Long-Term Prognosis of Autoimmune Pancreatitis in Terms of Glucose Tolerance

Kenji Hirano, MD, PhD, Akihiro Isogawa, MD, PhD,† Minoru Tada, MD, PhD,* Hiroyuki Isayama, MD, PhD,* Naminatsu Takahara, MD,* Koji Miyabayashi, MD,* Suguru Mizuno, MD,* Dai Mohri, MD, PhD,* Kazumichi Kawakubo, MD,* Takashi Sasaki, MD, PhD,* Hirofumi Kogure, MD, PhD,* Natsuyo Yamamoto, MD, PhD,* Naoki Sasahira, MD, PhD,* Nobuo Toda, MD, PhD,† Rie Nagano, MD,‡ Hiroshi Yagioka, MD, PhD,§ Yoko Yashima, MD, PhD,|| Tsuyoshi Hamada, MD,|| Yukiko Ito, MD, PhD,|| and Kazuhiko Koike, MD, PhD**

Objective: Glucose intolerance is often observed in autoimmune pancreatitis (AIP), although its long-term prognosis after steroid treatment (ST) is still unclear.

Methods: A total of 47 patients with AIP were enrolled. On the basis of the change in hemoglobin A1c (Hb_{A1c}) and the use of diabetic medication, prognosis was classified into 3 categories, namely, "improved," "aggravated," and "unchanged." The relation between the result of an initial glucagon tolerance test (ΔCPR) and the later use of insulin during maintenance ST was examined in 20 patients. The transitions of homeostasis model assessment β cell and insulin resistance (HOMA-β and HOMA-R) were analyzed in 16 patients.

Results: Glucose tolerance was improved in 6 patients (13%), aggravated in 9 patients (19%), and unchanged in 32 patients (68%). All patients with ΔCPR less than 0.6 ng/mL were obliged to use insulin even after long-term observation, whereas all patients with ΔCPR more than 1.0 ng/mL were free from insulin therapy. Moreover, HOMA-β showed significant improvement after ST (43.9%→56.0% in median, $P = 0.030$), and HOMA-R showed significant aggravation (1.30→1.78, $P = 0.039$).

Conclusions: Glucose tolerance that is too severely damaged may not recover fully even after ST. Thus, ST should be performed to preserve insulin secretion at the early stage of AIP.

Key Words: autoimmune pancreatitis, diabetes mellitus, glucose tolerance, steroid

Abbreviations: ST - steroid treatment, CPR - C-peptide response, HOMA-β - homeostasis model assessment β cell, HOMA-R - homeostasis model assessment insulin resistance, DM - diabetes mellitus, GTT - glucagon tolerance test, OHA - oral hypoglycemic agent, Hb_{A1c} - hemoglobin A1c

(*Pancreas* 2012;00: 00–00)

Autoimmune pancreatitis (AIP) is a unique pancreatic benign disease characterized by irregular narrowing of the pancreatic duct, swelling of the pancreas, lymphoplasmacytic infiltration and fibrosis, various extrapancreatic lesions, and favorable response to steroid treatment (ST).[1–9]

Diabetes mellitus (DM) is often observed in patients with AIP (43%–83%).[10–14] It is known that some of these patients can obtain improvement of DM after ST. The improvement rate is various (25%–64%).[11–14] In previous reports, the improvement of DM seemed to be judged based on the transition of Hb_{A1c}. However, definite criteria were not described in any previous reports, which might cause such difference. There is as yet no consensus criterion for judging improvement or aggravation of glucose tolerance, and it seems that a definite criterion needs to be established before the long-term outcome of glucose tolerance can be discussed. Therefore, we revealed our criterion first, despite it perhaps being an arbitrary one. Then, we tried to classify the long-term outcome of glucose tolerance in AIP.

In consideration of the long-term outcome of glucose tolerance, the eventual use or nonuse of insulin is of great interest. A glucagon tolerance test (GTT) is often used to assess the insulin response, and it is the most useful test for the prediction of failure of oral hypoglycemic agents (OHAs).[15,16] Focusing on the result of GTT, we examined whether the initial result of GTT can predict the later use of insulin during maintenance ST.

It is well known that steroid may induce insulin resistance, which causes glucose intolerance.[17–20] Therefore, in addition to deteriorated pancreatic β-cell function, glucose intolerance or DM after ST may be partly attributed to insulin resistance. It is clinically important to assess the influence of insulin resistance because it seems useful for choosing adequate OHA. In this study, the changes in homeostasis model assessment β cell (HOMA-β) and homeostasis model assessment insulin resistance (HOMA-R) were examined to evaluate the pathogenesis of glucose intolerance after ST.

MATERIALS AND METHODS

Patients

As of October 2010, 51 patients with AIP received ST and were followed up at the University of Tokyo Hospital or our affiliated hospitals. The diagnosis of AIP was made based on Asian diagnostic criteria,[2] and we believe all our patients are categorized as what is called type 1 AIP.[1] Among them, 47 patients with follow-up periods of more than 12 months were enrolled in this study. They consisted of 41 men and 6 women. The mean onset age of AIP was 63 years (range, 40–76 years). The mean follow-up period was 60 months (range, 12–151 months). According to the World Health Organization criteria,[21] 33 patients (70%) had DM at the time of the introduction of ST. Onset timing of DM is often emphasized in some previous reports.[11,22] In our series, DM occurred before the onset of AIP in 8 patients

From the *Department of Gastroenterology, Graduate School of Medicine, The University of Tokyo; †Department of Internal Medicine, Mitsui Memorial Hospital; ‡Department of Gastroenterology, Kanto Central Hospital of the Mutual Aid Association of Public School Teachers; §Department of Gastroenterology, JR Tokyo General Hospital; and ||Department of Gastroenterology, Japanese Red Cross Medical Center, Tokyo, Japan.

Received for publication May 19, 2011; accepted October 5, 2011.

Reprints: Kenji Hirano, MD, PhD, Department of Gastroenterology, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan (e-mail: khirano-tky@umin.ac.jp).

This work was supported in part by Health and Labor Sciences Research Grants for research on intractable diseases from the Ministry of Health, Labor and Welfare of Japan.

The authors declare no conflict of interest.

TABLE 1. Criterion for the Judgment of Long-Term Outcome of Glucose Tolerance

1) Patients who had already received diabetic medication (insulin therapy or oral antidiabetic agents) or began it at the introduction of ST
Improved:
• Relief from diabetic medication after steroid was decreased to maintenance dose, with decreased Hb_{A1c}
• Decrease of Hb_{A1c} by 2.0% or more with oral antidiabetic agents after steroid was decreased to maintenance dose
Aggravated: Switching from oral antidiabetic agents to insulin therapy after steroid was decreased to maintenance dose
Unchanged: Neither "Improved" nor "Aggravated"
2) Patients who did not receive diabetic medication at the introduction of ST,
Improved: Normalization of Hb_{A1c} (≤6.2%) or decrease of Hb_{A1c} by 0.6% or more without the introduction of diabetic medication
Aggravated: Elevation of Hb_{A1c} from normal value to abnormally high value (>6.2%); or elevation of Hb_{A1c} by 0.6% or more
Unchanged: Neither "Improved" nor "Aggravated"

and at the same time of AIP in 25 patients. As for ST, prednisolone at an initial dosage of 30 to 40 mg/d was administered for 2 to 4 weeks in most cases. It was then tapered by 5 mg every 2 to 6 weeks until reaching 10 mg/d, and 2.5 to 7.5 mg/d was continued as maintenance therapy in principle. Diabetes was controlled by DM specialists, and intensified insulin therapy was introduced at the same time as ST in patients in whom remarkable hyperglycemia was expected or already, in fact, observed. This retrospective study was approved by the review board of our institute.

Long-Term Outcome of Glucose Tolerance

There is no consensus criterion for judging improvement or aggravation of glucose tolerance, but to avoid inconsistent judgment, it seemed necessary to show a definite criterion in advance even if it was arbitrary. In this study, we proposed the criterion shown in Table 1, which was supported and approved by a DM specialist (I.A.), one of the coauthors. We suppose that this simple criterion may not be perfect, but it is not far from the common sense practiced by clinicians treating patients with pancreatic diabetes. In our criteria, the transition of Hb_{A1c} and the use of diabetic medication (insulin or OHA) were weighted, whereas the dose of medication was not considered. The value of Hb_{A1c} just before ST and the latest value were used in our analysis. According to the Japan Diabetes Society (JDS), the Hb_{A1c} value in Japan (Hb_{A1c} [JDS]) differs from the Hb_{A1c} value of the National Glycohemoglobin Standardization Program (Hb_{A1c} [NGSP]), which is used in many countries other than Japan. That is, Hb_{A1c} (JDS) is 0.4% lower than Hb_{A1c} (NGSP). In this study, Hb_{A1c} (NGSP) was adopted to express the value of Hb_{A1c}.[23]

Glucagon Tolerance Test

The GTT was performed as follows: blood samples were collected at 0 and 6 minutes after a bolus intravenous injection of glucagon (Glucagon Novo; Eizai Co, Ltd, Tokyo, Japan; 1.0 mg/body). C-peptide response (CPR) concentration was measured each time, and the ΔCPR value was reported as insulin secretion. A value of 1.5 ng/mL or less was diagnosed as dysfunction of insulin secretion by β cells. It is reported that a value of 0.35 nM (1.05 ng/mL) or less is almost always associated with OHA failure in normal-weight patients.[16] The GTT was performed before ST in 20 patients. Among them, GTT was also performed 1 month after the introduction of ST in 17. First, we examined the transition of the ΔCPR value before and 1 month after ST. Second, we examined the relation between ΔCPR before ST and the present use or nonuse of insulin.

