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.

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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

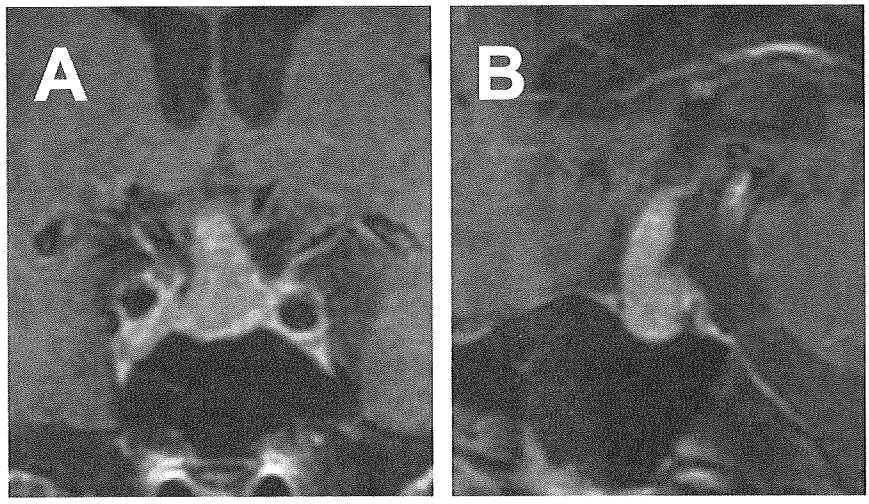

Fig. 1. MR imaging of the pituitary in a patient with IgG4-related infundibulo-hypophysitis [33]. T1-weighted gadolinium enhanced imaging (A: coronal section, B: saggital sections)

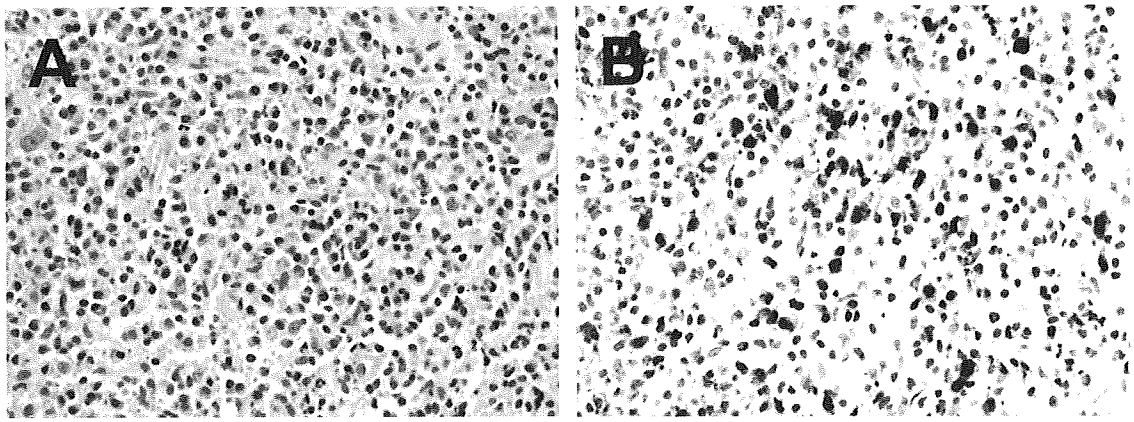

Fig. 2. Histopathology of the pituitary gland in a patient with IgG4-related infundibulo-hypophysitis [33]. A: HE staining, B: IgG4 immunostaining.

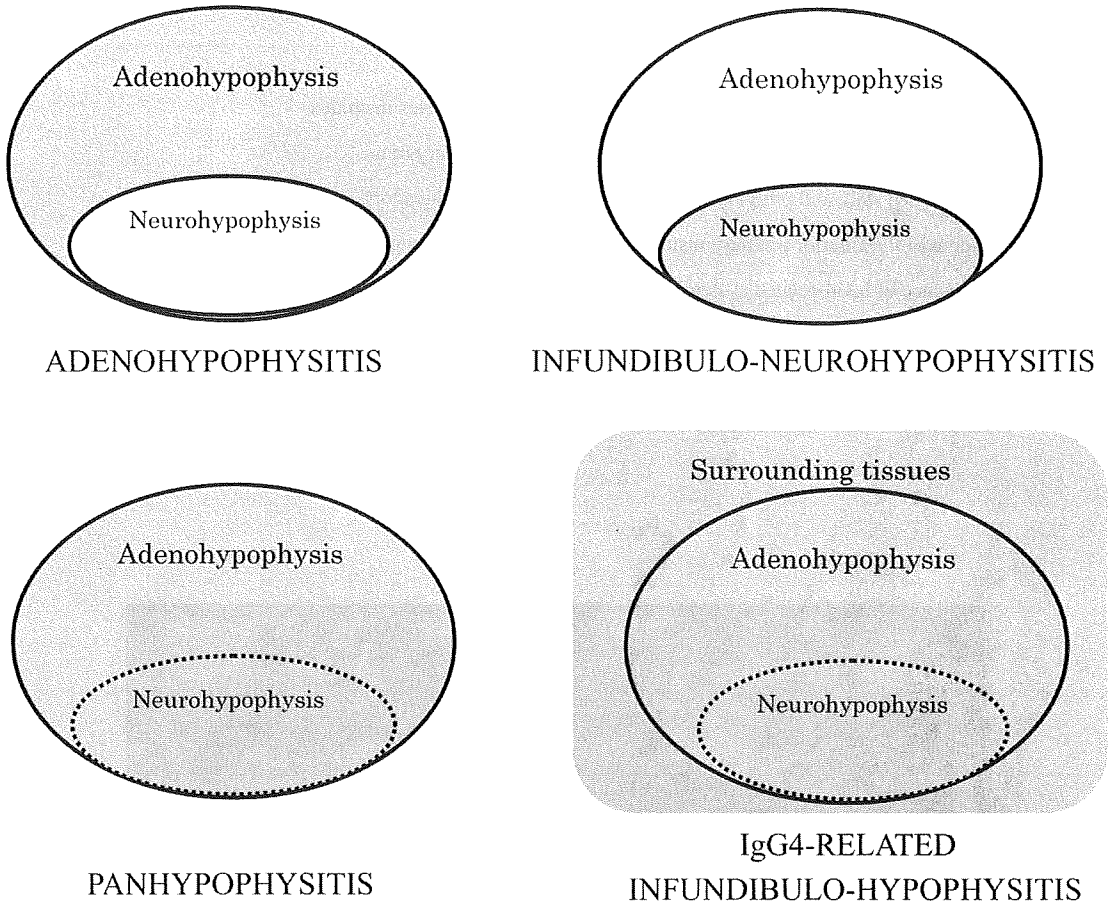

Fig. 3. Involvement of pituitary gland with hypophysitis (conceptual figures).
Shaded area represents the involved tissues.

Possible involvement of T helper type 2 responses to Toll-like receptor ligands in IgG4-related sclerosing disease

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ABSTRACT

We report a case of immunoglobulin G4 (IgG4)-related sclerosing disease involving the pancreas, liver and salivary glands. Massive infiltration of IgG4-expressing plasma cells was seen in the liver and submandibular lymph nodes. Interestingly, accumulation of IgG4-expressing plasma cells was also seen in the colon and terminal ileum. Peripheral blood mononuclear cells (PBMCs) isolated from this patient exhibited enhanced production of IgG4 and interleukin-10 upon stimulation with Toll-like receptor (TLR) ligands as compared with those from a healthy control. In contrast, production of tumour necrosis factor α and interferon γ by PBMCs from this patient was markedly reduced. Since colonic mucosa is always exposed to TLR ligands derived from commensal organisms, the results of immunological studies suggest that enhanced T helper type 2 responses to intestinal microflora may underlie the immunopathogenesis in this patient with IgG4-related sclerosing disease.

