8 and 85.8 ± 21.7, respectively; and IgG3, 776.7 ± 635.3 and 1177.4 ± 462.3, respectively (Fig. 5). The levels of IgG1 and IgG2b were significantly higher in the poly I:C–treated mice than in the control mice. The serum IgG2a and IgG3 levels were elevated in the poly I:C–treated mice, but they were not significantly higher than those of the control mice.

Autoantibody Production

The OD values for autoantibodies directed against CA-II in the poly I:C–administered mice (0.745 ± 0.222) were significantly higher than those in the PBS-treated mice (0.371 ± 0.299). Similarly, the titers of the anti-LF antibodies in the poly I:C–administered mice (0.613 ± 0.191) were higher than those in the control mice (0.288 ± 0.231). The titers of the anti-PSTI antibody in the poly I:C–administered mice (0.489 ± 0.177) were also higher than those in the PBS-treated mice (0.289 ± 0.271; Figs. 6A–C). When the cutoff index was set at a value equivalent to the mean absorbance + 3 SDs of the values for untreated C57BL/6 mice (0.347), 91.7% (22/24) of the poly I:C–treated mice were positive for anti-PSTI antibody. This is in contrast to the relatively low frequencies of anti–CA-II (33.3%, 8/24) and

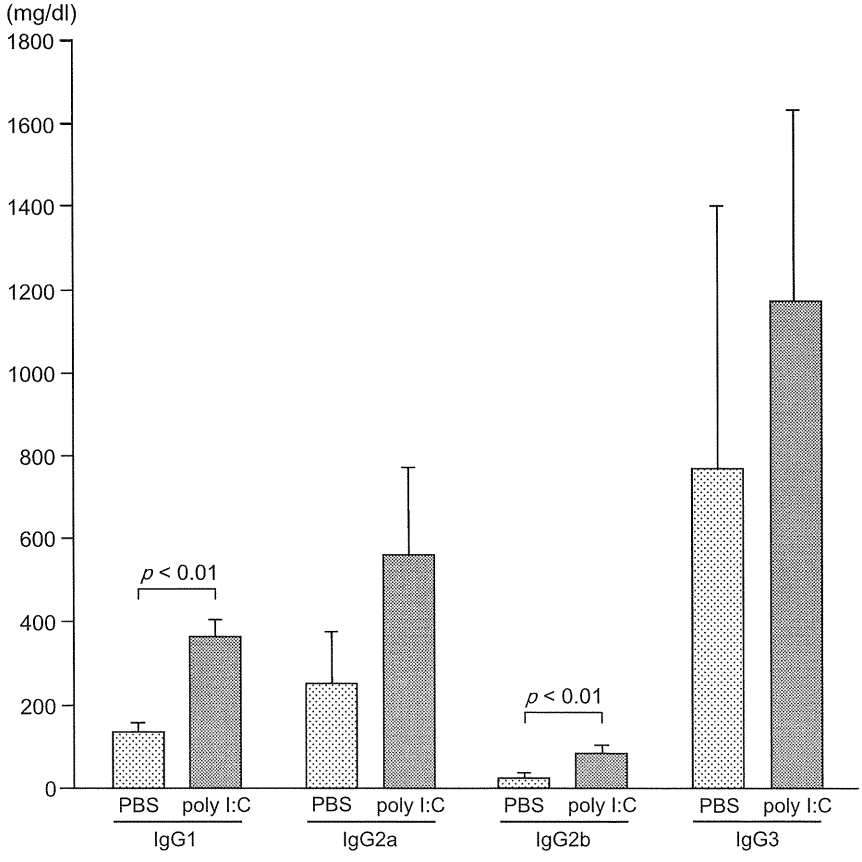

FIGURE 5. Measurement of serum IgG subclasses. The levels of the IgG subclasses were quantified by ELISA. The amounts of IgG1 and IgG2b were significantly higher in the poly I:C–treated mice than in the control mice ($P < 0.01$). The serum IgG2a and IgG3 levels were elevated in the poly I:C–treated mice but were not significantly higher than those in the control mice. The results are expressed as mean ± SD.