Transitions of HOMA-β and HOMA-R

We analyzed only patients who received neither insulin treatment nor OHA both before ST and at the time of the latest blood test. When the serum glucose level was more than 200 mg/dL, HOMA-β and HOMA-R were not calculated because their values might not be reliable. Finally, the values of HOMA-β and HOMA-R before ST and their latest ones were calculated in 16 patients, and their transitions were analyzed. In general, when HOMA-β is less than 40%, dysfunction of insulin secretion is suspected. When HOMA-R exceeds 2.5, the presence of insulin resistance is suspected.[24]

Statistical Analysis

Continuous variables were reported as mean (SD) or as median and compared by Mann-Whitney *U* test or Wilcoxon signed-rank test when appropriate. $P < 0.05$ was considered statistically significant. Statistical analyses were performed by statistical software JMP 7.0.1 (SAS Institute, Inc, Cary, NC).

RESULTS

Insulin therapy had already been introduced before ST in 8 patients. It was introduced concomitantly with ST in 13 patients, among whom 4 became insulin-free in 1, 1, 8, and 48 months, respectively. In 2 patients, insulin therapy was started during follow-up, namely, 49 and 112 months after ST. There were 24 patients with no history of insulin therapy. Limited to 17 patients whose Hb_{A1c} level before ST was more than 7%; there were only 2 patients who did not receive insulin therapy at the introduction of ST. The reason was refusal by the patients.

According to our criterion (Table 1), glucose tolerance was improved in 6 patients (13%), aggravated in 9 patients (19%), and unchanged in 32 patients (68%; Table 2). Comparing improved and aggravated patients, the onset age of AIP (61 [5.6] vs 60 [9.1] years, $P = 0.835$) and follow-up period (51 [33] vs 83 [37] months, $P = 0.112$) were not significantly different. Limited to the patients with DM at the introduction of ST (n = 33), glucose tolerance was improved in 5 patients (15%), aggravated in 4 patients (12%), and unchanged in 24 patients (73%). In 8 patients with preexistent DM, there was no case that showed improvement.

The changes in ΔCPR value before and 1 month after ST were assessed in 17 patients, with the results shown in Table 3

TABLE 2. Long-Term Outcome of Glucose Tolerance in 47 Patients With AIP

Presence of DM at the Introduction of ST	Improved	Aggravated	Unchanged
Patients with preexistent DM (n = 8)	0	0	8
Patients in whom DM and AIP occurred simultaneously (n = 25)	5	4	16
Patients without DM (n = 14)	1	5	8

TABLE 3. Results of GTT and Insulin Use in 20 Patients With AIP

Case No.	Age at Onset of AIP, y	DM*	Age at Onset of DM,† y	IgG4, mg/dL	ΔCPR Before ST, ng/mL	ΔCPR 1 mo After ST, ng/mL	Insulin Use at the Initiation of ST	Insulin Use After Follow-Up	Condition of Glucose Tolerance	Follow-Up Period After ST, mo
1	47	+	B	960	0	0.46	+	+	Unchanged	12
2	67	+	B	230	0.2	0.9	+	+	Unchanged	47
3	76	+	S	431	0.3	0.8	+	+	Unchanged	14
4	71	+	B	613	0.35	0.53	+	+	Unchanged	13
5	74	+	B	98	0.5	0.5	+	+	Unchanged	18
6	53	+	S	143	0.6	1.4	−	−	Improved	52
7	59	+	S	140	0.6	1.3	+	+	Unchanged	13
8	68	+	S	989	0.6	0.6	+	+	Unchanged	21
9	40	−		650	0.7	1.7	−	−	Unchanged	43
10	64	+	S	232	0.8	1.5	+	+	Unchanged	13
11	72	+	S	455	1	1.6	+	−	Unchanged	38
12	61	+	S	331	1	NT	+	+	Unchanged	44
13	65	+	S	1030	1.12	NT	−	−	Improved	14
14	73	+	S	1800	1.3	4.2	−	−	Unchanged	26
15	62	+	S	236	1.5	1.7	−	−	Unchanged	43
16	56	−		436	2.3	3.5	−	−	Aggravated	51
17	66	+	S	270	2.38	3.72	−	−	Unchanged	56
18	71	−		458	2.9	2.1	−	−	Aggravated	36
19	76	−		543	3	5	−	−	Unchanged	14
20	43	−		223	3.8	NT	−	−	Unchanged	43

*Existence of DM at the introduction of ST.

†B indicates that DM occurred before onset of AIP; S, DM and AIP occurred simultaneously.

NT indicates not tested.

and Figure 1. There was a significant increase of ΔCPR (1.20 [0.96] vs 1.85 [1.40] ng/mL, $P = 0.0041$). For the ΔCPR value 1 month after ST, 8 patients showed a value higher than 1.5 ng/mL. Among them, only 1 patient had ΔCPR before ST less than 1 ng/mL.

The relation between ΔCPR before ST and the later use of insulin during maintenance ST was also assessed. The result is shown in Table 3. In all 5 patients whose ΔCPR before ST was 0.5 ng/mL or less, the later use of insulin was inevitable. In all 8 patients whose ΔCPR before ST was more than 1 ng/mL, insulin use could be avoided after long-term follow-up. Comparing patients needing insulin therapy after long-term follow-up (n = 9) and not (n = 11), the former group showed lower ΔCPR before ST than the latter (0.48 [0.31] vs 1.88 [1.06] ng/mL, $P = 0.0008$). After excluding 4 patients with preexistent DM, this result did not change (0.66 [0.26] vs 1.88 [1.06] ng/mL, $P = 0.012$).

The changes in HOMA-β and HOMA-R in 16 patients are summarized in Table 4. Moreover, HOMA-β showed significant improvement after ST (43.9%→56.0% in median, $P = 0.030$), and HOMA-R showed significant aggravation (1.30→1.78, $P = 0.039$), although Hb_{A1c} did not change (5.8→5.9%, $P = 0.313$).

DISCUSSION

Diabetes mellitus is often associated with AIP, and it may show improvement with ST. However, it is difficult to document how often such improvement is observed because there is no definite criterion for judging the improvement or aggravation of DM or glucose tolerance. In addition, improvement of DM does not necessarily mean the recovery of pancreatic endocrine function if insulin or OHA was initiated or increased. In previous reports, the outcome of DM or glucose tolerance was discussed without showing concrete judgment criteria. Despite its difficulty, this situation is nonetheless undesirable. Although our criterion in the present study may be imperfect, it will be still useful for grasping the general trend of the outcome of glucose tolerance associated with AIP.

Improvement of glucose tolerance was observed in 13% (6/47), and that of DM in 15% (5/33), which seemed to be inferior to previous reports (25%–64%). Although this difference might be due to our strict judgment criterion, we think it is partly affected by the longer follow-up periods in our series. In our analysis, the follow-up period was shorter in improved patients than in aggravated ones (51 vs 81 months), despite the difference not being statistically significant. The improvement rate of 64% (7/11) reported by Ito et al[14] was not evaluated with long-term follow-up. Thus, on a long-term basis, the prognosis of glucose tolerance may actually be worse than previously reported. However, if the absence of aggravation can be regarded as acceptable, the nonaggravation rate of 81% (38/47) does not seem so bad, although the exact natural history of glucose tolerance in AIP without ST is still rather unclear. It is difficult to discuss the natural history of glucose tolerance because many patients receive ST later due to the relapse of pancreatic or extrapancreatic lesions.[9] As for our experience, we had 21 patients with AIP whose follow-up period without ST was longer than 12 months (82 months in average). We compared the conditions at diagnosis and just before ST in 10 patients who did finally receive ST and in those at diagnosis and at the latest visit in 11 patients without a history of ST. Among the 21 patients, at least in 9 (43%), aggravation of glucose tolerance was confirmed

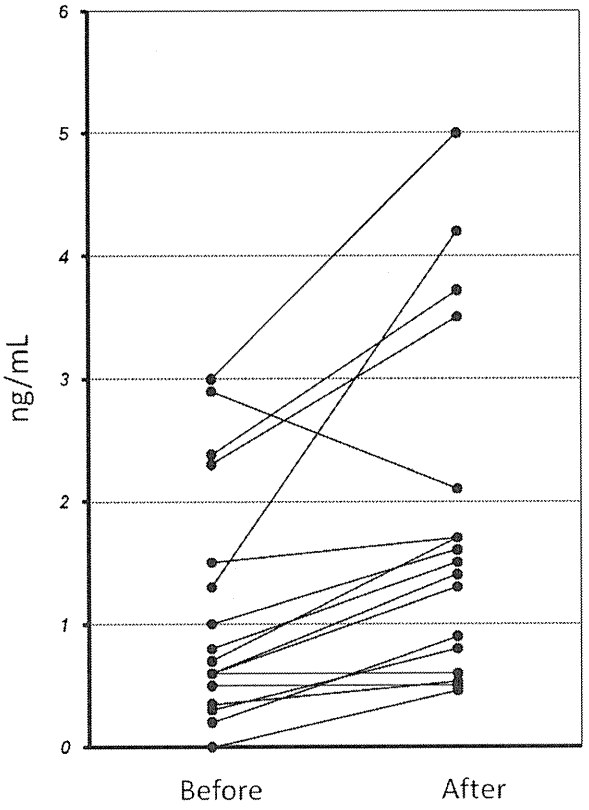

FIGURE 1. Transition of ΔCPR before and 1 month after ST in 17 patients is shown.

during the period without ST. Among these 9 patients, there were 5 patients in whom pancreatic lesion was localized and obstructive jaundice was not recognized at the diagnosis of AIP. Thus, we think that the natural history of glucose tolerance in AIP will be much worse than that of the patients treated with steroid.

To assess the pure pancreatic endocrine function, GTT with results little affected by insulin therapy or OHA was performed after January 2006. As shown in Figure 1, on a short-term basis, ΔCPR increased after ST in 82% (14/17). However, in patients with more mildly damaged insulin secretion, the extent of the recovery was more remarkable. This may also be true of the evaluation on a long-term basis. All the patients with too severely damaged insulin secretion (ΔCPR ≤ 0.5 ng/mL) did not become insulin free even after ST, whereas all the patients with mildly damaged insulin secretion (ΔCPR > 1.1 ng/mL) remained insulin independent after ST (Table 3). These results may indicate that too severely damaged insulin secretion cannot recover enough with ST. To preserve endocrine function, the earlier ST is started, the more effective it is likely to be. We believe this fact to be very important in considering the indication of ST for AIP. At present, the major indication for ST is the presence of symptoms.[25] However, if asymptomatic patients wait for ST until symptoms become clear, they may experience exacerbation of endocrine function and come to need insulin therapy, which cannot then be recovered easily even with ST. Thus, in light of the pancreatic endocrine function, the indication for ST should be extended to asymptomatic patients at least in the case of impaired glucose tolerance. In connection with ST, earlier introduction of insulin therapy may be necessary to prevent glucose toxicity. Because we experienced 4 patients who became insulin therapy-free during the course, we believe that hesitation is ill placed when considering introduction of insulin therapy as well as ST.