INTRODUCTION

Autoimmune pancreatitis (AIP) is an inflammatory disorder which is characterised by increased serum levels of immunoglobulin G4 (IgG4) or by an IgG4-positive plasmacytic infiltrate into the inflamed tissue.[1] Another important feature of AIP is a wide variety of extrapancreatic manifestations such as sialadenitis, cholangitis, retroperitoneal fibrosis and inflammatory pseudotumour of the liver and lung.[2] Since these extrapancreatic and pancreatic lesions share common histopathological findings (ie, abundant infiltration by $IgG4^+$ plasmacytes), Kamisawa *et al* proposed a new clinicopathological entity: 'IgG4-related sclerosing disease'.[2] However, little is understood regarding the role played by this IgG subtype in the inflammatory process. In this regard, IgG4 itself does not seem to be responsible for the development of tissue damage since this IgG subtype does not cause cell-mediated lysis due to poor binding activity to complement.[3] In addition, anti-inflammatory activity of IgG4 has been shown.[4] Consistent with these biological functions of IgG4, clinical manifestations of immune complex disease such as arthritis and glomerulonephritis are rarely seen in patients with IgG4-related sclerosing disease.[5] These facts suggest that abnormal immunological environments leading to enhanced IgG4 responses, rather than IgG4 antibody itself, underlie the pathogenesis of this disease.

Distribution of IgG4-expressing plasmacytes in the gastrointestinal tract of patients with AIP has been observed.[6 7] However, it is unknown whether this distribution of $IgG4^+$ cells is directly induced by immune reactions occurring in the gastrointestinal tract or is indirectly induced by systemic IgG4 responses. Given the fact that mucosa of the gastrointestinal tract is always exposed to antigens derived from intestinal microflora, it is tempting to speculate that immune responses against microbial antigens create abnormal environments leading to enhanced IgG4 responses in the gut. Indeed, we experienced a case of IgG4-related sclerosing disease in which accumulation of IgG4-expressing plasmacytes was visualised as colonic inflammatory pseudopolyps.[8] Here we report a case with IgG4-related sclerosing disease whose ileal and colonic mucosa bore a marked infiltration of IgG4-expressing plasma cells. Interestingly, peripheral blood mononuclear cells (PBMCs) isolated from this case show enhanced T helper type 2 (Th2) and IgG4 responses upon stimulation with Toll-like receptor (TLR) ligands. These results indicate possible involvement of excessive Th2 responses against intestinal microflora in some cases with IgG4-related sclerosing disease.

CASE REPORT

A 70-year-old asymptomatic man was admitted for further investigation of swelling of the pancreas and submandibular lymph nodes. He had a history of systemic lymphadenopathy of unknown aetiology at the age of 45. Laboratory tests revealed elevation of serum levels of amylase (229 IU/l; normal range <129 IU/l) and IgG (2144 mg/dl; normal range <1840 mg/dl). Abdominal CT using contrast reagent showed focal swelling of the pancreatic head without an enhancement effect. Endoscopic retrograde cholangiopancreatography revealed irregular narrowing of the main pancreatic duct and the stricture of the lower bile duct. These radiographic findings were consistent with those of AIP.[1] A hypoechoic tumour was detected in the lateral segment of the liver on abdominal ultrasonography. Since a marked elevation of serum IgG4 level was detected (918 mg/dl; normal range <135 mg/dl), this patient was strongly suspected to have IgG4-related scrclosing disease involving the pancreas, bile duct and liver. Biopsy of the liver tumour revealed massive infiltration of plasmacytes expressing IgG and IgG4 around the bile duct (figure 1). More than 50% of IgG-expressing cells

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were positive for IgG4 staining, which suggests that this liver tumour was a pseudotumour due to IgG4-associated cholangitis. Similar histological findings were obtained in the immunohistochemical analyses using biopsy specimens from submandibular lymph nodes (figure 1). Based on these results, this patient was finally diagnosed as having IgG4-related sclerosing disease.

Colonoscopy was performed to exclude the involvement of the gastrointestinal tract before starting prednisolone treatment. Although no inflammatory mucosa was seen from the terminal ileum to the rectum on colonoscopic examination, biopsy specimens taken from the intact mucosa of the terminal ileum and colon revealed a marked infiltration of plasmacytes expressing IgG into the submucosa without destruction of crypt architecture or fibrosis (figure 1). Interestingly, >50% of IgG-expressing cells were positive for IgG4 staining. Accumulation of IgG4-expressing plasma cells in the colonic mucosa led us to hypothesise that abnormal immunological responses to gut microbial antigens might underlie the development of enhanced IgG4 responses. PBMCs from this case and healthy controls were stimulated with TLR ligands to see immune responses against antigens derived from intestinal microflora.[9] Ethical permission for this study was granted by the review board of Kyoto University. As shown in figure 2, production of IgG4 as well as interleukin-10 (IL-10) was enhanced upon stimulation with TLR4 (lipopolysaccharide (LPS)) and TLR5 (flagellin) ligands. Production of IgG4 was also enhanced by stimulation of a TLR2 ligand (peptidoglycan (PGN)). In contrast, production of Th1 cytokines (interferon γ (IFNγ) and tumour necrosis factor α (TNFα)) in response to TLR ligands by the patient's PBMCs was impaired as compared with that by control PBMCs. No difference was seen in the production of IL-8 or IL-17 upon stimulation with TLR ligands or TNFα. These data suggest that activation of TLRs generates both IgG4 and Th2 responses in PBMCs from this case since IFNγ and IL-10 are prototypical Th1 and Th2 cytokines, respectively.[9] We determined the type of immune cells producing these cytokines by cell depletion study

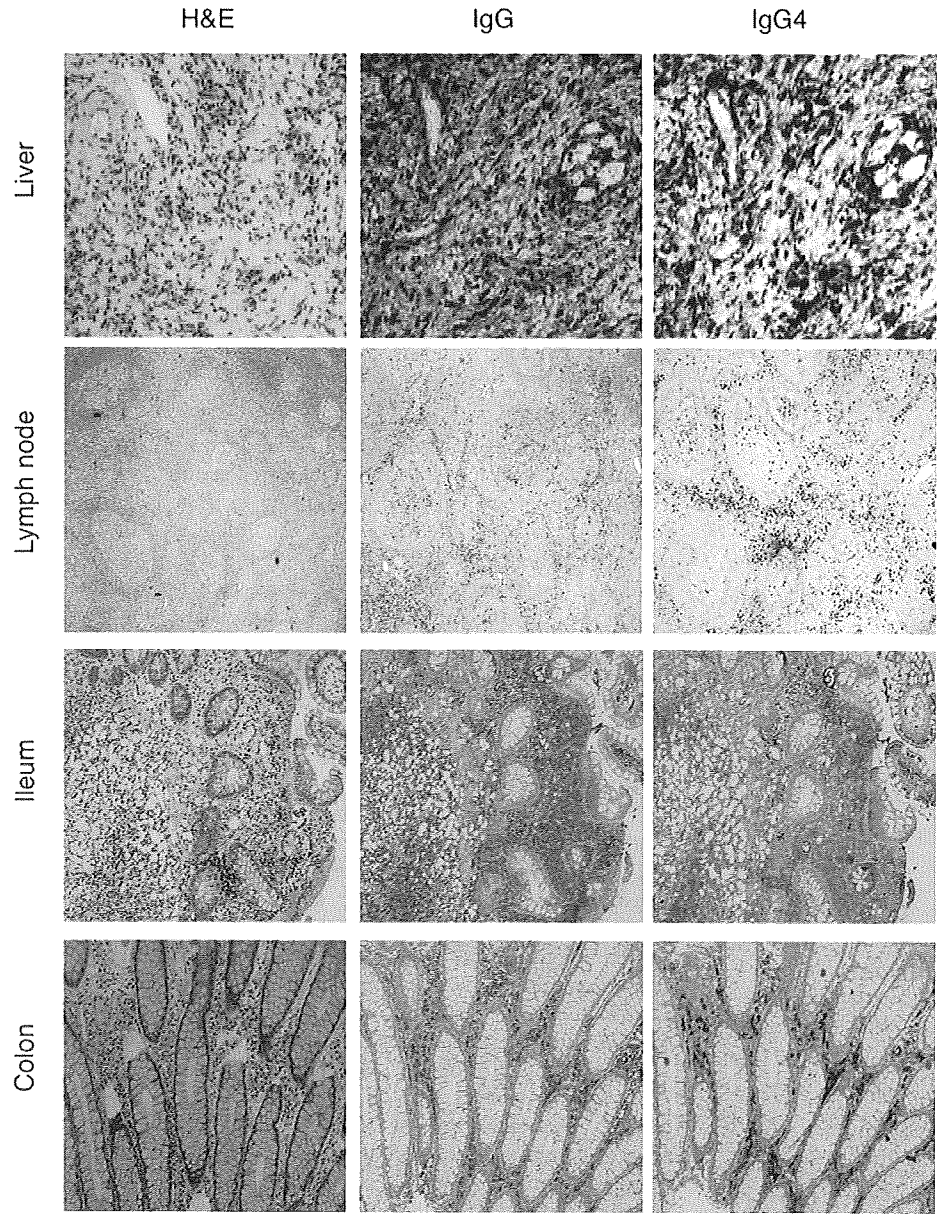

Figure 1 Immunohistochemical staining of immunoglobulin G4 (IgG4) and IgG. Biopsy specimens obtained from the liver, submandibular lymph nodes, terminal ileum and colon were stained with anti-IgG4 or anti-IgG antibody for visualisation of plasma cells expressing IgG4 or IgG.