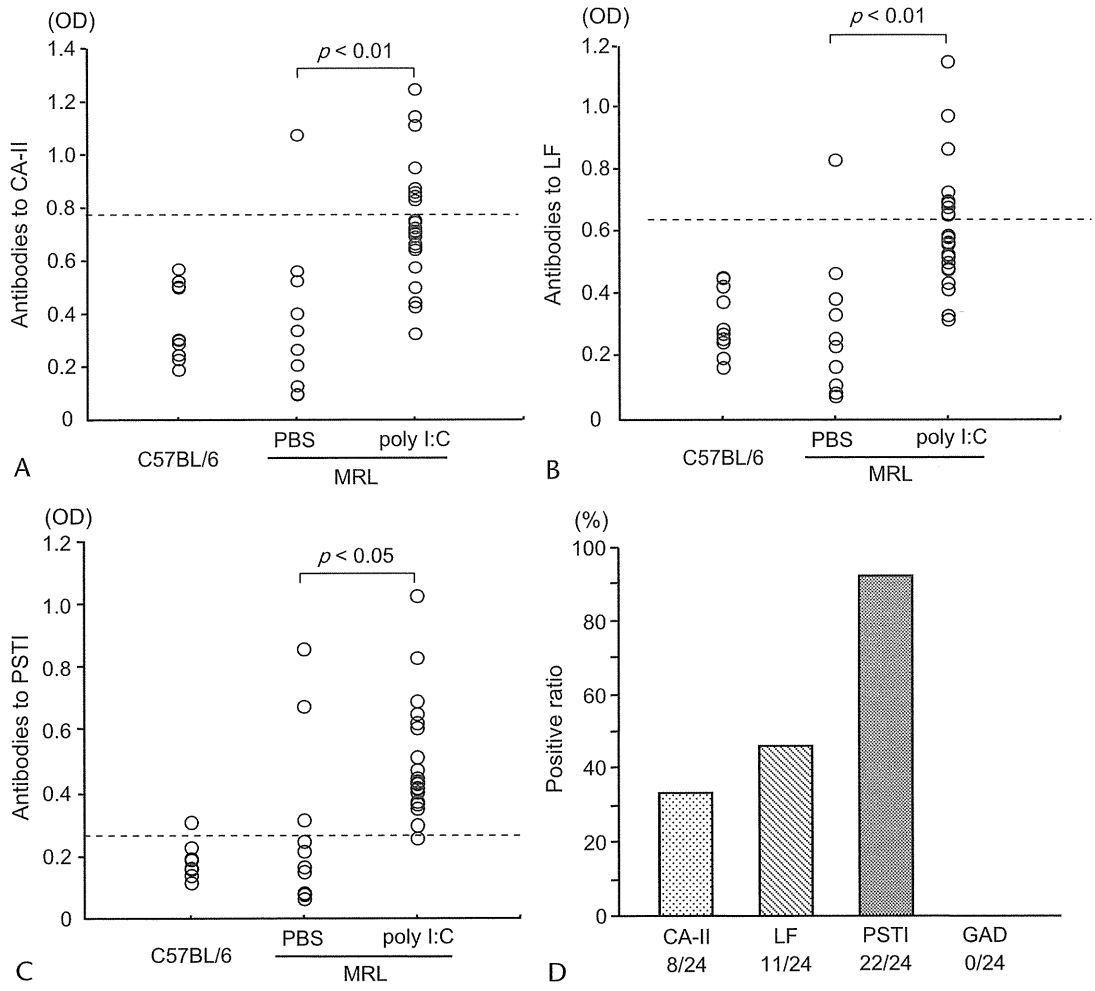

FIGURE 6. Titers of autoantibodies in the sera of mice treated with poly I:C for 12 weeks: anti–CA-II (A), anti-LF (B), anti-PSTI antibodies (C), and prevalence of autoantibodies (D). The titers of these antibodies were all significantly higher than those of the control mice. When the cutoff values were equivalent to the means + 3 SDs of the values for the untreated C57BL/6 mice, the prevalence of the anti-PSTI antibody (91.7%) was higher than that of the anti–CA-II (33.3%) and anti-LF antibodies (45.8%). No anti-GAD antibodies were detected in the mice with AIP. The dotted line indicates the means + 3 SDs of the values for the untreated C57BL/6 mice.

anti-LF antibodies (45.8%, 11/24). No sera were positive for anti-GAD antibody (Fig. 6D).