TABLE 4. Transitions of HOMA-β and HOMA-R in 16 Patients With AIP

Case No.*	Age at Onset of AIP, y	DM	IgG4, mg/dL	HOMA-β Before ST	HOMA-β After ST	HOMA-R Before ST	HOMA-R After ST	Hb_{A1c} Before ST	Hb_{A1c} After ST	Condition of Glucose Tolerance	Follow-Up Period After ST, mo
21	58	+	481	15.8	86.9	1.97	4.18	6.2	6.5	Aggravated	69
6	53	+	143	24.8	27.7	0.45	1.58	8.6	7.4	Improved	52
19	76	−	543	27.0	72.0	0.76	2.03	5.7	5.9	Unchanged	14
22	72	−	1400	32.7	37.9	0.42	1.00	5.6	5.9	Unchanged	85
23	61	−	578	32.7	53.6	0.71	1.90	5.4	5.6	Unchanged	75
24	64	−	670	34.6	97.7	1.42	6.24	5.8	5.8	Unchanged	55
9	40	−	650	35.1	45.0	1.03	0.64	5.7	5.7	Unchanged	43
14	73	+	1800	42.4	112.8	0.96	6.74	5.8	5.7	Unchanged	26
25	66	+	310	45.5	44.1	4.68	1.66	6.3	6.4	Unchanged	99
26	49	−	222	49.1	92.3	1.59	2.52	5.4	5.9	Unchanged	117
27	65	+	1260	49.7	129.0	2.39	7.70	5.5	5.7	Unchanged	60
28	55	+	320	52.4	58.4	2.33	1.48	5.8	5.9	Unchanged	101
20	43	−	223	53.3	58.9	0.89	2.62	5.5	5.8	Unchanged	43
18	71	−	458	56.3	43.9	1.17	1.28	5.9	5.4	Aggravated	36
13	65	+	1030	58.4	47.4	1.48	1.25	6.4	6.0	Improved	14
16	56	−	436	60.0	30.0	1.47	1.10	5.5	6.4	Aggravated	51

*Case numbers 6 to 20 correspond to those of Table 3.

In all 7 patients with DM in this table, DM and AIP occurred simultaneously.

HOMA-β(%) = (IRI × 360)/(FPG-63).

HOMA-R = IRI × FPG/405.

FPG indicates fasting plasma glucose (mg/dL); IRI, immunoreactive insulin (μU/mL).

In patients without medication for DM, HOMA-β and HOMA-R are useful for the evaluation of insulin secretion and insulin resistance, respectively. They were assessed in 16 patients in whom glucose tolerance was improved in 2 patients, aggravated in 3 patients, and unchanged in 11 patients. In contrast with the little change in Hb_{A1c} level, the changes in HOMA-β and HOMA-R were very impressive. The result of GTT revealed a short-term effect for improved insulin secretion, whereas the change in HOMA-β indicated that the effect was sustained for a long period at least in those with nonsevere glucose intolerance. Despite improved insulin secretion, the Hb_{A1c} level showed little change, perhaps partly due to the significantly aggravated insulin resistance. Because aggravated insulin resistance may be explained as an adverse effect of steroid, tapering steroid may be effective for glucose intolerance if the condition of AIP is stable. Although "improved insulin secretion and aggravated insulin resistance" is a general trend, it is not necessarily true for all cases. For example, in case 16 (Table 4), aggravated glucose tolerance seems to be attributable to aggravated insulin secretion. Thus, needless to say, when medication is started for DM associated with AIP, HOMA-β and HOMA-R should be assessed on a case-by-case basis for the adequate choice of OHA.

The major weak point of this study was that it was not a prospective study. The GTT was not performed routinely before 2006, and the serum insulin level was not measured routinely before 2010 during follow-up. As a result, we could not analyze HOMA-β and HOMA-R in many patients. In addition, HOMA-β, as well as HOMA-R, is a variable index, and it is inferior to GTT in reliability. In fact, deviation between HOMA-β and ΔCPR before ST was observed in a few cases. In addition, because we have no comparative data about pancreatic endocrine function before and at the onset of AIP, it may be difficult to attribute all dysfunction of insulin secretion to AIP only, which is another limitation of the present study.

In summary, glucose tolerance that is too severely damaged may not recover fully even after ST. Thus, ST should be performed to preserve insulin secretion at the early stage of AIP.

ACKNOWLEDGMENTS

The authors acknowledge of the medical specialists for DM at the Department of Metabolic Diseases, Graduate School of Medicine, at the University of Tokyo Hospital, and at our affiliated hospitals for the control of blood glucose levels.

REFERENCES

1. Shimosegawa T, Chari ST, Frulloni L, et al. International consensus diagnostic criteria for autoimmune pancreatitis: guidelines of the International Association of Pancreatology. *Pancreas*. 2011;40:352–358.
2. Otsuki M, Chung JB, Okazaki K, et al. Asian diagnostic criteria for autoimmune pancreatitis: consensus of the Japan-Korea Symposium on Autoimmune Pancreatitis. *J Gastroenterol*. 2008;43:403–408.
3. Klöppel G, Lüttges J, Löhr M, Zamboni G, et al. Autoimmune pancreatitis: pathological, clinical, and immunological features. *Pancreas*. 2003;27:14–19.
4. Hirano K, Fukushima N, Tada M, et al. Diagnostic utility of biopsy specimens for autoimmune pancreatitis. *J Gastroenterol*. 2009;44:765–773.
5. Hirano K, Shiratori Y, Komatsu Y, et al. An involvement of the biliary system in autoimmune pancreatitis: a follow-up study. *Clin Gastroenterol Hepatol*. 2003;1:453–464.
6. Hirano K, Tada M, Isayama H, et al. Endoscopic evaluation of factors contributing to intrapancreatic biliary stricture in autoimmune pancreatitis. *Gastrointest Endosc*. 2010;71:85–90.
7. Hirano K, Kawabe T, Komatsu Y, et al. High-rate pulmonary involvement in autoimmune pancreatitis. *Intern Med J*. 2006;36:58–61.
8. Nishi H, Tojo A, Onozato ML, et al. Anti–carbonic anhydrase II antibody in autoimmune pancreatitis and tubulointerstitial nephritis. *Nephrol Dial Transplant*. 2007;22:1273–1275.
9. Hirano K, Tada M, Isayama H, et al. Long-term prognosis of autoimmune pancreatitis without and with corticosteroid treatment. *Gut*. 2007;56:1719–1724.
10. Ito T, Kawabe K, Arita Y, et al. Evaluation of pancreatic endocrine and exocrine function in patients with autoimmune pancreatitis. *Pancreas*. 2007;34:254–259.
11. Nishimori I, Tamakoshi A, Kawa S, et al. Research Committee on Intractable Pancreatic Diseases, the Ministry of Health and Welfare of Japan. Influence of steroid therapy on the course of diabetes mellitus in patients with autoimmune pancreatitis: findings from a nationwide survey in Japan. *Pancreas*. 2006;32:244–248.
12. Nishino T, Toki F, Oyama H, et al. Long-term outcome of autoimmune pancreatitis after oral prednisolone therapy. *Intern Med*. 2006;45:497–501.
13. Kamisawa T, Yoshiike M, Egawa N, et al. Treating patients with autoimmune pancreatitis: results from a long-term follow-up study. *Pancreatology*. 2005;5:234–238.
14. Ito T, Nishimori I, Inoue N, et al. Treatment for autoimmune pancreatitis: consensus on the treatment for patients with autoimmune pancreatitis in Japan. *J Gastroenterol*. 2007;42(suppl 180):50–58.
15. Vague P, Nguyen L. Rationale and methods for the estimation of insulin secretion in a given patient: from research to clinical practice. *Diabetes*. 2002;51(suppl 1):S240–S244.
16. Prando R, Odetti P, Melga P, et al. Progressive deterioration of beta-cell function in nonobese type 2 diabetic subjects. Postprandial plasma C-peptide level is an indication of insulin dependency. *Diabetes Metab*. 1996;22:185–191.
17. Weinstein SP, Wilson CM, Pritsker A, et al. Dexamethasone inhibits insulin-stimulated recruitment of GLUT4 to the cell surface in rat skeletal muscle. *Metabolism*. 1998;47:3–6.
18. Dimitriadis G, Leighton B, Parry-Billings M, et al. Effects of glucocorticoid excess on the sensitivity of glucose transport and metabolism to insulin in rat skeletal muscle. *Biochem J*. 1997;321:707–712.
19. Saad MJ, Folli F, Araki E, et al. Regulation of insulin receptor, insulin receptor substrate-1 and phosphatidylinositol 3-kinase in 3T3-F442A adipocytes. Effects of differentiation, insulin, and dexamethasone. *Mol Endocrinol*. 1994;8:545–557.
20. Sakoda H, Ogihara T, Anai M, et al. Dexamethasone-induced insulin resistance in 3T3-L1 adipocytes is due to inhibition of glucose transport rather than insulin signal transduction. *Diabetes*. 2000;49:1700–1708.
21. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*. 1997;20:1183–1197.
22. Ito T, Nakamura T, Fujimori N, et al. Characteristics of pancreatic diabetes in patients with autoimmune pancreatitis. *J Dig Dis*. 2011;12(3):210–216.
23. Seino Y, Nanjo K, Tajima N, et al. Report of the committee on the classification and diagnostic criteria of diabetes mellitus. *Diabetol Int*. 2010;1:2–20.
24. Kishitani Y. Clinical laboratory examination of diabetic patients in conjunction with metabolic syndrome [in Japanese]. *Rinsho Byori*. 2009;57:1066–1074.
25. Kamisawa T, Shimosegawa T, Okazaki K, et al. Standard steroid treatment for autoimmune pancreatitis. *Gut*. 2009;58:1504–1507.