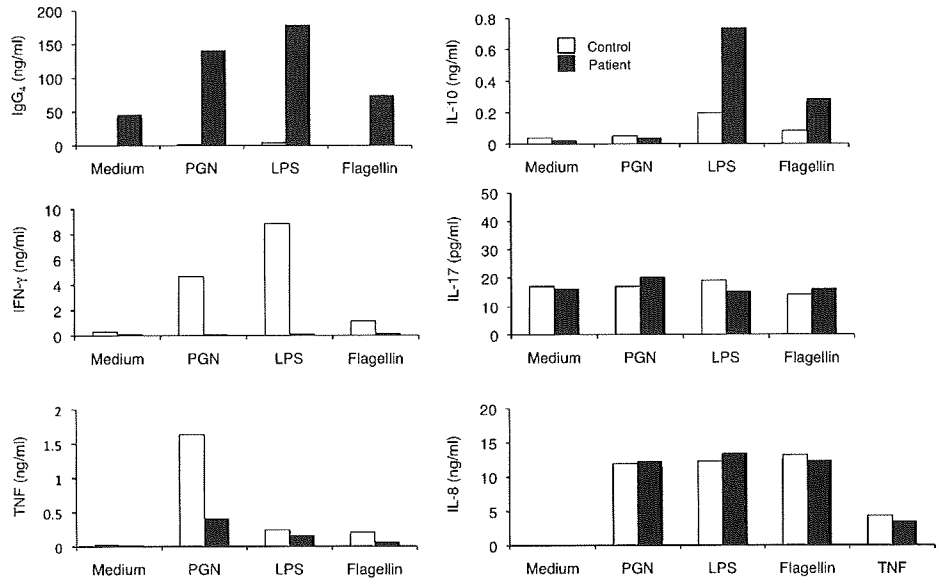

Figure 2 Enhanced T helper type 2 (Th2) responses to Toll-like receptor (TLR) ligands by peripheral blood mononuclear cells (PBMCs) isolated from the patient. PBMCs (2×10^6/ml) isolated from the patient and healthy controls were stimulated with peptidoglycan (PGN, 10 μg/ml), lipopolysaccharide (LPS, 1 μg/ml), flagellin (1 μg/ml) or tumour necrosis factor (TNF, 20 ng/ml). PBMCs were cultured for 48 h for interleukin-8 (IL-8) and TNF assay, and for 14 days for IgG4, interferon γ (IFNγ), IL-10 and IL-17 assay. Results shown are means of triplicate wells.

using control samples. We found that $CD3^+$ T cells produced IFNγ and IL-10 whereas $CD14^+$ monocytes produced IL-10 and TNFα (data not shown).

DISCUSSION

An interesting observation in this case with IgG4-related sclerosing disease was a marked infiltration of IgG4-expressing plasmacytes into the colonic mucosa which appeared to be normal on endoscopic examination. It remains unclear whether we can regard this case as IgG4-related sclerosing disease involving the colonic mucosa since no pathological findings were present in colonic biopsy specimens other than marked infiltration of $IgG4^+$ cells. Thus, unlike our previous case in which infiltration of IgG4-expressing plasmacytes was visualised as colonic polypoidal lesions,[8] we have to be cautious in the interpretation of infiltration of IgG4-expressing plasma cells into endoscopically normal colonic mucosa in the setting of IgG4-related sclerosing disease.

Immune responses leading to accumulation of IgG4-expressing plasmacytes in the gastrointestinal tract are poorly understood. PBMCs isolated from this case exhibited enhanced production of IgG4 and Th2 cytokines upon stimulation with TLR ligands, suggesting that enhanced immune reactions against microbial antigens cause infiltration of lymphocytes as in the case of inflammatory bowel disease (IBD).[10] In fact, this idea is supported by clinical evidence that a significant population of patients with AIP have a diagnosis of IBD.[11] Importantly, IgG4 responses induced by TLR activation are associated with enhanced IL-10 production. In this regard, two different groups report involvement of regulatory T cells (Tregs) producing IL-10 in IgG4-related sclerosing disease.[12 13] Thus, enhanced IL-10 production seen in this case may be partially mediated by circulating Tregs. Given the fact that IL-10 is an important cytokine for IgG4 class switching,[14] we assume that excessive Th2 responses triggered by TLR ligands together with activation of Tregs create abnormal immunological environments leading to enhanced IgG4 responses. This idea partially explains immunological mechanisms of enhanced Th2 responses in patients with IgG4-related sclerosing disease.[12]

Although storiform fibrosis is a characteristic pathological finding of IgG4-related sclerosing disease,[2] molecular mechanisms of fibrosis in this disorder are poorly understood. Th2 cytokines mediated by activation of TLRs on macrophages have been identified as positive regulators of tissue fibrosis in the liver and lung.[15] Thus, enhanced Th2 responses to TLR ligands might be involved in the development of storiform fibrosis in IgG4-related sclerosing disease. However, analysis of expression of both Th2 cytokines and TLRs using fibrotic tissue samples is necessary to address this issue.

What is the mechanism by which enhanced Th2 responses against intestinal microflora cause IgG4-related sclerosing disease without the development of colitis? In this regard, immune reactions causing tissue injury and those causing IgG4 responses should be considered separately as shown by the fact that IgG4 antibody itself has anti-inflammatory properties.[4] Indeed, tissue destruction was not seen in the lower gastrointestinal tract of this case despite a marked infiltration of IgG4-expressing plasmacytes into the submucosa. Several mechanisms for preventing hyper-responsiveness to microbial antigens operate in the gut. For example, the intestine is the preferential site where naïve T cells differentiate into Tregs.[10] Thus, one possible explanation is that pathogenic immune reactions causing tissue injury are suppressed in the gut by regulatory mechanisms, whereas such reactions cause tissue injury in other sterile organs such as the pancreas and bile duct due to the lack of regulatory mechanisms. Based on this, it is tempting to speculate that the gastrointestinal tract is an induction site for systemic IgG4 responses and functions as a reservoir for IgG4-expressing plasmacytes even if disease-associated pathogenic changes are absent. Alternatively, distribution of IgG4-expressing plasmacytes into the colonic mucosa may be an epiphenomenon associated with systemic IgG4 responses.

In conclusion, the results of immunological studies using PBMCs from this case suggest involvement of excessive Th2 responses to intestinal microflora in the development of IgG4-related sclerosing disease. Confirmation of this idea awaits further studies using a large number of patients with IgG4-related sclerosing disease.

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Competing interests None.

Ethics approval This study was conducted with the approval of the Kyoto University review board.

Patient consent Obtained.

Provenance and peer review Not commissioned; externally peer reviewed.

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CLINICAL STUDIES

Identification and characterization of IgG4-associated autoimmune hepatitis

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Keywords
autoimmune hepatitis – IgG4 – steroid treatment

Abbreviations
AIH, autoimmune hepatitis; AIP, autoimmune pancreatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyl transpeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus; HPF, high-power field; PBC, primary biliary cirrhosis; PSC, primary sclerosing cholangitis; Th1, T helper type 1; Th2, T helper type 2.

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Abstract

Background: Autoimmune hepatitis (AIH) and autoimmune pancreatitis (AIP) share clinical and pathological features such as high serum levels of immunoglobulin (Ig) G and autoantibodies, and lymphoplasmacytic infiltration, suggesting the presence of common immunological abnormalities. However, little is known about the possible involvement of IgG4, a hallmark of AIP, in AIH. *Aims:* In this study, we examined whether the IgG4 response contributes to the histopathological and clinical findings in AIH. *Methods:* Liver sections from 26 patients with AIH, 10 patients with primary biliary cirrhosis (PBC), three patients with primary sclerosing cholangitis (PSC) and 20 chronic hepatitis patients with hepatitis C virus (HCV) infection were immunostained for IgG4. We investigated the relationship among the histopathology, the responses to steroid therapy and the IgG4 staining. *Results:* Nine of the 26 liver specimens from patients with AIH showed positive staining for IgG4 whereas none of the 10 samples from patients with PBC, the three samples from patients with PSC or the 20 samples from patients with HCV hepatitis were positive. Patients with IgG4-positive AIH also showed increased serum levels of IgG. The numbers of T cells, B cells and plasma cells were significantly increased in the livers of patients with IgG4-positive AIH as compared with those patients with IgG4-negative AIH. Patients with IgG4-positive AIH also showed a marked response to prednisolone therapy. *Conclusions:* AIH may be classified into either an IgG4-associated type or an IgG4 non-associated type with the former showing a marked response to prednisolone treatment.