Immunoglobulin G Subclass and Epitope Mapping of Anti-PSTI Antibody

Anti-PSTI antibody was shown by ELISA to be predominantly present as the IgG2a subclass (data not shown). The reactivity of the sera from the AIP mice was tested against overlapping peptides corresponding to PSTI. When the cutoff index was set at a value equivalent to the mean absorbance + 3 SDs of untreated C57BL/6 mice, 82.3% (20/24) and 75% (18/24) of the sera from poly I:C–treated mice reacted with peptides 1 and 2, respectively. However, none of the sera reacted with peptide 3.

DISCUSSION

Although the clinical and histopathological features of AIP have been well documented, the precise pathogenesis of the disease is poorly understood.[22] This is partly because of the difficulty in making a diagnosis and obtaining pancreatic samples in the early stage of the disease. Therefore, the early cellular events underlying the pathogenesis of the disease are difficult to clarify in patients with AIP. To overcome the difficulty, we used MRL/Mp mice, in which pancreatitis spontaneously develops via an autoimmune mechanism.[15] The development of pancreatitis was accelerated by the administration of poly I:C, a synthetic double-stranded RNA.[19] Because of its structural resemblance to double-stranded viral RNA, poly I:C should accelerate the development of autoimmune diseases in several animals with genetically susceptible backgrounds.[23,24]

The observation of increased serum IgG levels or the presence of autoantibodies supports the diagnosis, whereas elevated serum IgG4 levels are nearly diagnostic.[25,26] We found that serum IgG levels were increased in mice treated with poly I:C compared with those of the control mice. However, we found no increase in specific serum IgG subclass levels, although immunoglobulin production was highly increased by the administration of poly I:C, a polyclonal activator of B cells.

Interestingly, various autoantibodies, including anti–CA-II antibody and anti-LF antibody, were also detected in the MRL/Mp mice with pancreatitis, confirming the hypothesis that CA-II and LF are target antigens in autoimmune-mediated pancreatitis as reported previously.[10,12,14] In addition to the

production of autoantibodies directed against CA-II and LF, we found that the prevalence of anti-PSTI antibodies was markedly increased relative to the autoantibodies directed against CA-II or LF. This is in contrast to the prevalence of anti-PSTI antibodies in human AIP, in which anti-PSTI antibodies are detected in 30% to 40% of patients. This may be explained by a difference in genetic background because MRL mice are considered genetically homogeneous, whereas humans constitute a genetically heterogeneous population. Therefore, although the analysis of a mouse model is useful for studying the pathogenesis of AIP, the results obtained may not be applicable to all patients with AIP.

In our previous study, anti-PSTI antibodies in the sera of patients with AIP were of the IgG1 subclass, not of the IgG4 subclass. Moreover, there was no significant correlation between the serum levels of IgG4 and anti-PSTI IgG antibodies in patients with AIP. This is in contrast to a recent study that found a strong association between increased serum IgG4 and anti–CA-II antibody levels in patients with AIP.[26] In the mice with pancreatitis, anti-PSTI antibodies were of the IgG2a subclass, not of the IgG1 or IgG4 subclass, although the roles of the corresponding IgG subclasses are considered to be different between mice and human.

Pancreatic secretory trypsin inhibitor (serine protease inhibitor, Kazal type 1), a 56–amino acid peptide, is synthesized in pancreatic acinar cells and colocalizes with trypsinogen granules. It inhibits approximately 20% of trypsin activity within the pancreas by physically blocking the active site on trypsin.[27,28] In addition to its protective role in acinar cells, PSTI inhibits the activation of trypsinogen in the pancreatic duct.[29] Such protective role of PSTI has been demonstrated in several animal models of experimental pancreatitis.[29–32] Recent studies have suggested that N34S, an exonic mutation of PSTI, is closely associated with the pathogenesis of hereditary pancreatitis and idiopathic chronic pancreatitis.[33,34]