ARTHRITIS & RHEUMATISM
Vol. 64, No. 3, March 2012, pp 914–924
DOI 10.1002/art.33386

Involvement of Activation of Toll-like Receptors and Nucleotide-Binding Oligomerization Domain–like Receptors in Enhanced IgG4 Responses in Autoimmune Pancreatitis

Tomohiro Watanabe,[1] Kouhei Yamashita,[1] Saori Fujikawa,[1] Toshiharu Sakurai,[2] Masatoshi Kudo,[2] Masahiro Shiokawa,[1] Yuzo Kodama,[1] Kazushige Uchida,[3] Kazuichi Okazaki,[3] and Tsutomu Chiba[1]

Objective. **IgG4-related disease is a recently recognized entity affecting multiple organs, including the pancreas, biliary tracts, and salivary glands. Although IgG4-related disease is characterized by systemic IgG4 antibody responses and by infiltration of IgG4-expressing plasma cells, the innate immune responses leading to adaptive IgG4 antibody responses are poorly understood. The aim of this study was to clarify the innate immune responses leading to IgG4 antibody production.**

Methods. **IgG4 and cytokine responses to various nucleotide-binding oligomerization domain (NOD)–like receptor (NLR) and Toll-like receptor (TLR) ligands were examined using peripheral blood mononuclear cells (PBMCs) from healthy control subjects and patients with IgG4-related autoimmune pancreatitis.**

Results. **Activation of NOD-2 in monocytes from healthy control subjects induced IgG4 production by B cells in a BAFF-dependent and T cell–independent manner. In addition, PBMCs from patients with IgG4-related disease produced a large amount of IgG4 upon stimulation with NLR and TLR ligands; this enhanced IgG4 production was associated with the induction of BAFF by NLR and TLR ligands. Monocytes from patients with IgG4-related disease induced IgG4 production by B cells from healthy control subjects upon stimulation with NLR and TLR ligands.**

Conclusion. **The results of these studies suggest that abnormal innate immune responses against microbial antigens may underlie the immunopathogenesis of IgG4-related disease.**

Autoimmune pancreatitis is a unique form of chronic pancreatitis, and autoimmune reactions are considered to be involved in its pathogenesis (1,2). Currently, the diagnosis of autoimmune pancreatitis is based on characteristic clinical findings such as hypergammaglobulinemia, irregular narrowing of the main pancreatic duct, and lymphoplasmacytic infiltration of the pancreas associated with fibrosis (3). Another important feature of autoimmune pancreatitis is the variety of extrapancreatic manifestations (4). Sclerosing cholangitis, sialoadenitis, and retroperitoneal fibrosis are sometimes seen in patients with autoimmune pancreatitis. In addition to the clinicopathologic findings described above, most patients with autoimmune pancreatitis have elevated serum levels of IgG4 (5). Moreover, abundant infiltration of IgG4-expressing plasmacytes is observed in both pancreatic and extrapancreatic lesions (4,6). Based on these recent findings, it is generally accepted that an enhanced systemic IgG4 response is one of the most important immunologic features in autoimmune

Supported in part by the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research (21590532), the Takeda Science Foundation, the Astellas Foundation for Research on Metabolic Disorders, the Yakult Bioscience Foundation, the Cell Science Research Foundation, the Kato Memorial Trust for Nambyo Research, the Pancreas Research Foundation of Japan, the Uehara Memorial Foundation (to Dr. Watanabe), and the Health and Labor Sciences Research Grants for Research on Intractable Diseases from the Ministry of Health, Labor and Welfare, Japan (to Dr. Chiba).

[1]Tomohiro Watanabe, MD, PhD, Kouhei Yamashita, MD, PhD, Saori Fujikawa, BS, Masahiro Shiokawa, MD, Yuzo Kodama, MD, PhD, Tsutomu Chiba, MD, PhD: Kyoto University Graduate School of Medicine, Kyoto, Japan; [2]Toshiharu Sakurai, MD, PhD, Masatoshi Kudo, MD, PhD: Kinki University Graduate School of Medicine, Osaka-Sayama, Osaka, Japan; [3]Kazushige Uchida, MD, PhD, Kazuichi Okazaki, MD, PhD: Kansai Medical University, Hirakata, Osaka, Japan.

Address correspondence to Tomohiro Watanabe, MD, PhD, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoin Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: tmhrwtnb@kuhp.kyoto-u.ac.jp.

Submitted for publication April 27, 2011; accepted in revised form September 27, 2011.

pancreatitis, and that autoimmune pancreatitis is considered to be a pancreatic manifestation of systemic IgG4-related disease (3,4) and IgG4-positive multiorgan lymphoproliferative syndrome (7).

Our present understanding of the immunopathogenesis of autoimmune pancreatitis is limited. Although the presence of autoantibodies against carbonic anhydrase and lactoferrin in the serum of patients with autoimmune pancreatitis suggests the involvement of excessive autoimmune responses (1,2), pathogenic antigens have not been identified. In addition, it remains unknown whether an enhanced IgG4 response is responsible for the development of autoimmune pancreatitis, or whether it is an epiphenomenon associated with inflammatory reactions. Given the fact that a significant population of patients with autoimmune pancreatitis have a diagnosis of inflammatory bowel disease, the development of which requires excessive immune reactions to intestinal microflora (8–10), it is possible that enhanced immune responses toward microbial antigens underlie the immunopathogenesis of autoimmune pancreatitis. Consistent with this idea, an accumulation of plasma cells expressing IgG4 was observed in the gastrointestinal tract of patients with autoimmune pancreatitis (11). Moreover, our group recently reported 2 cases of autoimmune pancreatitis in patients whose peripheral blood mononuclear cells (PBMCs) exhibited enhanced Th2 cytokine responses upon stimulation with microbial antigens (12,13).

Antigens derived from the intestinal microflora activate the host innate immune system via pattern recognition receptors such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization (NOD)–like receptors (NLRs) (10,14). Antigen-presenting cells (APCs) interact with B cells to induce IgG and IgA production (14). Interestingly, the activation of TLRs and NLRs in APCs, including dendritic cells, monocytes, and macrophages, leads to immunoglobulin class switching through production of BAFF and APRIL (15,16). However, the roles played by APCs expressing TLRs and NLRs in IgG4 responses are poorly defined.

In this study, we sought to determine how the activation of TLRs and NLRs in APCs enhances IgG4 responses linked to autoimmune pancreatitis. NOD-2 is an intracellular NLR for small peptides derived from the bacterial cell wall component peptidoglycan (PGN) (10). Here, we provide evidence that NOD-2 activation in monocytes enhances production of IgG4 from B cells via secretion of BAFF in a T cell–independent manner, in healthy control subjects. Moreover, we report that PBMCs from patients with autoimmune pancreatitis exhibited enhanced production of IgG4 upon stimulation with NLR and TLR ligands. Thus, we propose that excessive innate immune responses to microbial antigens may underlie the immunopathogenesis of IgG4-related autoimmune pancreatitis.

PATIENTS AND METHODS

Stimulation of cells with a broad range of microbial antigens. PBMCs (2×10^6/ml) from healthy control subjects were stimulated with FK-156 (20 μg/ml; Astellas Pharma), muramyldipeptide (MDP) (20 μg/ml; InvivoGen), PGN (10 μg/ml; Sigma-Aldrich), palmitoyl-3-cysteine-serine-lysine-4 (Pam_3CSK_4) (1 μg/ml; InvivoGen), double-stranded RNA (dsRNA) (25 μg/ml; InvivoGen), lipopolysaccharide (LPS) (1 μg/ml; Sigma-Aldrich), flagellin (1 μg/ml; InvivoGen), and R837 (50 μg/ml; InvivoGen) for 14 days in complete RPMI medium, as previously described (17,18). In some experiments, CD3+ T cells, CD14+ monocytes, and CD19+ B cells (1×10^6/ml) were isolated from PBMCs by positive selection using anti-human CD3, CD14, and CD19 microbeads, respectively (Miltenyi Biotech) (19). Depletion of CD14+ monocytes was performed by negative selection, using anti-CD14 microbeads. The isolated cell populations were cultured with the indicated doses of a neutralizing anti-BAFF monoclonal antibody (R&D Systems) or a mouse IgG control antibody (eBioscience). CD14+ monocytes were incubated with 10 μ*M* SB203580 (p38 and RICK inhibitor), PD98059 (ERK inhibitor), SP600125 (JNK inhibitor), or BAY 11-7082 (NF-κB inhibitor) (all from InvivoGen) for 1 hour, followed by overnight stimulation with MDP (20 μg/ml). Culture supernatants were collected at the indicated time points.

Enzyme-linked immunosorbent assays (ELISAs). Culture supernatants were assayed for the measurement of interleukin-6 (IL-6), IL-8, IL-12p40, IL-17, tumor necrosis factor (TNF), interferon-γ (IFNγ), APRIL, and BAFF, using ELISA kits (PharMingen and eBioscience). The production of IgG1 and IgG4 was determined by ELISAs. Anti-human IgG1 and IgG4 antibodies (PharMingen) were used as capture antibodies, and horseradish peroxidase (HRP)–labeled anti-human IgG antibody (PharMingen) was used for detection. The production of IgA and IgE was determined using an ELISA (Bethyl Laboratories).