Autoimmune hepatitis (AIH) is an organ-specific disease of the liver that is characterized by hypergammaglobulinaemia, autoantibodies in the serum and by the presence of interface hepatitis and plasma cell infiltration on histological examination (1). The pathogenic mechanisms accounting for the development of AIH are still unknown. Because AIH is associated with certain human leukocyte antigen subtypes (2) and with the presence of various autoantibodies (2, 3), it is plausible to assume that adaptive immune responses mediated by T cells and B cells are involved. This notion is supported by research showing that antibodies to autoantigens such as soluble liver antigen/liver–pancreas antigen and cytochrome P-450 2D6 are involved in the progression of the disease (3). In addition, recent findings suggest that T-cell-mediated immune responses play a major role in the development of AIH. For example, a predominant infiltration of $CD4^+$ and $CD8^+$ T cells is seen in the livers of patients with AIH (4). These liver-infiltrating T cells produce both T-helper type 1 (Th1) and type 2 (Th2) cytokines that mediate liver damage (5). On the other hand, the number of $CD4^+CD25^+$ regulatory T cells that function to suppress the effector Th1 and Th2 responses is decreased in peripheral blood samples taken from patients with AIH (6). Thus, the pathogenic mechanisms of AIH may partially be explained by both an enhanced effector T-helper response and an impaired regulatory T-cell response. In contrast to the role played by T cells, our knowledge regarding the role of B cells and plasma cells infiltrating the AIH lesions is very limited.

Recent studies of the immunopathogenesis of autoimmune pancreatitis (AIP) have shown results that may potentially be applicable to AIH (7). For example, as in the case of AIH, patients with AIP show elevated levels of serum immunoglobulin (Ig) G and autoantibodies (8). Lymphoplasmacytic infiltration of T cells and plasma cells is seen in the pancreas of patients with AIP, findings that are similar to those of the liver of patients with AIH (9). Furthermore, both AIP and AIH respond well to steroid therapy (1, 10). Thus, AIH and AIP appear to share clinical and histological features. It is now generally accepted that patients with AIP have elevated levels of serum IgG4 (11) and that plasma cells expressing IgG4 are abundantly seen not only in the pancreas but also in the other involved organs (12). More importantly, the presence of IgG4-expressing plasma cells in the liver has now been convincingly linked to cholangitis and hepatitis (13–15). In this respect, IgG4 itself does not seem to be responsible for the development of liver damage because this IgG subtype does not cause cell-mediated lysis owing to poor binding activity to complement (16). It is possible, however, that abnormal immunological environments leading to enhanced IgG4 responses, rather than IgG4 itself, underlie the pathogenesis of the liver damage seen in AIH. Given the similar clinical and pathological features between AIH and AIP, we asked the question as to whether these two autoimmune disorders share a common pathophysiology. To address this, we examined the IgG4 expression in the livers of patients with AIH. Our results identify a subtype of AIH that is characterized by the infiltration of IgG4-expressing plasma cells and by a marked response to steroid therapy.

Methods

Patients

Twenty-six AIH patients who met the international criteria for the diagnosis of AIH (17) were enrolled in this study from October 2002 to May 2007. All patients were admitted to Kinki University Hospital or two affiliated hospitals. Ten primary biliary cirrhosis (PBC) patients, three primary sclerosing cholangitis (PSC) patients and 20 chronic hepatitis patients with hepatitis C virus (HCV) infection were also studied. The diagnosis of PBC was made based on established criteria [i.e. at least three of the following: alkaline phosphatase (ALP)-γ-glutamyl transpeptidase (γ-GTP) above the upper limit of normal; antimitochondrial antibodies positive at a titre of 1:20; increased serum levels of IgM; the absence of biliary obstruction as assessed by ultrasonography, computed tomography or cholangiography; or a compatible liver biopsy] (18). The diagnosis of PSC was made based on the findings of endoscopic retrograde cholangiography and liver biopsy (19). The status of hepatitis B virus (HBV) and HCV infection was determined by HB surface antigen and HCV antibody tests. None of the patients with AIH, PBC or PSC were positive for HBV or HCV infection. Ethical permission for this study was granted by the review board of Kinki University.

Histopathology and immunohistochemistry

Liver specimens were obtained percutaneously with an 18 G needle under ultrasound guidance before starting the treatment. The mean length of the specimen was 1.5 cm and each contained six to 15 portal tracts. Liver tissues were fixed in 10% buffered formalin phosphate and embedded in paraffin, after which 4 μm sections were cut and then stained with haematoxylin and eosin, and elastica van Gieson. The sections were evaluated by experienced pathologists blinded to the laboratory and clinical data. Fibrosis was graded as 0 (absent), 1 (periportal fibrosis), 2 (bridging fibrosis), 3 (bridging fibrosis with lobular distortion) or 4 (cirrhosis). For the histological analysis, the following were categorized as either positive or negative (+, −): canalicular cholestasis, portal inflammation, interface hepatitis, ductular proliferation, chronic non-suppurative destructive cholangitis, bile duct loss, rosette formation and collapse of hepatocytes, i.e. dropout of hepatocytes because of massive necrosis. The following were classed as positive or negative depending on the number of events visible per high-power field (HPF): lobular hepatitis (− = 0–3 focal necrosis/HPF; + = > 3 focal necrosis/HPF), plasma cell infiltration (− = 0–9 cells/HPF; + = > 9 cells/HPF); steatosis [(− = 0–30% cells with fatty change/HPF; + = > 30 cells with fatty change/HPF); and eosinophil infiltration (− = 0–4 cells/HPF, + = > 4 cells/HPF)]. Portal inflammation was quantitatively analysed according to the Ishak scoring system (20). Immunostaining for IgG4 was performed in 26 patients with AIH, 10 patients with PBC, three patients with PSC and 20 patients with chronic HCV infection. Liver biopsy specimens from patients with IgG4-associated cholangitis were also stained with anti-IgG4 as a positive control. After deparaffinization and rehydration, all sections on silane-coated slides were pretreated with proteinase K (Dako, Kyoto, Japan) for 20 min. Endogenous peroxidase was blocked in 1% hydrogen peroxide for 3 min using a microwave oven. After a second blocking step with 2% bovine serum albumin, the sections were incubated with a monoclonal antibody to IgG4 (Zymed Laboratories, San Francisco, CA, USA) for 10 min using a microwave oven. Immunostaining for IgG, IgG1, CD3, CD20 and CD38 was performed on samples from the 26 AIH patients as described previously (21, 22). Briefly, sections were incubated with biotinylated antibodies to IgG, IgG1, CD3, CD20 or CD38. Antibodies to IgG, CD3 and CD20 were purchased from Dako , the antibody to CD38 from Novocastra Laboratories Ltd (Newcastle, UK) and the antibody to IgG1 from The Binding Site (Birmingham, UK). An avidin–biotin technique was used for all the immunostaining experiments, with diaminobenzidine tetrahydrochloride used for visualization and haematoxylin for nuclear counterstaining.

Treatment and follow-up

All AIH patients were initially treated with 30–40 mg/day prednisolone, except for four elderly patients with low-grade activity [as judged by histology and serum alanine aminotransferase (ALT) levels]. None of the patients were treated with azathioprine, 6-mercaptopurine or cyclosporine. Tapering of the prednisolone dose was performed according to an established protocol (1). Serum levels of ALT were monitored every 2 weeks before normalization and every 3 months after normalization. The dose of prednisolone was increased in some patients who showed elevated levels of serum ALT because of the result of a flare-up of their condition. Treatment continued during the observation period and no patients were lost.