However, the role of serum anti-PSTI antibodies in the development of human AIP has not yet been determined. One possibility is that anti-PSTI antibodies neutralize and inhibit the action of PSTI, resulting in the excessive activation of trypsin in the pancreas. To clarify the role of anti-PSTI in the pathogenesis of AIP, we synthesized overlapping peptides of the serine protease inhibitor, Kazal type 3 (Spink3), which is considered to be homologous to human PSTI, and investigated the epitopic region of the anti-PSTI antibodies. Interestingly, the sera of mice with pancreatitis reacted with synthetic peptide 1 (amino acids 1–25) and peptide 2 (amino acids 17–43) but not to peptide 3 (amino acids 32–56). Because previous studies have reported that the active site of PSTI necessary for its binding to trypsin occurs in the amino acid sequence 18 to 21,[35] such skewed reactivity may indicate that the anti-PSTI antibody inhibits the activity of PSTI in vivo. Alternatively, the presence of anti-PSTI antibody may merely be a secondary immune response against antigens released from the destroyed pancreatic tissue. It is necessary to study whether the anti-PSTI antibody inhibits the function of PSTI, leading to the progression of pancreatitis, although the cell-mediated immune response is considered to play the major role in the pathogenesis of murine AIP.[15] This would clarify the autoimmunity in human AIP, in which both humoral and cellular immune response may be involved.[36]

In conclusion, we have demonstrated the increased production of various autoantibodies in mice with AIP. Notably, autoantibody production directed against PSTI was more prevalent than that against CA-II and LF, and this immunoreactivity was predominantly directed to the active site of PSTI. These findings suggest that the autoimmune response to PSTI protein accelerates AIP disease progression through the inhibition of PSTI activity.

REFERENCES

1. Steer ML, Waxman I, Freedman S. Chronic pancreatitis. *N Eng J Med.* 1995;332:1482–1490.
2. Sarles H, Sarles JC, Muratore R, et al. Chronic inflammatory sclerosis of the pancreas—an autonomous pancreatic disease? *Am J Dig Dis.* 1961;6:688–698.
3. Okazaki K, Uchida K, Matsushita M, et al. Autoimmune pancreatitis. *Intern Med.* 2005;44:1215–1223.
4. Yoshida K, Toki F, Takeuchi T, et al. Chronic pancreatitis caused by an autoimmune abnormality. Proposal of the concept of autoimmune pancreatitis. *Dig Dis Sci.* 1995;40:1561–1568.
5. Okazaki K, Chiba T. Autoimmune related pancreatitis. *Gut.* 2005;40:1–4.
6. Chari ST, Smyrk TC, Levy MJ, et al. Diagnosis of autoimmune pancreatitis: the Mayo Clinic experience. *Clin Gastroenterol Hepatol.* 2006;4:1010–1016.
7. Okazaki K, Kawa S, Kamisawa T, et al. Clinical diagnostic criteria of autoimmune pancreatitis: revised proposal. *J Gastroenterol.* 2006; 41:626–631.
8. Kim MH, Kwon S. Diagnostic criteria for autoimmune chronic pancreatitis. *J Gastroenterol.* 2007;42(suppl 18):42–49.
9. Klöppel G, Sipos B, Zamboni G, et al. Autoimmune pancreatitis: histo- and immunopathological features. *J Gastroenterol.* 2007;42 (suppl 18):28–31.
10. Uchida K, Okazaki K, Konishi Y, et al. Clinical analysis of autoimmune-related pancreatitis. *Am J Gastroenterol.* 2000;95: 2788–2794.
11. Okazaki K, Uchida K, Chiba T. Recent concept of autoimmune-related pancreatitis. *J Gastroenterol.* 2001;36:293–302.
12. Uchida K, Okazaki K, Nishi T, et al. Experimental immune-mediated pancreatitis in neonatally thymectomized mice immunized with carbonic anhydralse II and lactoferrin. *Lab Invest.* 2002;82:411–424.
13. Nishimori I, Miyaji E, Morimoto K, et al. Serum antibodies to carbonic anhydrase IV in patients with autoimmune pancreatitis. *Gut.* 2005; 54:274–281.
14. Asada M, Nishio A, Uchida K, et al. Identification of a novel autoantibody against pancreatic secretory trypsin inhibitor in patients with autoimmune pancreatitis. *Pancreas.* 2006;33:20–26.
15. Kanno H, Nose M, Itoh J, et al. Spontaneous development of pancreatitis in the MRL/Mp strain of mice in autoimmune mechanism. *Clin Exp Immunol.* 1992;89:68–73.
16. Hosaka N, Nose M, Kyogoku M, et al. Thymus transplantation, a critical factor for correction of autoimmune disease in aging MRL/+ mice. *Proc Natl Acad Sci U S A.* 1996;93:8558–8562.
17. Tsubata R, Tsubata T, Hiai H, et al. Autoimmune disease of exocrine organs in immunodeficient alymphoplasia mice: a spontaneous model of Sjögren's syndrome. *Eur J Immunol.* 1996;26:2742–2748.
18. Valance BA, Hewlett BR, Snider DP, et al. T cell-mediated exocrine pancreatic damage in major histocompatibility complex class II-deficient mice. *Gastroenterology.* 1998;115:978–987.
19. Qu WM, Miyazaki T, Terada M, et al. A novel autoimmune pancreatitis model in MRL mice treated with polyinosinic: polycytidylic acid. *Clin Exp Immunol.* 2002;129:27–34.
20. Davidson TS, Longnecker DS, Hickey WF. An experimental model of autoimmune pancreatitis in the rat. *Am J Pathol.* 2005;166:729–736.
21. Meagher C, Tang Q, Fife BT, et al. Spontaneous development of a pancreatic exocrine disease in CD28-deficient NOD mice. *J Immunol.* 2008;180:7793–7803.
22. Nahon UK, Levy P, O'Toole D, et al. Is idiopathic chronic pancreatitis an autoimmune disease? *Clin Gastroenterol Hepatol.* 2005;3:903–909.
23. Steinberg AD, Baron S, Talal N. The pathogenesis of autoimmunity in New Zealand mice, I. Induction of antinucleic acid antibodies by polyinosinic-polycytidylic acid. *Proc Natl Acad Sci U S A.* 1969;63: 1102–1107.