Fluorescence-activated cell sorter analysis. PBMCs were preincubated with Fc-blocking solution (Miltenyi Biotech), stained with fluorescein isothiocyanate–conjugated anti-human CD14 or CD19 antibody (both from eBioscience) and phycoerythrin-labeled anti-human BAFF (R&D Systems), APRIL (BioLegend), B cell maturation antigen (BCMA; R&D Systems), HLA–DR, CD40, CD80, CD86 (all from eBioscience), inducible costimulator (ICOS) ligand (eBioscience), CD163 (BioLegend), BAFF receptor (BAFF-R; BioLegend), and TACI (BioLegend). An intracellular staining kit (PharMingen) was used for intracellular staining of BAFF and APRIL. Stained cells were analyzed on an Accuri C6 cytometer (Accuri Cytometers).

Immunoblotting. CD14+ monocytes from healthy control subjects were stimulated with MDP (20 μg/ml). At the indicated time points, protein extracts were isolated and

subjected to immunoblotting as described previously (18). The primary antibodies used in this study were as follows: actin (Santa Cruz Biotechnology), phospho-p38, total p38, phospho-ERK, total ERK, phospho-JNK, total JNK, and total IκBα (Cell Signaling Technology). To detect NOD-2 expression, cells were first immunoprecipitated with monoclonal NOD-2 antibody (eBioscience) followed by immunoblotting with polyclonal NOD-2 antibody (Santa Cruz Biotechnology).

Small interfering RNA (siRNA) study. MonoMac6 cells (1×10^6/ml), a human monocytic cell line, were transfected with control siRNA or NOD-2–specific siRNA (25 n*M*; Santa Cruz Biotechnology), using TransIT-TKO transfection reagent (Mirus). Cells were stimulated with MDP (20 μg/ml) for 36 hours after transfection and then incubated for an additional 24 hours.

NF-κB activation assay. Nuclear extracts were prepared using an extraction kit (Clontech), and the binding activity of nuclear extract to NF-κB p50, p65, c-Rel, p52, and RelB was measured using a TransAM NF-κB kit (Active Motif). Nuclear extracts (10 μg) isolated from monocytes stimulated with MDP (20 μg/ml) for 30 minutes were applied to each well coated with NF-κB consensus oligonucleotides and then incubated with rabbit anti-p50, anti-p65, anti–c-Rel, anti-p52, and anti-RelB antibodies followed by HRP-labeled anti-rabbit IgG.

Studies using peripheral blood cells from patients with autoimmune pancreatitis. Ethics permission for this study was granted by the review board of Kyoto University. Healthy control subjects (n = 24) and treatment-naive patients with IgG4-related autoimmune pancreatitis (n = 8) were enrolled in this study after informed consent was obtained. PBMCs, CD14+ monocytes, and CD19+ B cells were isolated from the patients and stimulated with a broad range of microbial antigens, as described above.

Statistical analysis. Student's *t*-test was used to evaluate statistical significance. Statistical analysis was performed with the StatView version 4.5 program (Abacus Concepts). *P* values less than 0.05 were considered significant.

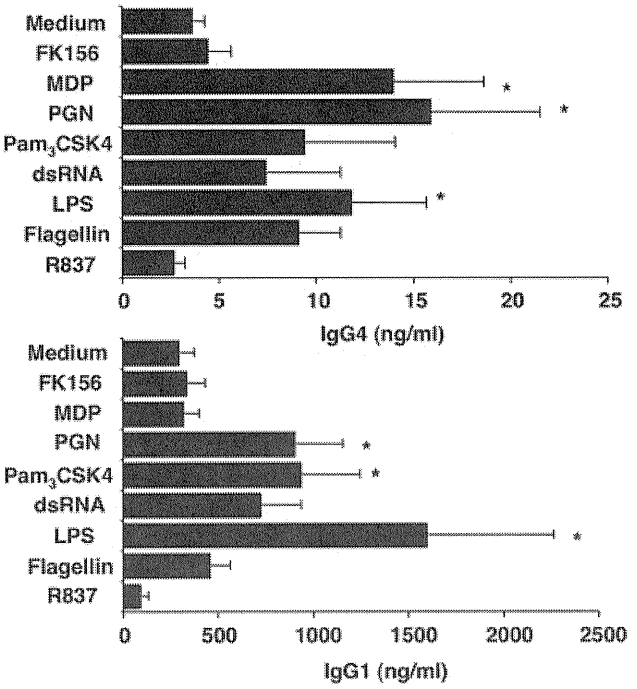

Figure 1. Muramyldipeptide (MDP)–induced activation of nucleotide-binding oligomerization domain 2 (NOD-2) enhances IgG4 production by peripheral blood mononuclear cells (PBMCs) from healthy control subjects. PBMCs (2×10^6/ml) from healthy control subjects (n = 14) were stimulated with a broad range of NOD-like receptor (NLR) and Toll-like receptor (TLR) ligands (FK-156, 20 μg/ml; MDP, 20 μg/ml; peptidoglycan [PGN], 10 μg/ml; palmitoyl-3-cysteine-serine-lysine-4 [Pam_3CSK_4], 1 μg/ml; double-stranded RNA [dsRNA], 25 μg/ml; lipopolysaccharide [LPS], 1 μg/ml; flagellin, 1 μg/ml; R837, 50 μg/ml) for 14 days. Production of IgG4 and IgG1 in the culture supernatants was determined by enzyme-linked immunosorbent assay. Bars show the mean ± SEM. * = $P < 0.05$ versus medium.

RESULTS

NOD-2–induced IgG4 production by PBMCs from healthy controls. Although an enhanced IgG4 response is one of the most important immunologic features in patients with autoimmune pancreatitis (4,5), the molecular mechanisms accounting for increased production of this IgG subtype in patients with autoimmune pancreatitis are poorly understood. To address the role of microbial antigens in the production of IgG4, PBMCs from healthy control subjects were stimulated with a broad range of TLR and NLR ligands (FK-156 [NOD-1 ligand], MDP [NOD-2 ligand], Pam_3CSK_4 [TLR-1/2 ligand], PGN [TLR-2/6 ligand], dsRNA [TLR-3 ligand], LPS [TLR-4 ligand], flagellin [TLR-5 ligand], and R837 [TLR-7 ligand]) for 14 days.

Activation of NOD-2 by MDP induced the production of IgG4, but not IgG1, by PBMCs from healthy control subjects (Figure 1). In contrast, activation of TLR-2 and TLR-4 induced the production of both IgG1 and IgG4. Thus, these data suggest that activation of NOD-2 enhances the IgG4 response rather than the IgG1 response. Because the regulation of IgG4 production is dependent on Th2 cytokines (20), we evaluated the profiles of cytokines induced by MDP. Stimulation with MDP did not induce production of Th1-associated cytokines such as IFNγ and IL-12p40, Th2-associated cytokines such as IL-4 and IL-13, or Th17-associated cytokines such as IL-6 and IL-17 (data not shown). These results suggested that IgG4 production by MDP is not associated with typical Th1, Th2, or Th17 cell responses. Significant production of IL-10 was induced in PBMCs stimulated with MDP. All of the TLR and NLR ligands enhanced IL-8 production, which suggests that the doses of microbial antigens used in this study were sufficient to cause innate immune responses.

T cell–independent IgG4 production by NOD-2. We next determined the types of cells that are involved in enhanced IgG4 production through NOD-2 activation. Initially, we isolated CD3+ T cells, CD19+ B cells, and CD14+ monocytes and then examined the types of cells expressing NOD-2 at the protein level. Immunoprecipitation followed by immunoblotting with anti–NOD-2 antibody revealed that NOD-2 expression was limited to CD14+ monocytes (Figure 2A). Consistent with this NOD-2 expression in monocytes, the depletion of CD14+ cells from PBMCs reduced IgG4 production stimulated by MDP-induced activation of NOD-2 (data not shown). These results suggested that activation of monocytes expressing NOD-2 is responsible for MDP-induced IgG4 production.

To confirm these results, we assessed IgG4 production by CD19+ B cells cocultured with CD14+ monocytes or CD3+ T cells. MDP-induced IgG4 production was observed in the coculture containing both B cells and monocytes (Figure 2B). In contrast, the addition of CD3+ T cells to the culture reduced MDP-induced IgG4 production. These results showed that the presence of monocytes and B cells is sufficient for MDP-induced IgG4 production, and that NOD-2 activation induces IgG4 production in a T cell–independent manner. The production of IgG1, IgA, and IL-10 was comparable in the coculture containing monocytes and B cells, regardless of whether cells were activated with MDP. Furthermore, the optimal induction of IL-10 by MDP required B cells, T cells, and monocytes, suggesting that T cells are the main producers of this cytokine. Taken together, these data suggested that stimulation of MDP activates NOD-2 in monocytes and then induces IgG4 production by B cells in a T cell–independent manner.

We examined regulation of the expression of surface markers in monocytes stimulated with MDP. MDP-induced activation of NOD-2 enhanced cell surface expression of HLA–DR and costimulatory molecules such as CD40, CD80, and CD86, whereas no significant change was observed in the cell surface expression of CD163 or ICOS ligand (data not shown).

IgG4 production by NOD-2 activation of BAFF signaling pathways. We next examined the effects of NOD-2 activation in monocytes on the survival of B cells. The percentage of annexin V–positive apoptotic B cells was significantly reduced in PBMCs stimulated with MDP (Figure 3A). These results suggested that MDP-induced activation of NOD-2 in monocytes enhances the survival of CD19+ B cells by inhibiting apoptotic cell death.