Statistical analysis

Fisher's exact test was used to assess the differences in the patient distribution of variables such as gender and concurrent autoimmune diseases. Analysis of variance (ANOVA) was used to compare variables among the three groups. If the ANOVA was significant, the Bonferroni procedure was used for multiple comparisons. Normally distributed variables were compared using Student's *t*-test and non-normally distributed variables were compared using the Mann–Whitney *U*-test. The Wilcoxon signed rank test was used to compare the degree of infiltration by $IgG4^+$ plasma cells. Correlations were expressed by the Spearman rank correlation coefficient. $P < 0.05$ was considered to be statistically significant. All statistical analyses were performed using SPSS software version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Identification of immunoglobulin G4-associated autoimmune hepatitis

All the AIH patients in this study met the international criteria for the diagnosis of definite AIH. All the AIH patients were negative for serum antibodies against liver–kidney microsome 1 and were diagnosed as type I. Patients who did not meet the international criteria were excluded from the study. These data are shown in Table 1. Liver specimens were obtained from the AIH patients before starting steroid therapy.

Immunoglobulin G4 immunostaining of the liver tissues was performed to assess the degree of infiltration by IgG4-producing plasma cells. We counted the number of $IgG4^+$ cells in at least three portal tracts and calculated the average number of $IgG4^+$ cells in each specimen. We regarded the specimens as IgG4 positive when more than five $IgG4^+$ plasma cells were identified per HPF according to the report by Zhang *et al.* (23). As shown in Figure 1A and B, nine of the 26 AIH patients (34.6%) showed positive staining for IgG4 and were classified into IgG4-associated AIH. In contrast, all 10 patients with PBC, three patients with PSC and 20 patients with HCV hepatitis were negative.

We also examined the IgG4 expression in the livers of two patients with IgG4-associated cholangitis as a positive control. As shown in Figure 1B, the liver sample is heavily infiltrated by $IgG4^+$ cells as compared with the samples from the IgG4-associated AIH patients.

Clinical profile of immunoglobulin G4-associated autoimmune hepatitis

As shown in Figure 2, there were no significant differences in age, serum levels of ALT, ALP, γGTP, antinuclear antibody or in the degree of liver fibrosis between the IgG4-associated and the IgG4 non-associated AIH patients. The IgG4-associated AIH patients had significantly higher total serum IgG levels and AIH scores as compared with the IgG4 non-associated patients. In contrast, there was no difference in serum IgG4 levels between the IgG4-associated AIH and the IgG4 non-associated AIH patients. In addition, we did not find a difference in any of the factors in the AIH scoring system, except for the total serum IgG levels.

Histological analysis of immunoglobulin G4-associated autoimmune hepatitis

We performed an extensive histological analysis of the liver samples taken from the IgG4-associated AIH patients using haematoxylin and eosin-stained tissue. As shown in Figure 3A, portal inflammation and interface hepatitis were present in all liver samples from patients with either IgG4-associated or IgG4 non-associated AIH. In contrast, plasma cell infiltration and lobular hepatitis were detected more frequently in the livers of IgG4-associated AIH than in those of IgG4 non-associated AIH. Although portal inflammation was seen in all the liver samples from AIH patients, the degree of inflammation was more severe in the patients with IgG4-associated AIH (Fig. 3B). Most of the AIH patients were negative for cholangitis on histological analysis. In addition, none of the AIH patients showed any abnormalities of the bile duct as assessed by ultrasonography or computed tomography.

Immunohistochemical analysis of immunoglobulin G4-associated autoimmune hepatitis

Infiltration of T cells, B cells and plasma cells was examined by immunostaining for CD3, CD20, CD38 and IgG using semiconsecutive sections taken from the livers of AIH patients. As shown in Figures 4 and 5, the numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells and IgG^+ cells in the livers of patients with IgG4-associated AIH were greater than those in IgG4 non-associated AIH patients. Therefore, IgG4-associated AIH is characterized by the infiltration of T cells, B cells and plasma cells into the liver tissue of these patients. No difference was seen in the number of $IgG1^+$ cells or the ratio of $IgG1^+/IgG^+$ cells between the two subgroups,

Table 1. Clinical characteristics of patients at the time of diagnosis

	AIH ($n = 26$)	PBC ($n = 10$)	HCV ($n = 20$)
Age (years)	60 ± 9 (42–78)	57 ± 13 (28–72)	56 ± 11 (29–78)
Gender (female/male; %female)	24/2; 92%	9/1; 90%	10/10; 50%*
Concurrent autoimmune diseases	7/26; 27%	0/10; 0%	1/20; 5%
Laboratory data			
AST (IU/L)	196 ± 604 (48–2350)	65 ± 56 (25–210)	58 ± 49 (35–240)†
ALT (IU/L)	216 ± 510 (57–1776)	93 ± 78 (28–234)‡	78 ± 55 (38–229)†
ALP (IU/L)	558 ± 274 (191–1126)	857 ± 269 (480–1217)	244 ± 128 (147–663)†,§
Total bilirubin (mg/dl)	0.9 ± 5.3 (0.4–20)	0.5 ± 0.5 (0.4–2.1)	0.7 ± 0.2 (0.5–1.1)
Albumin (g/dl)	3.9 ± 0.6 (2.3–4.7)	4.0 ± 0.2 (3.7–4.3)	4.1 ± 0.3 (3.3–4.8)†
Immunoglobulin G (g/dl)	2.2 ± 0.7 (1.1–4.1)	1.4 ± 0.8 (1.2–3.9)	1.8 ± 0.7 (0.9–2.8)
IAHG score	19 (16–22)	–	–

Data are expressed as median ± standard deviation (range) or frequency.

*$P < 0.05$ (Fischer's exact test).

†,‡,§$P < 0.05$ between AIH and HCV, between AIH and PBC, and between PBC and HCV respectively.

AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCV, hepatitis C virus; IAHG, International Autoimmune Hepatitis Group; PBC, primary biliary cirrhosis.

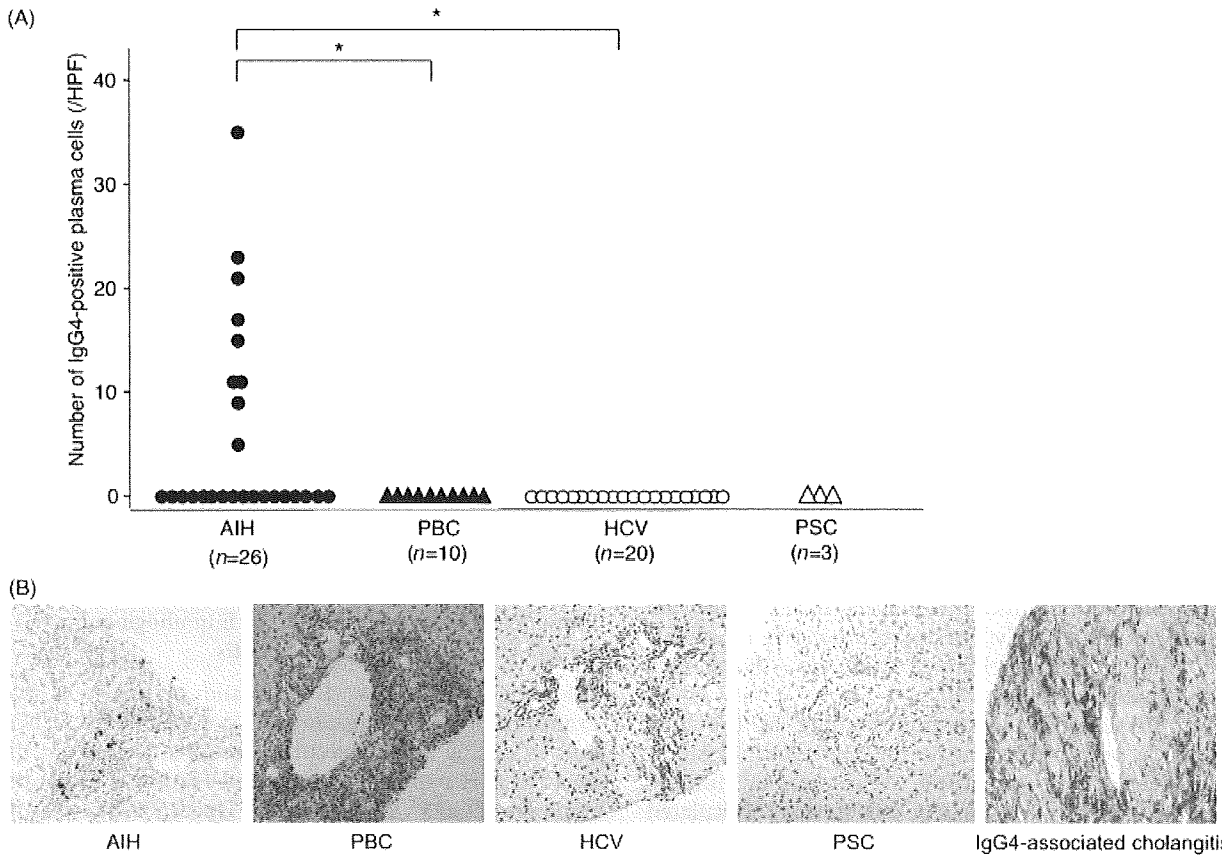

Fig. 1. Identification of immunoglobulin (Ig) G4-associated autoimmune hepatitis (AIH). (A) Number of $IgG4^+$ plasma cells/high-power field in liver specimens from 26 patients with AIH, 10 patients with primary biliary cirrhosis (PBC), three patients with primary sclerosing cholangitis (PSC) and 20 patients with hepatitis C virus (HCV) hepatitis. Nine of the 26 (34.6%) samples from the AIH patients showed positive staining for IgG4. All 10 patients with PBC, 20 patients with HCV and three patients with PSC were negative. *$P < 0.05$. (B) Representative sections showing the immunohistochemical staining of IgG4 in the livers of patients with IgG4-associated AIH, PBC, HCV hepatitis, PSC and IgG4-associated cholangitis.