24. Kobayashi Y, Murakami H, Akbar SMF, et al. A novel and effective approach of developing aggressive experimental autoimmune gastritis in neonatal thymectomized BALB/c mouse by polyinosinic: polycytidylic acid. *Clin Exp Immunol*. 2004;136:423–431.
25. Hamano H, Kawa S, Horiuchi A, et al. High serum IgG4 concentrations in patients with sclerosing pancreatitis. *N Eng J Med*. 2001;344: 732–738.
26. Aparisi L, Farre A, Gomez-Cambronero L, et al. Antibodies to carbonic anhydrase and IgG4 levels in idiopathic chronic pancreatitis: relevance for diagnosis of autoimmune pancreatitis. *Gut*. 2005;54: 703–709.
27. Yamamoto T, Nakamura Y, Nishide J, et al. Molecular cloning and nucleotide sequence of human pancreatic trypsin inhibitor (PSTI) cDNA. *Biochem Biophys Res Commun*. 1985;132:605–612.
28. Bartelt DC, Shapanka R, Greene LJ. The primary structure of the human pancreatic secretory trypsin inhibitor. Amino acid sequence of the reduced *S*-aminoethylated protein. *Arch Biochem Biophys*. 1977; 179:189–199.
29. Ohlsson K, Olsson R, Björk P, et al. Local administration of human pancreatic secretory inhibitor prevents the development of experimental acute pancreatitis in rats and dogs. *Scand J Gastroenterol*. 1989;24: 693–704.
30. Funakoshi A, Miyasaka K, Jimi A, et al. Protective effect of human pancreatic secretory trypsin inhibitor on cerulein-induced acute pancreatitis in rats. *Digestion*. 1992;52:145–151.
31. Chen YZ, Ikei S, Yamaguchi Y, et al. The protective effects of long-acting recombinant human trypsin inhibitor on cerulein-induced pancreatitis. *J Int Med Res*. 1996;24:59–68.
32. Nathan JD, Romac J, Peng RY, et al. Transgenic expression of pancreatic secretory trypsin inhibitor-I ameliorates secretagogue-induced acute pancreatitis in mice. *Gastroenterology*. 2005;128:717–727.
33. Böldicke T, Kindt S, Maywald F, et al. Production of specific monoclonal antibodies against the active sites of human pancreatic secretory trypsin inhibitor variants by in vitro immunization with synthetic peptides. *Eur J Biochem*. 1988;175:259–264.
34. Witt H, Luck W, Hennies HC, et al. Mutations in the gene encoding the serine protease inhibitor, Kazal type 1 are associated with chronic pancreatitis. *Nat Genet*. 2000;25:213–216.
35. Pfutzer RH, Barmada MM, Brunskill AP, et al. SPINK/PSTI polymorphisms act as disease modifiers in familial and idiopathic chronic pancreatitis. *Gastroenterology*. 2000;119:615–623.
36. Okazaki K, Uchida K, Ohana M, et al. Autoimmune-related pancreatitis is associated with autoantibodies and a Th1/Th2-type cellular immune response. *Gastroenterology*. 2000;118:573–581.