The TNF family members BAFF and APRIL are crucial survival factors for peripheral B cells (21,22). Innate immune cells such as APCs and epithelial cells produce BAFF and APRIL to cause T cell–independent immunoglobulin responses (15,16). Therefore, we ad-

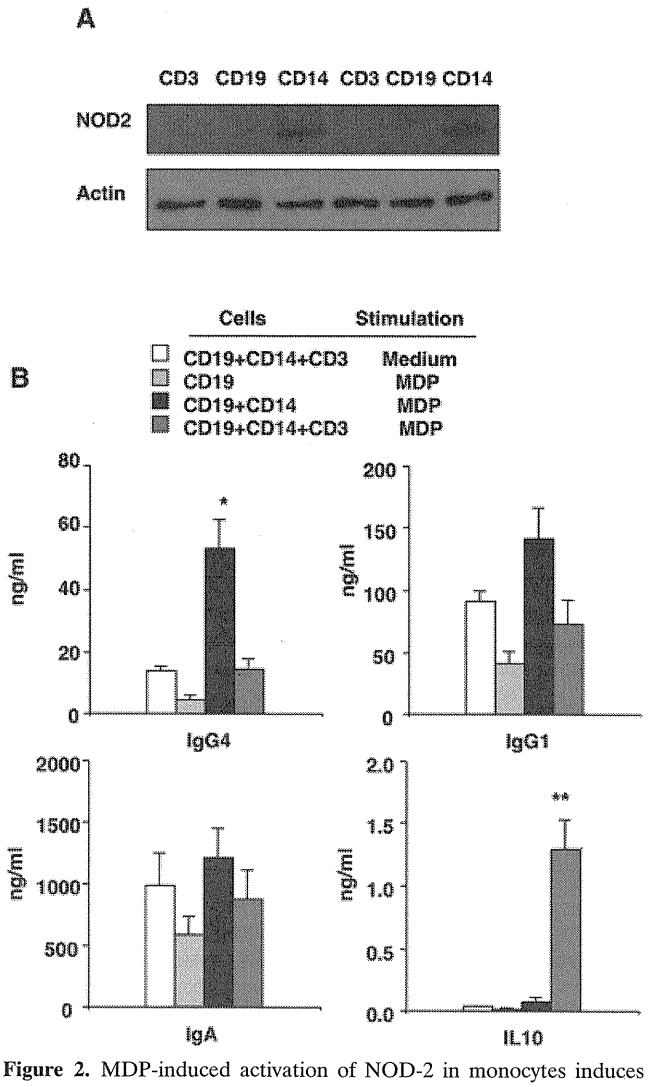

Figure 2. MDP-induced activation of NOD-2 in monocytes induces IgG4 production by B cells in a T cell–independent manner. **A,** CD3+ T cells, CD14+ monocytes, and CD19+ B cells were purified from the PBMCs of healthy control subjects. Protein extracts were isolated from CD3+ T cells, CD19+ B cells, and CD14+ monocytes and then subjected to immunoprecipitation with anti–NOD-2 antibody, followed by immunoblotting with anti–NOD-2 antibody. Expression of NOD-2 by immunoblotting using 2 healthy controls is shown. **B,** CD19+ B cells (1×10^6/ml) from healthy control subjects (n = 6) were cocultured with CD14+ monocytes (1×10^6/ml) and/or CD3+ T cells (1×10^6/ml) in the presence of MDP (20 μg/ml) for 14 days. Culture supernatants were analyzed for the production of IgG4, IgG1, IgA, and interleukin-10 (IL-10). Bars show the mean ± SEM. * = $P < 0.05$; ** = $P < 0.01$ versus the 3 other coculture conditions. See Figure 1 for other definitions.

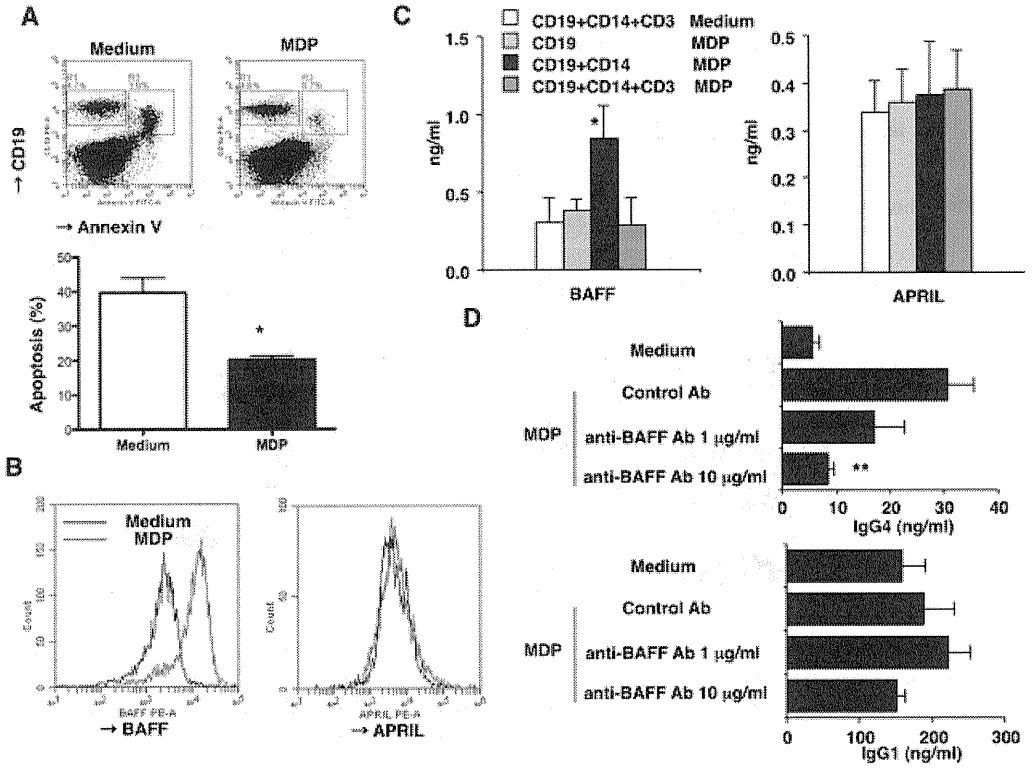

Figure 3. MDP-induced activation of NOD-2 in monocytes induces IgG4 production in a BAFF-dependent manner. **A,** PBMCs from healthy control subjects were stimulated with MDP for 24 hours and then stained with phycoerythrin (PE)–conjugated anti-CD19 antibody (Ab) and fluorescein isothiocyanate (FITC)–conjugated annexin V. The percentage of apoptotic annexin V–positive CD19+ B cells was decreased by stimulation with MDP. * = $P < 0.05$ versus medium. **B,** CD14+ monocytes from healthy control subjects were stimulated with MDP for 24 hours, and intracellular expression of BAFF and APRIL was determined by flow cytometry. **C,** CD19+ B cells from healthy control subjects (n = 6) were cocultured with CD14+ monocytes and/or CD3+ T cells in the presence of MDP for 24 hours. Culture supernatants were analyzed for the production of BAFF and APRIL. * = $P < 0.05$ versus other coculture conditions. **D,** CD19+ B cells from healthy control subjects (n = 6) were cocultured with CD14+ monocytes in the presence of neutralizing anti-BAFF antibody or control antibody for 14 days. Culture supernatants were analyzed for the production of IgG4 and IgG1. ** = $P < 0.01$ versus control. Bars in **A, C,** and **D** show the mean ± SEM. See Figure 1 for other definitions.

dressed whether MDP-induced activation of NOD-2 stimulates production of BAFF and/or APRIL and thereby enhances IgG4 production. Intracellular staining revealed a marked increase in BAFF, but not APRIL, expression in monocytes stimulated with MDP (Figure 3B). We next determined whether MDP-mediated BAFF production depends on NOD-2 activation. For this purpose, we used MonoMac6 cells, a human monocytic cell line, because of the very low efficiency of gene transfection in primary monocytes. MDP-mediated BAFF production was reduced in MonoMac6 cells transfected with NOD-2–specific siRNA, which suggested NOD-2–dependent BAFF production upon stimulation with MDP (data not shown).

The production of BAFF in the coculture containing B cells and monocytes was enhanced upon stimulation with MDP (Figure 3C). More importantly, abrogation of BAFF signaling by its neutralizing antibody inhibited the production of IgG4 in a dose-dependent manner (Figure 3D). BAFF binds to the receptors BCMA, TACI, and BAFF-R, which are expressed on the cell surface of B cells (21,22). Stimulation of PBMCs with MDP enhanced cell surface expression of TACI on B cells (data not shown), suggesting that the interaction between TACI and BAFF is involved in enhanced IgG4 production mediated by NOD-2 activation. Consistent with these data, the addition of BAFF alone led to a marked increase in IgG4 production by CD19+ B cells (data not shown). These results suggested a novel mechanism of IgG4 production mediated by NOD-2 activation: monocytes produce BAFF upon

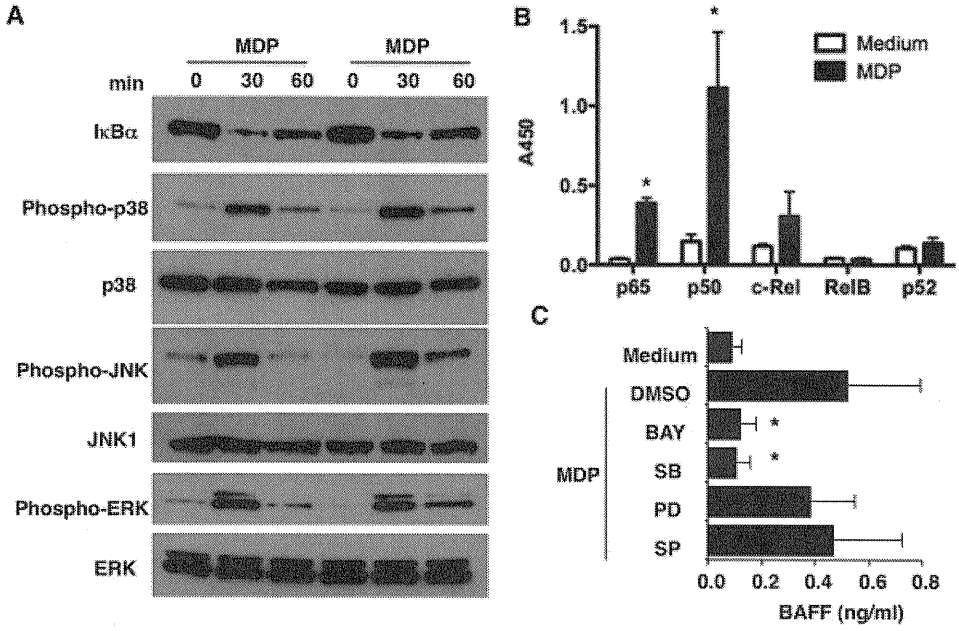

Figure 4. MDP-induced activation of NOD-2 in monocytes stimulates BAFF production via NF-κB activation. **A,** CD14+ monocytes from 2 healthy control subjects were stimulated with MDP, and protein extracts were isolated at the indicated time points. Whole extracts were subjected to immunoblotting with anti-IκBα, phospho-p38, p38, phospho-JNK, JNK-1, phospho-ERK, and ERK antibodies. **B,** Nuclear extracts were isolated from monocytes stimulated with MDP (20 μg/ml) for 30 minutes. The activation of NF-κB subunits in the nuclear extracts was determined. * = $P <$ 0.05 versus medium. **C,** CD14+ monocytes (1×10^6/ml) from healthy control subjects (n = 4) were preincubated with 10 μ*M* BAY 11-7082 (BAY, NF-κB inhibitor), SB203580 (SB; p38 and RICK inhibitor), PD98059 (PD; ERK inhibitor), or SP600125 (SP; JNK inhibitor) for 1 hour, followed by overnight stimulation with MDP (20 μg/ml). Culture supernatants were analyzed for production of BAFF. * = $P < 0.05$ versus DMSO. Bars in **B** and **C** show the mean ± SEM. See Figure 1 for other definitions.