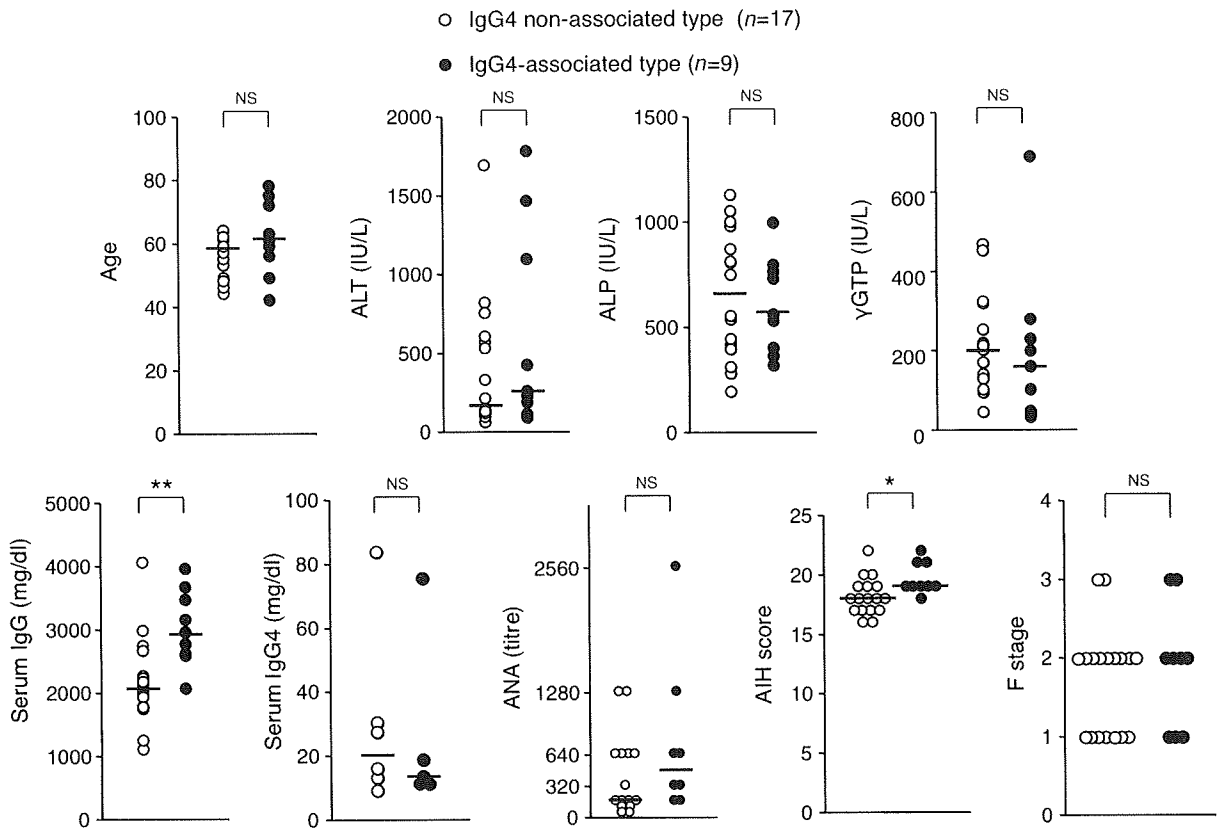

Fig. 2. Comparison of the clinical characteristics between immunoglobulin (Ig) G4-associated and IgG4 non-associated autoimmune hepatitis (AIH). Horizontal bars represent median values in each plot. Serum IgG levels and AIH scores were significantly higher in IgG4-associated AIH patients than in IgG4 non-associated AIH patients ($^*P < 0.05$, $^{**}P < 0.01$). NS, not significant.

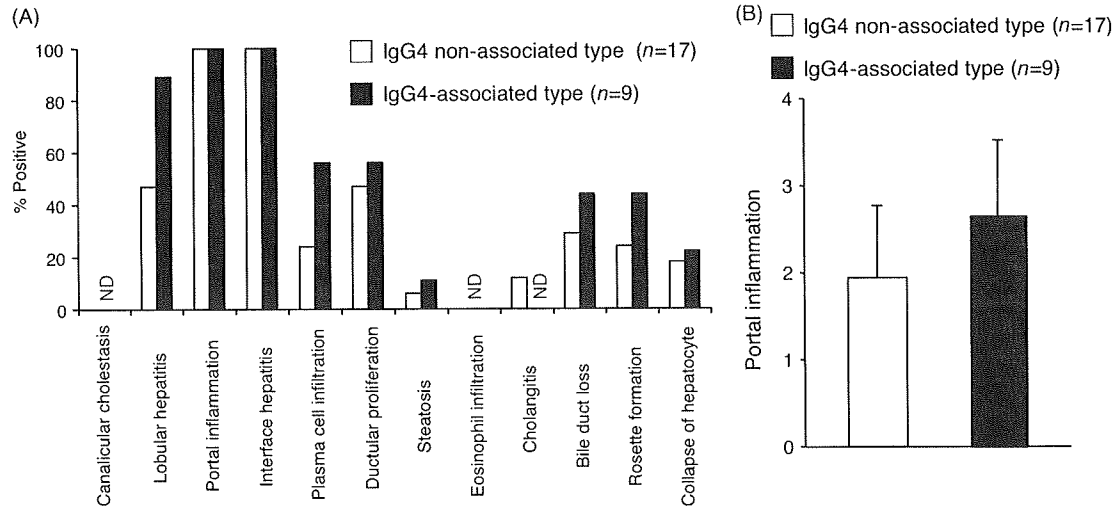

Fig. 3. Comparison of the histological findings between immunoglobulin (Ig) G4-associated and IgG4 non-associated autoimmune hepatitis (AIH). (A) Haematoxylin and eosin-stained liver tissues obtained from AIH patients were analysed. Plasma cell infiltration and lobular hepatitis were more frequently detected in the livers of IgG4-associated AIH patients than in IgG4 non-associated AIH patients, whereas portal inflammation and interface hepatitis were seen in all liver samples. ND, not detected. (B) The degree of portal inflammation as assessed by the Ishak scoring system.