J Gastroenterol
DOI 10.1007/s00535-010-0361-y

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Comparison of steroid pulse therapy and conventional oral steroid therapy as initial treatment for autoimmune pancreatitis

Takashi Tomiyama · Kazushige Uchida · Mitsunobu Matsushita · Tsukasa Ikeura · Toshiro Fukui · Makoto Takaoka · Akiyoshi Nishio · Kazuichi Okazaki

Received: 2 June 2010 / Accepted: 2 December 2010

Abstract
Background The efficacy of oral steroid therapy for autoimmune pancreatitis (AIP) is well known, and oral prednisolone treatment is most usually commenced at 30–40 mg/day, but there have been few reports about comparative studies of oral steroid therapy and steroid pulse therapy as the initial treatment for AIP. We studied the clinical course and image findings to estimate the utility of steroid pulse therapy for AIP, comparing it with oral steroid therapy.
Methods Laboratory and image findings were assessed retrospectively in 11 patients who received steroid pulse therapy, and the findings were compared to those in 10 patients who received conventional oral steroid therapy.
Results Change in pancreatic size showed no significant difference between the therapies after 2 weeks of treatment. Significant improvement of lower bile duct strictures after 2 weeks of treatment and that of immunoglobulin values within 6 months were shown with both therapies. However, steroid pulse therapy showed significant improvement of γ-guanosine triphosphate (GTP) in 2 weeks and of alanine aminotransferase (ALT) in 2 and 8 weeks, compared with oral steroid therapy. Moreover, there was one patient in whom the lower bile duct stricture was not improved by oral steroid therapy, but it did show improvement with steroid pulse therapy.
Conclusions Initial steroid pulse therapy is a beneficial alternative to oral steroid therapy for the improvement of bile duct lesions. In future, the accumulation of a larger number of patients receiving steroid pulse therapy is needed, and prospective studies will be required.

Keywords Autoimmune pancreatitis (AIP) · Steroid pulse therapy · Bile duct stricture · Diabetes mellitus · Pancreatic cancer

Introduction

Sarles et al. [1] reported a case of chronic pancreatitis with hypergammaglobulinemia, but the clinical entity was not confirmed thereafter. Autoimmune pancreatitis (AIP), which was first proposed as a clinical entity by Yoshida et al. [2] from Japan, is now generally accepted as a distinctive type of pancreatitis [1]. AIP is characterized by diffuse irregular narrowing of the main pancreatic duct, sausage-like diffuse swelling of the pancreas, high serum levels of IgG or IgG4, and steroid responsiveness [3–6]. Since the fibroinflammatory process of AIP responds well to steroids, autoimmune mechanisms are thought to be involved in the development of AIP. A recent large Japanese study of AIP and guidelines for treatment recommend standard oral steroid therapy with an initial dose of 0.5–0.6 mg/kg/day [7, 8].

While steroid responsiveness as a diagnostic component is not included in the revised Japanese criteria, it is included in the Korean criteria, Mayo Clinic HISORt (Histology, Imaging, Serology, Other organ involvement, and Response to steroids) criteria, and recently proposed Asian criteria [8–11]. The most important issue in AIP management is making the diagnosis of AIP, especially the

T. Tomiyama · K. Uchida · M. Matsushita · T. Ikeura · T. Fukui · M. Takaoka · A. Nishio · K. Okazaki (✉)
Division of Gastroenterology and Hepatology, the Third Department of Internal Medicine, Kansai Medical University, 10-15 Fumizono, Moriguchi, Osaka 570-8507, Japan
e-mail: okazaki@hirakata.kmu.ac.jp

Published online: 25 December 2010

mass-forming type, differentiating it from pancreatic or biliary cancers [12–15], although some cases of pancreatic cancer are accompanied by AIP [16–19]. In some tumor-forming AIP cases, the efficacy of a steroid trial has been reported as useful in diagnosing AIP by criteria other than the Japanese diagnostic criteria [20]. Moreover, Korean investigators have reported the usefulness of a 2 weeks' trial of oral steroids in differentiating AIP from malignancy, with continuing administration if AIP is diagnosed [20]. However, it has not yet been established whether or not withdrawal of steroids in reconsidering malignancy presents a risk of postoperative adrenal insufficiency [21–23]. Therefore, it is desirable to have an alternative to the discontinuation of steroid administration immediately after surgery.