NOD-2 activation by MDP, and BAFF induce IgG4 production via interaction between BAFF and TACI.

NF-κB–dependent BAFF production by NOD-2. We next examined the signaling pathway involved in NOD-2–induced BAFF production in monocytes. Previous studies have established that NOD-2 activation results in interactions between the CARD domain of the NOD molecule and the CARD domain of a downstream effector molecule, the serine/threonine kinase RICK; activated RICK generated in this manner activates NF-κB and MAP kinases (MAPKs) (10). Stimulation of monocytes with MDP led to degradation of IκBα and enhanced expression of the phosphorylated forms of p38, JNK, and ERK (Figure 4A). In addition, the binding of NF-κB subunits p65 and p50 to consensus sequences was markedly enhanced in nuclear extracts isolated from monocytes upon stimulation with MDP (Figure 4B). In contrast, there was no significant difference in the binding of c-Rel, RelB, or p52 in the nuclear extracts either with or without MDP stimulation. These results suggested that MDP-induced activation of NOD-2 results in the activation of NF-κB and MAPK in monocytes.

In subsequent studies to determine which of the previously established components of NOD-2 signaling were relevant to BAFF production, monocytes were preincubated with various inhibitors specific to these pathways, including PD98059 (ERK inhibitor), SB203580 (p38 and RICK inhibitor), SP600125 (JNK inhibitor), and BAY 11-7082 (NF-κB inhibitor) and then stimulated with MDP. The addition of BAY 11-7082 or SB203580 to cultures led to reduced BAFF production by monocytes (Figure 4C). In contrast, the addition of PD98059 or SP600125 had no inhibitory effects on BAFF production. To exclude toxic effects of these inhibitors, we performed a gene-silencing study. Transfection of p65 siRNA into MonoMac6 cells led to a substantial reduction in BAFF production upon stimulation with MDP as compared with cells transfected with control siRNA (data not shown). Taken together, these results suggested that BAFF production by MDP is induced by the activation of NF-κB through RICK.

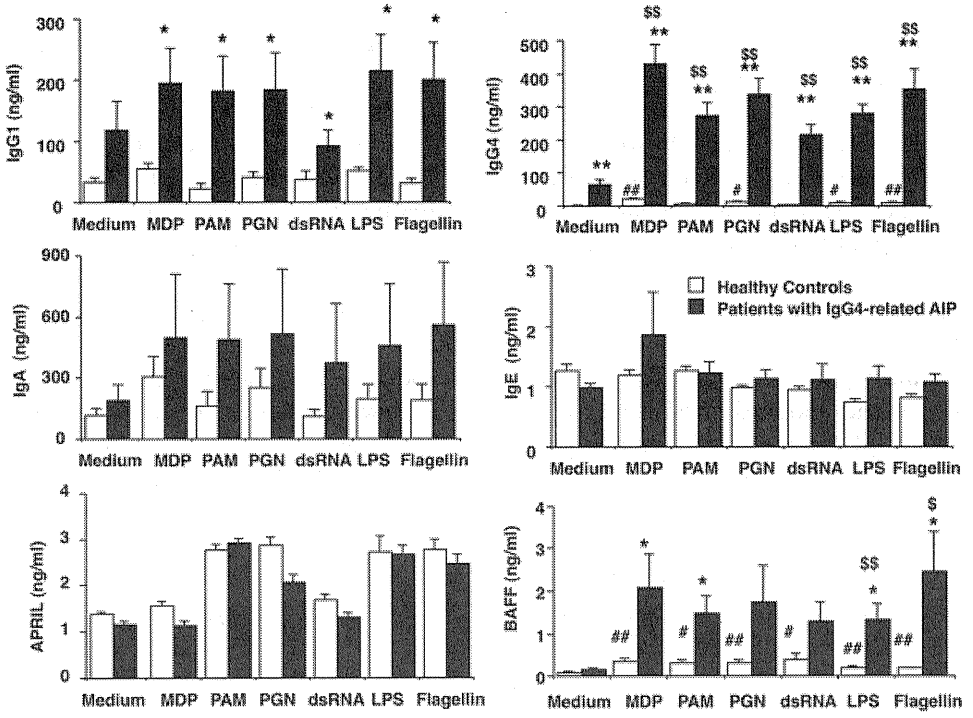

Figure 5. Nucleotide-binding oligomerization domain–like receptor (NLR) and Toll-like receptor (TLR) ligands induce production of BAFF and IgG4 by peripheral blood mononuclear cells (PBMCs) from patients with IgG4-related autoimmune pancreatitis (AIP). PBMCs (1×10^6/ml) from healthy control subjects (n = 10) and patients with IgG4-related autoimmune pancreatitis (n = 8) were stimulated with a broad range of NLR and TLR ligands for 14 days, as described in Figure 1. Culture supernatants obtained at 14 days were assayed for the presence of IgG1, IgG4, IgA, and IgE. Culture supernatants obtained at 2 days were assayed for the presence of APRIL and BAFF. Bars show the mean ± SEM. * = $P < 0.05$ and ** = $P < 0.01$ versus healthy controls; # = $P < 0.05$ and ## = $P < 0.01$ versus medium; $ = $P < 0.05$ and $$ = $P < 0.01$ versus medium. MDP = muramyldipeptide; PAM = palmitoyl-3-cysteine-serine-lysine-4; PGN = peptidoglycan; dsRNA = double-stranded RNA; LPS = lipopolysaccharide.

IgG4 production by PBMCs from patients with IgG4-related autoimmune pancreatitis via activation of NLRs and TLRs. As mentioned above, we identified a novel pathway of NOD-2–mediated IgG4 production by using PBMCs from healthy control subjects. To establish the significance of this finding in the immunopathogenesis of IgG4-related disease, we compared the immune responses of PBMCs from patients with IgG4-related autoimmune pancreatitis with those of PBMCs from healthy controls. Patients enrolled in this study met the criteria for the diagnosis of autoimmune pancreatitis (23,24) and were diagnosed as having lymphoplasmacytic sclerosing pancreatitis. The mean ± SD serum levels of IgG4 and IgG1 in patients enrolled in this study were 665 ± 232 mg/dl (normal range 4.8–105) and 1,050 ± 567 mg/dl (normal range 320–748), respectively. PBMCs from healthy control subjects and patients were stimulated with NLR and TLR ligands to measure the production of immunoglobulins and cytokines.

Basal production of IgG4 by PBMCs was markedly elevated in patients compared with controls, and this enhanced IgG4 production by patient PBMCs was augmented in the presence of a broad range of NLR and TLR ligands (Figure 5, top). Although the activation of NOD-2 preferentially induces IgG4 production in healthy PBMCs, not only NOD-2 ligands (MDP) but also TLR ligands (Pam_3CSK_4 and PGN [TLR-2 ligands], dsRNA [TLR-3 ligand], LPS [TLR-4 ligand], and flagellin [TLR-5 ligand]) efficiently induced IgG4 production by PBMCs from patients. Similar results were observed for IgG1, although the difference between control subjects and patients was much smaller than the differences observed for IgG4 (Figure 5, top).

PBMCs from patients and control subjects produced comparable levels of IgA and IgE upon stimulation with NLR and TLR ligands (Figure 5, middle). Interestingly, BAFF production by PBMCs from patients was much higher than that by PBMCs from

control subjects upon stimulation with NOD-2 and TLR ligands (Figure 5, bottom). In contrast, stimulation of PBMCs with NLR and TLR ligands induced comparable levels of APRIL in control subjects and patients (Figure 5, bottom). These results suggested that PBMCs from patients with IgG4-related autoimmune pancreatitis produce a large amount of IgG4 upon stimulation with NLR and TLR ligands, and that such enhancement of IgG4 production is associated with BAFF induced by NLR and TLR ligands.