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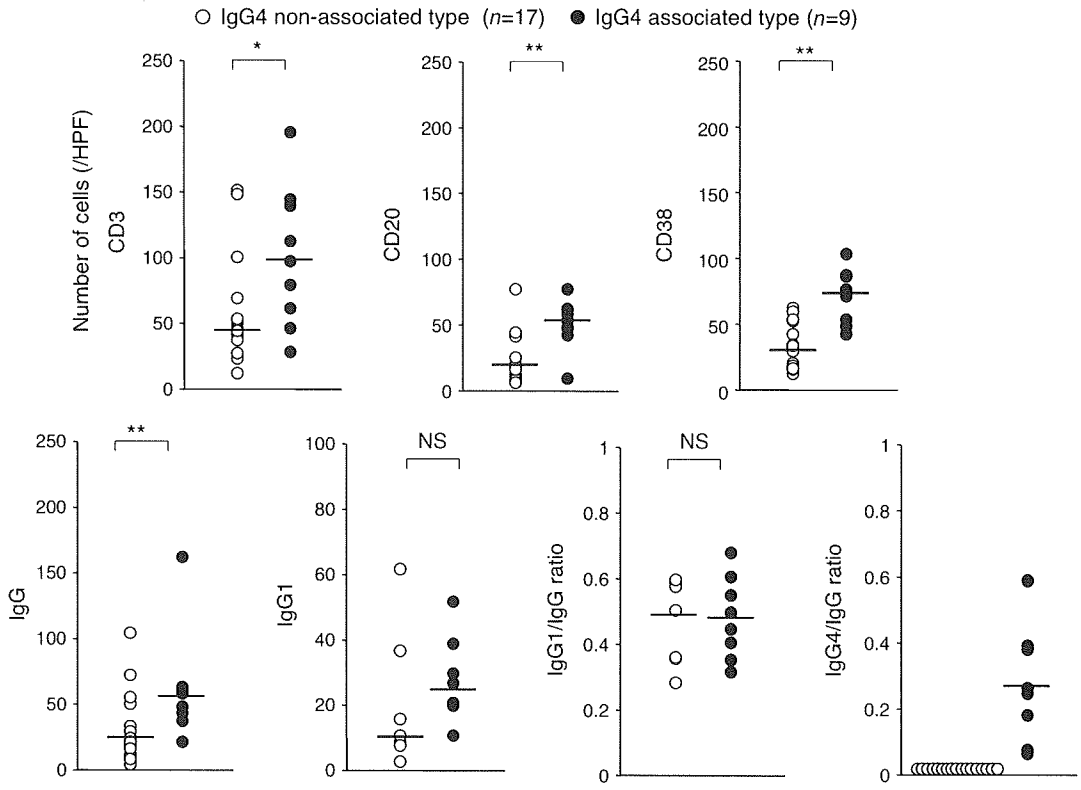

Fig. 4. Numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells, immunoglobulin (Ig) G^+ cells and $IgG1^+$ cells in the liver specimens from patients with autoimmune hepatitis (AIH). Horizontal bars represent median values in each plot. The numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells and IgG^+ cells were greater in the livers of patients with IgG4-associated AIH than in patients with IgG4 non-associated AIH ($^*P < 0.05$, $^{**}P < 0.01$). NS, not significant.

suggesting a selective accumulation of $IgG4^+$ cells in IgG4-associated AIH. No correlation was seen between the number of $IgG4^+$ plasma cells and the number of $CD3^+$ T cells, $CD20^+$ B cells or $CD38^+$ plasma cells in the livers of patients with IgG4-associated AIH (data not shown). Moreover, the number of $IgG4^+$ plasma cells did not correlate with the AIH score or the serum ALT level, suggesting that the number of $IgG4^+$ cells is not associated with the severity of AIH (data not shown).

Response to steroid therapy in patients with immunoglobulin G4-associated autoimmune hepatitis

Finally, we compared the response of IgG4-associated and IgG4 non-associated AIH patients to steroid therapy. Six patients with IgG4-associated AIH and 16 patients with IgG4 non-associated AIH were treated with prednisolone at an initial dose of 30–40 mg/day, followed by maintenance therapy at 5–10 mg/day (Fig. 6). No difference was seen in the treated dose of prednisolone between IgG4-associated and IgG4 non-associated AIH patients. As shown in Figure 6, a significant decrease of serum ALT levels was seen in patients with IgG4-associated AIH and those with IgG4 non-associated AIH at 4 weeks after starting prednisolone therapy. Importantly, the reduced serum ALT levels in IgG4-associated AIH were maintained at 48, 72 and 96 weeks after starting prednisolone therapy. This was not the case for the IgG4 non-associated AIH patients. None of the patients with IgG4-associated AIH showed elevated levels of serum ALT on prednisolone therapy. In contrast, eight patients (50.0%) with IgG4 non-associated AIH did show elevated levels of serum ALT, even on prednisolone therapy. Furthermore, no relapse was seen in the patients with IgG4-associated AIH, whereas six patients with IgG4 non-associated AIH (37.5%) required incremental doses of prednisolone because of relapse. Thus, IgG4-associated AIH shows a marked response to prednisolone not only in the initial phase but also in the maintenance phase of treatment.

Discussion

In this study, we describe a novel subtype of AIH characterized by infiltration of IgG4-expressing plasma cells. Our data show that infiltration by $IgG4^+$ plasma cells does occur in a subpopulation of AIH patients but not in PBC or PSC patients. Thus, tissue staining for IgG4 can be used to classify AIH into either an IgG4-associated type or an IgG4 non-associated type. Patients with IgG4-associated AIH have increased levels of serum IgG and AIH scores as compared with patients with the

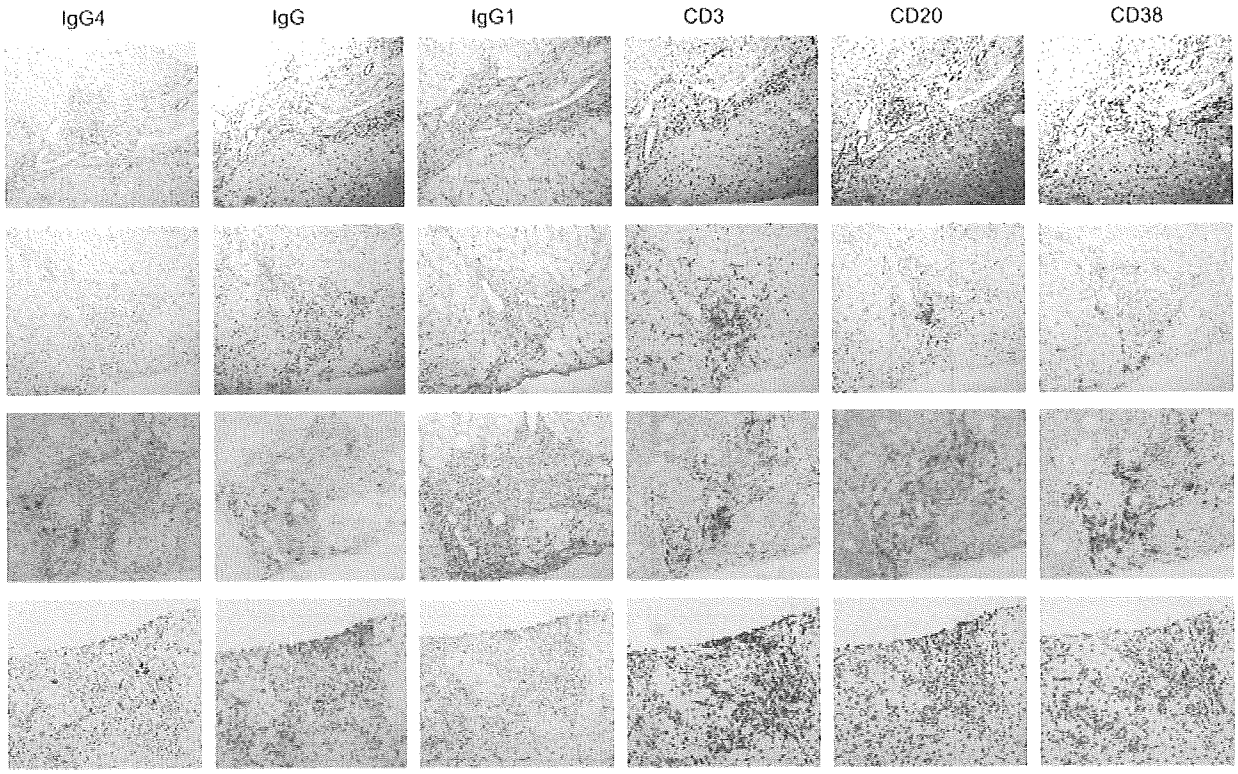

Fig. 5. Immunohistochemical staining of immunoglobulin (Ig) G4, IgG, IgG1, CD3, CD20 and CD38 in the livers of patients with autoimmune hepatitis (AIH). The top two and bottom two panels show liver sections from IgG4 non-associated and IgG4-associated AIH patients respectively. The numbers of $CD3^+$ T cells, $CD20^+$ B cells, $CD38^+$ plasma cells and IgG^+ plasma cells in the livers of the patients with IgG4-associated AIH were greater than in those of the patients with IgG4 non-associated AIH.

IgG4 non-associated type. More importantly, prednisolone therapy is very effective in patients with IgG4-associated AIH for both induction and maintenance of remission. We regarded the tissue specimens as IgG4 positive when more than five $IgG4^+$ plasma cells were identified per HPF as reported by Zhang *et al.* (23), although significant differences in the response to prednisolone and in serum IgG levels were still observed when we set a positive threshold of 10 $IgG4^+$ plasma cells per HPF as reported by Ghazale *et al.* (13) (data not shown). Our data suggest that positive IgG4 staining in the liver can be used as a surrogate marker for the subtype of AIH that responds well to prednisolone therapy.