Steroid pulse therapy is widely used to initiate treatment in patients with rapidly progressive and immunologically mediated disorders such as acute graft rejection, Graves ophthalmopathy, pemphigus, and severe systemic lupus erythematosus [24–27]. Moreover, high doses of systemic steroid can be given with comparative safety within a period of 1 week [28]. We therefore evaluated the efficacy of short-term steroid pulse therapy, in comparison with oral steroid therapy, in patients with AIP.

Methods

Patients and treatment

For this study, we retrospectively examined the records of all 21 AIP patients treated in our hospitals from November 2004 to May 2009. All patients were diagnosed with AIP according to the clinical diagnostic criteria for AIP proposed by the Research Committee of Intractable Diseases of the Pancreas supported by the Japanese Ministry of Health, Labor, and Welfare, and the Japan Pancreas Society. Following diagnosis, 20 patients with AIP were randomly distributed to two treatment groups by their attending physicians. One patient (case 10) was referred to our hospital after the withdrawal of oral steroid for AIP because his bile duct lesion had not responded to the treatment. Eleven patients (cases 1–11; 5 male and 6 female; aged 47–80 years, with a mean age of 66 years, named the "pulse group") (Table 1) received steroid pulse therapy, and ten patients (cases 12–21; 8 male and 2 female, aged 49–72 years, with a mean age of 69 years, named the "oral group") (Table 2) received oral steroid therapy. For the pulse group, the initial dose of methylprednisolone was 500 mg/day for 3 days each week as 1 course, and we treated them with 2 weekly courses. Then oral prednisolone at 20 mg/day was prescribed as maintenance therapy and the dose was tapered off. For the oral group, ten patients commenced oral prednisolone at 30–40 mg/day. Two weeks after the start of the treatment, oral prednisolone at 20 mg/day was prescribed, and the dose was tapered off. This study was approved by the Kansai Medical University ethics committee.

Serological study

We analyzed immunological findings for the following: IgG, IgG4, antinuclear antibodies (ANA), rheumatoid factor (RF), antimitochondrial antibodies (AMA), myeloperoxidase-antineutrophil cytoplasmic antibodies (MPO-ANCA), anti-Sjögren's syndrome A antibodies (SS-A), anti-Sjögren's syndrome B antibodies (SS-B), anti-thyroid peroxidase antibodies (TPOAb), and anti-thyroglobulin antibodies (TgAb). To compare liver and endocrine function in both groups, we evaluated the serum levels of γ-guanosine triphosphate (GTP) and alanine aminotransferase (ALT) on day 0 (data just before the treatment), and at weeks 2 and 8 after therapy, and checked glycosylated hemoglobin values (HbA1c) at months 1, 3, and 7 after therapy, which closely reflected glucose tolerance at months 0, 2, and 6, respectively. In each evaluation, patients who did not show abnormal values during the clinical course were excluded in order to evaluate the therapeutic effect strictly.

Radiological study

All the patients were examined by contrast-enhanced helical computed tomography (CT), magnetic resonance imaging (MRI), and endoscopic retrograde cholangiopancreatography (ERCP) and underwent liver function tests, combined with bile duct drainage and pathological tests as necessary.

For morphological changes, CT, MRI, and ERCP were studied. The width of the pancreas along its longest axis was measured on CT or MRI images and compared with the transverse diameter of the vertebral body according to the method of Heuck et al. [29]. The pancreas size on the first image was defined as 100%.

Cases showing lower bile duct stricture were classified as follows: 0 = absent, 1 = <0–25%, 2 = <25–50%, 3 = <50–75%, and 4 = <75–100%, according to the method of Craig et al. [30]. Using the method described above, pancreas size was evaluated after 2 weeks on steroids, and stricture of the distal third of the common bile duct was measured after 2 weeks and after 8 weeks.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test, Wilcoxon signed-ranks test, paired *t*-test, and