Some of the patients with IgG4-related AIP have various extrapancreatic lesions. Because these extrapancreatic and pancreatic lesions share common histopathological findings (i.e. abundant infiltration by $IgG4^+$ plasma cells), Kamisawa and Okamoto (10) proposed a new clinicopathological entity: 'IgG4-related sclerosing disease'. Portal inflammation and lobular hepatitis characterized by abundant infiltration of $IgG4^+$ plasma cells is often seen in the livers of AIP patients (12, 15). This AIP-associated liver inflammation is called IgG4 hepatopathy (15). One important issue arising from our study is whether IgG4-associated AIH is a hepatic manifestation of this systemic disease rather than a subtype of classical AIH. It should be noted that none of the AIH patients in this study showed any swelling of the pancreas or abnormality of the bile or pancreatic ducts as assessed by ultrasonography or computed tomography findings that are characteristic of IgG4-associated AIP and cholangitis (7). In addition, extrapancreatic manifestations of IgG4-related systemic disease such as sialadenitis, retroperitoneal fibrosis and inflammatory liver pseudotumour (10) were absent in all AIH patients studied. More importantly, serum IgG4 levels in patients with IgG4-associated AIH were not so high as those in patients with IgG4-related systemic disease. These results suggest that IgG4-associated AIH is distinct from IgG4-related systemic disease, including AIP and IgG4 hepatopathy, although all show marked responses to prednisolone therapy. Further examination of the pancreatobiliary system using endoscopic retrograde cholangiopancreatography or magnetic resonance cholangiopancreatography may be useful to confirm that IgG4-associated AIH is not a hepatic manifestation of IgG4-related systemic disease including IgG4-associated cholangitis.

Although enhanced IgG4 antibody responses are linked to AIP, the role of this antibody in disease development and progression is poorly understood (7). Enhanced IgG4 antibody responses may be an epiphenomenon associated with inflammatory reactions (7). One possibility is that

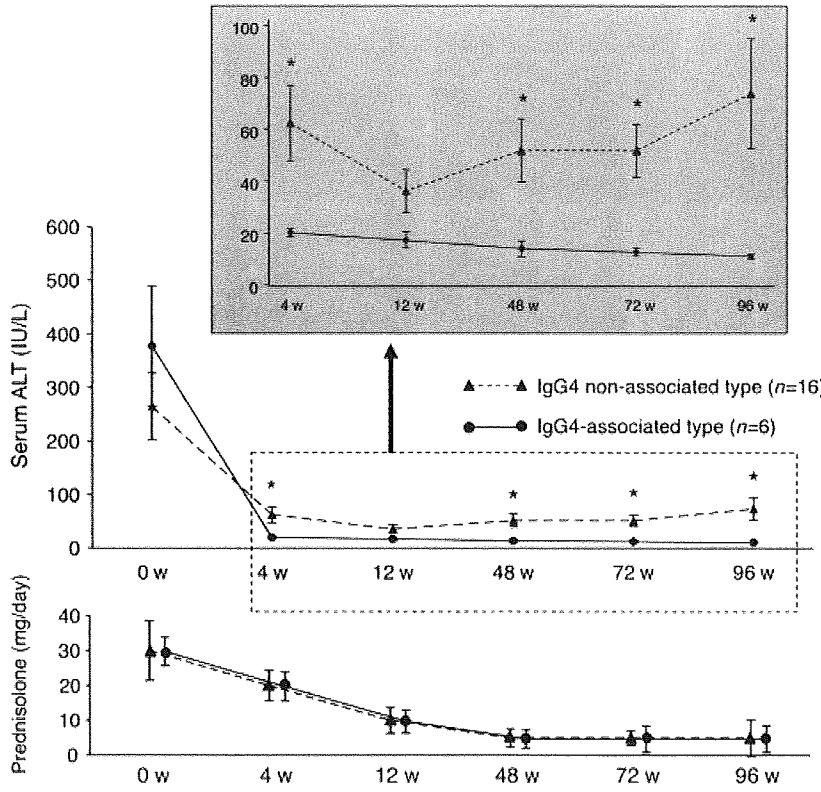

Fig. 6. Serial changes of serum alanine aminotransferase (ALT) levels in patients with autoimmune hepatitis (AIH) during treatment with prednisolone. Serum levels of ALT were monitored in six patients with immunoglobulin (Ig) G4-associated AIH and in 16 patients with IgG4 non-associated AIH. These patients were treated with prednisolone. The doses of prednisolone at each time point are shown in the bottom panel. The results are shown as mean ± standard error ($^*P < 0.05$).

the increase in tissue-infiltrating $IgG4^+$ plasma cells in IgG4-associated AIH simply reflects the degree of migration and accumulation of B cells and IgG^+ plasma cells. In fact, we found that the numbers of $CD20^+$ B cells and $CD38^+$ plasma cells are greater in the livers of IgG4-associated AIH patients than in those of IgG4 non-associated AIH patients. However, we found no difference in the number of $IgG1^+$ cells or in the ratio of $IgG1^+$/IgG^+ cells between the two subgroups. Although the possibility of a non-selective increase in the migration and accumulation of B cells cannot be completely excluded, the results of IgG1 expression suggest that selective augmentation of IgG4 production occurs in the livers of patients with IgG4-associated AIH. Therefore, the involvement of factors driving IgG4 class switching may be considered in the immunopathogenesis of IgG4-associated AIH. Further studies are necessary to elucidate the molecular mechanisms for the selective accumulation of $IgG4^+$ plasma cells in the liver.

Serum levels of IgG4 were not increased in patients with IgG4-associated AIH. This finding suggests that tissue staining of IgG4 is more sensitive than serum IgG4 assays for the diagnosis of IgG4-associated AIH. In support of this, Zhang *et al.* (23) reported that visualization of $IgG4^+$ plasma cells is useful for the diagnosis of AIP in patients with normal levels of serum IgG4. However, Umemura *et al.* (14) reported the case of IgG4-associated AIH with a marked elevation in serum IgG4 levels. The reason for this discrepancy remains unknown. It should be noted that Umemura's case was characterized by liver infiltration by many $IgG4^+$ cells (> 40/HPF). This is much higher than we observed in any of our cases. This suggests that it is the degree of accumulation of $IgG4^+$ cells in the liver that determines the serum IgG4 response in patients with IgG4-associated AIH. Another possibility is that immune environments, which affect IgG4 responses, may be different in the peripheral blood and the liver of patients with IgG4-associated AIH. Indeed, predominant Th1 responses have been reported in the peripheral blood of patients with AIP (24), whereas Th2 responses are enhanced in the pancreas and liver (25). However, further studies using a large number of AIH patients are necessary to establish the relationship between serum IgG4 levels and IgG4 expression in the liver.

Our data show that $IgG4^+$ cells are distributed in the liver of IgG4-associated AIH in a scattered manner rather than in a densely packed manner. In addition, there is no difference in serum IgG4 levels between the IgG4-associated AIH and the non-associated AIH patients. More

importantly, the number of AIH patients enrolled in this study may not be sufficient to enable definite conclusions to be drawn. Thus, one might concern that the difference between these two groups is small. However, our results clearly show that the effectiveness of steroid therapy was different in the two groups of patients (Fig. 6). Future studies using a larger number of AIH patients would be required to confirm the difference between IgG4-associated AIH and non-associated AIH.

Patients with classical AIH usually show marked responses to steroid therapy (1). We have shown that the serum levels of ALT were reduced in most AIH patients 4 weeks after starting prednisolone treatment. An interesting observation is that the reduction in the serum ALT level in the IgG4-associated AIH was greater than that in the IgG4 non-associated AIH. Furthermore, remission was maintained in the patients with IgG4-associated AIH. Therefore, IgG4-associated AIH is characterized by a marked response to prednisolone treatment similar to that seen in patients with IgG4-related systemic disease (26). We found no relapse in patients with IgG4-associated AIH, whereas six patients with IgG4 non-associated AIH required incremental doses of prednisolone because of a relapse.

In conclusion, our data suggest that the immunostaining of liver biopsy specimens for IgG4 can be used to predict the prognosis of AIH patients by identifying the subpopulation that shows a marked response to steroid treatment. Further studies using larger patient groups are required to confirm these findings.

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