Table 1 Background of AIP patients who received steroid pulse therapy

Patient ID	Age (years)/ sex	Symptoms	IgG <1,700 (mg/dl)	IgG 4 <135 (mg/dl)	Amy <130 (IU/L)	T-Bil <0.9 (mg/dl)	ALT <30 (IU/L)	γ-GTP <35 (IU/L)	ANA	PFD <73.4 (%)	DM	Extrapancreatic lesion	Stenosis on ERCP	Morphological change of the pancreas
1	80/F	Jaundice	2,604	1,230	52	0.9	43	260	−	52.1	−	Sialoadenitis	Head, CBD	FS in head
2	63/M	Epigastralgia	1,714	354	66	0.9	56	222	−	29.7	−	Warthin tumor	Head, CBD	DS
3	54/F	Epigastralgia	1,828	324	561	1.0	100	403	−	NT	−	Hypothyroidism	Body, CBD	DS
4	71/F	None	1,916	295	78	0.6	60	101	−	97.9	−	Sialoadenitis, mediastinum LNS	Head	FS in head
5	66/F	Nausea	1,535	235	77	2.2	118	1,311	−	NT	−	Hypothyroidism, retroperitoneal fibrosis	Head to tail, CBD	FS in head
6	66/M	Epigastralgia, jaundice	2,695	1,790	164	12.5	98	137	−	58.1	+	None	Body, CBD	DS
7	47/F	Jaundice	2,453	629	15	1.0	190	65	−	NT	−	None	Head, body to tail, CBD	DS
8	72/M	Epigastralgia	1,692	452	66	1.3	721	1,352	+	30.9	+	None	Head to tail CBD	FS in head
9	72/M	Epigastralgia, jaundice	1,513	411	32	14.1	114	352	−	NT	+	None	Head, CBD	DS
10	63/M	Malaise	1,514	394	76	0.5	20	82	−	NT	−	None	Head to tail, CBD	FS in head
11	73/F	Epigastralgia	1,598	373	55	0.6	33	588	−	59.7	+	None	Head to tail, CBD	FS in head

AIP autoimmune pancreatitis, *T Bil* total bilirubin, *ALT* alanine aminotransferase, *ID* identification, *γ-GTP* γ-guanosine triphosphate, *Amy* amylase, *ANA* antinuclear antibody, *PFD* pancreatic functional diagnostic test, *DM* diabetes mellitus, *ERCP* endoscopic retrograde cholangiopancreatography, *CBD* common bile duct, *FS* focal swelling, *DS* diffuse swelling, *NT* not tested, *LNS* lymph node swelling

Table 2 Background of AIP patients treated with oral prednisolone

Patient ID	Age (years)/sex	Symptoms	IgG <1,700 (mg/dl)	IgG4 <135 (mg/dl)	Amy <130 (IU/L)	T-Bil <0.9 (mg/dl)	ALT <30 (IU/L)	γ-GTP <35 (IU/L)	ANA	PFD <73.4 (%)	DM	Extrapancreatic lesion	Stenosis on ERCP	Morphological change of the pancreas
12	71/M	Jaundice	3,274	1,870	58	1.0	24	76	−	32.7	−	Mediastinum LNS	Head to tail, CBD	DS
13	66/M	Thirst	4,060	1,170	64	0.4	18	116	−	65.8	−	Sialoadenitis, mediastinum LNS	Tail, CBD	DS
14	58/F	Epigastralgia	2,754	1,110	95	0.9	13	10	−	NT	−	Thyroiditis, mediastinum LNS	Body to tail	FS in head
15	52/F	None	2,190	661	435	0.6	15	15	−	69.7	−	Interstitial pneumonia, Mikulicz tumor	Tail	FS in tail
16	68/M	Fever	1,622	407	34	0.6	30	30	−	73.4	−	Thyroiditis	Head, body, CBD	DS
17	72/M	Vomiting	2,010	773	51	0.3	34	67	+	NT	−	None	Head	FS in head
18	55/M	Epigastralgia	1,461	659	53	0.6	178	240	−	17.9	+	None	Head to tail, CBD	DS
19	63/M	Jaundice	2,073	487	41	1.1	52	149	−	NT	+	None	Head, CBD	FS in head
20	49/M	Jaundice	2,065	479	62	0.9	52	503	−	35.7	−	None	Body, tail, CBD	DS
21	66/M	Malaise	1,607	200	47	1.0	41	189	−	77.9	+	None	Head, CBD	FS in head

Amy amylase, *ANA* antinuclear antibody, *PFD* pancreatic functional diagnostic test, *DM* diabetes mellitus, *ERCP* endoscopic retrograde cholangiopancreatography, *LNS* lymph node swelling, *CBD* common bile duct, *DS* diffuse swelling, *NT* not tested, *FS* focal swelling