Defective Th1 cell responses in patients with IgG4-related autoimmune pancreatitis. We next evaluated the profiles of cytokines induced by NLR and TLR ligands in patients with IgG4-related autoimmune pancreatitis. In patients compared with controls, IFNγ production by PBMCs was markedly reduced upon stimulation with TLR-2, TLR-3, TLR-4, and TLR-5 ligands (for PAM, mean ± SEM 229 ± 61 pg/ml versus 1,184 ± 290 pg/ml; for dsDNA, 260 ± 82 pg/ml versus 3,810 ± 1,400 pg/ml; for LPS, 1,690 ± 974 pg/ml versus 4,530 ± 929 pg/ml; for flagellin, 872 ± 530 pg/ml versus 4,110 ± 960 pg/ml). Stimulation with MDP induced comparable levels of IFNγ production by PBMCs from controls and those from patients (data not shown). Given the fact that IFNγ is a prototypical Th1 cytokine, these results suggested that PBMCs from patients with IgG4-related autoimmune pancreatitis show defective Th1 cell responses to TLR ligands. Consistent with this reduced IFNγ production, the production of IL-12p40, which is associated with Th1 cell responses, was significantly reduced in patients compared with controls upon stimulation with TLR-2 or TLR-4 ligand (for PAM, mean ± SEM 130 ± 18 pg/ml versus 456 ± 91 pg/ml; for LPS, 344 ± 58 pg/ml versus 670 ± 128 pg/ml). In addition, IL-6 production by TLR-2 and TLR-5 ligands was reduced in PBMCs from patients compared with controls (for PAM, mean ± SEM 3,491 ± 554 pg/ml versus 7,664 ± 848 pg/ml; for

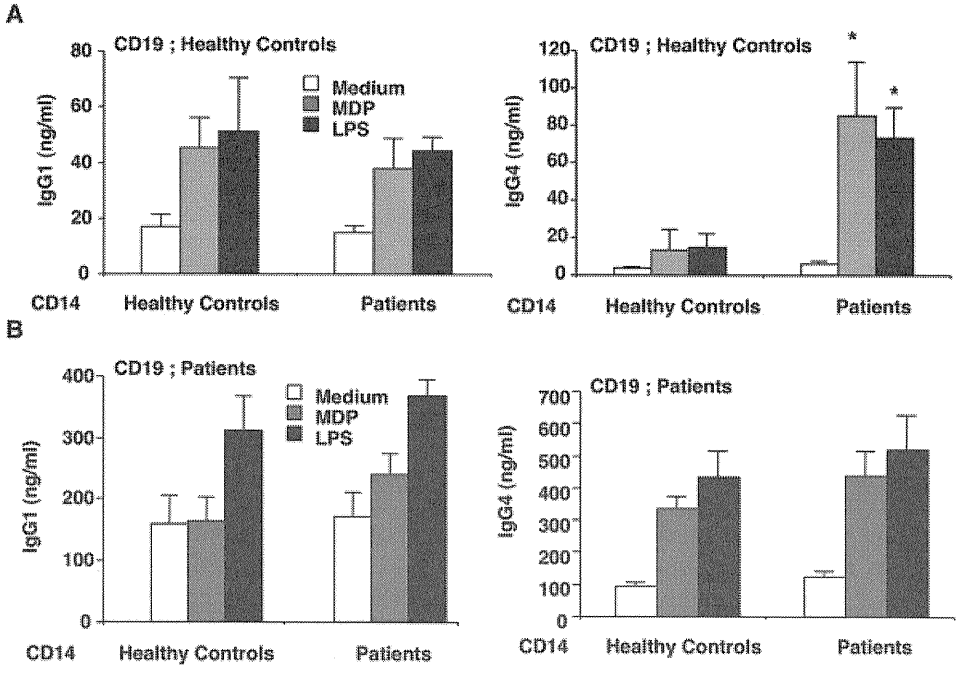

Figure 6. Monocytes isolated from patients with IgG4-related autoimmune pancreatitis induce IgG4 production by B cells from healthy controls upon stimulation with MDP and LPS. **A,** CD19+ B cells (1×10^6/ml) from healthy control subjects (n = 4) were cocultured with CD14+ monocytes (1×10^6/ml) from control subjects (n = 4) or patients with IgG4-related autoimmune pancreatitis (n = 4) in the presence of MDP (20 μg/ml) or LPS (1 μg/ml) for 14 days. **B,** CD19+ B cells (1×10^6/ml) from patients with IgG4-related autoimmune pancreatitis (n = 4) were cocultured with CD14+ monocytes (1×10^6/ml) from control subjects (n = 4) or patients (n = 4) in the presence of MDP (20 μg/ml) or LPS (1 μg/ml) for 14 days. Culture supernatants obtained at 14 days were assayed for the presence of IgG1 and IgG4. Bars show the mean ± SEM. * = $P < 0.05$ versus the respective controls. See Figure 5 for definitions.

flagellin, 4,403 ± 345 pg/ml versus 7,207 ± 750 pg/ml). In contrast, no significant difference between patients and control subjects was observed in the production of IL-17 or IL-10 by PBMCs stimulated with NLR and TLR ligands (data not shown). Thus, these studies clearly showed that defective Th1 cell responses to TLR ligands, but not NLR ligands, are associated with enhanced IgG4 production by PBMCs from patients with IgG4-related autoimmune pancreatitis.

Induction of IgG4 responses by monocytes from patients with IgG4-related autoimmune pancreatitis. In a final series of experiments, we determined the type of cells that play a critical role in the generation of IgG4 responses in patients with IgG4-related autoimmune pancreatitis. Because PBMCs from patients with autoimmune pancreatitis produced a large amount of BAFF upon stimulation with TLR and NLR ligands, we hypothesized that monocyte-induced activation of TLRs and NLRs is responsible for enhanced IgG4 production in patients with IgG4-related autoimmune pancreatitis. To this end, we conducted coculture experiments in which CD19+ B cells and CD14+ monocytes isolated from the PBMCs of healthy control subjects and patients were stimulated with MDP or LPS in various combinations.

First, we set up a coculture system in which CD19+ B cells from healthy control subjects were incubated with monocytes from healthy control subjects or patients. Monocytes isolated from patients, but not control subjects, induced production of IgG4 by healthy B cells upon stimulation with MDP or LPS (Figure 6A). In contrast, no differences were observed between patients and control subjects in IgG1 production upon stimulation with MDP or LPS (Figure 6A). Enhanced IgG4 production by monocytes from patients was significantly reduced by IFNγ (data not shown), suggesting that impaired Th1 cell responses are involved in enhanced IgG4 responses in patients with IgG4-related disease.

Next, we set up a coculture system in which CD19+ B cells from patients were incubated with monocytes from healthy control subjects or patients. B cells from patients produced comparable levels of IgG1 and IgG4 regardless of whether these cells were cocultured with monocytes from healthy controls or patients (Figure 6B). These data suggested that activation of NOD-2 or TLR-4 in the monocytes of patients triggers IgG4 production by healthy CD19+ B cells, and that monocyte activation of TLRs and NLRs underlies the immunopathogenesis of enhanced IgG4 production in IgG4-related disease.

DISCUSSION

Although autoimmune pancreatitis is characterized by systemic IgG4 antibody responses and is considered to be a pancreatic manifestation of IgG4-related disease and IgG4-positive multiorgan lymphoproliferative syndrome, the innate immune responses leading to adaptive IgG4 antibody responses are poorly understood. In the current study, we examined the innate immune responses inducing IgG4 antibody production. In our initial studies, we used healthy PBMCs to show that B cells produce a large amount of IgG4 in a T cell–independent manner when B cells are cocultured with monocytes stimulated with MDP, a NOD-2 ligand. Furthermore, we showed that BAFF produced by MDP-activated monocytes induces IgG4 production by B cells. Thus, we characterized T cell–independent IgG4 production induced by NOD-2 signaling, which operates in monocytes. With respect to the role of NOD-2 in IgG4-related autoimmune pancreatitis, we showed that NOD-2 activation enhances the production of BAFF and IgG4 by PBMCs from patients with autoimmune pancreatitis. More importantly, we showed that MDP-stimulated monocytes isolated from patients with autoimmune pancreatitis induce production of IgG4 by B cells from healthy controls. Therefore, these data suggest that NOD-2–dependent BAFF production in monocytes plays an important role in the generation of an IgG4 response in autoimmune pancreatitis.

NOD-2–mediated BAFF production by monocytes is an indispensable step for T cell–independent IgG4 production, as shown in this study. We showed enhanced activation of NF-κB and MAPKs in monocytes upon stimulation with MDP. Moreover, BAFF production mediated by MDP was markedly decreased by the addition of pharmacologic inhibitors for NF-κB and RICK, which strongly suggests that MDP-induced activation of NOD-2 elicits BAFF production by monocytes via NF-κB activation. Compatible with this idea, the BAFF gene promoter contains many NF-κB binding sites (25). In this study, however, the molecular mechanisms by which NOD-2–mediated BAFF production enhances IgG4 production remain unknown. BAFF is a crucial survival factor for peripheral B cells (21,22). In fact, the percentage of apoptotic B cells was reduced in the presence of monocytes stimulated with MDP, which suggests an expansion of B cells due to BAFF production induced by NOD-2 activation. Thus, one possible mechanism for BAFF-dependent IgG4 production may be promotion of the activation or expansion of B cells that are already committed to IgG4 production. In this

regard, the survival of B cells committed to IgG4 production is enhanced in the presence of IL-21 (26). Therefore, it is possible that BAFF, in concert with IL-21 and/or other unidentified factors, enhances IgG4 production via promoting the expansion of IgG4-committed B cells. Alternatively, BAFF may directly induce IgG4 class-switch DNA recombination. Although Litinskiy et al reported that BAFF induces IgG4 class-switch DNA recombination in the presence of IL-4 (15), we failed to detect IL-4 in cells stimulated with MDP.

Although the activation of NOD-2, but not TLRs, preferentially induces IgG4 production by PBMCs from healthy control subjects, not only MDP but also many TLR ligands induce IgG4 production by PBMCs from patients with IgG4-related autoimmune pancreatitis. This enhanced IgG4 production by TLR ligands is associated with the reduced production of proinflammatory Th1 cytokines and with the enhanced production of BAFF. Thus, in contrast to NOD-2–induced IgG4 production, which is dependent on BAFF production by monocytes, the reduced production of Th1 cytokines may also be involved in enhanced TLR-induced IgG4 production in autoimmune pancreatitis. Consistent with this idea, IgG4 production by healthy B cells upon coculture with monocytes from patients with IgG4-related disease is reduced in the presence of IFNγ. Therefore, it seems likely that not only NOD-2–mediated BAFF production but also TLR-mediated production of BAFF and reduced production of Th1 cytokines are involved in the generation of systemic IgG4 responses in patients with autoimmune pancreatitis. In addition, our preliminary data show that basophils isolated from patients with autoimmune pancreatitis, but not those from healthy control subjects, induce production of a large amount of IgG4 by B cells upon stimulation with TLR-2 and TLR-4 ligands (Watanabe T, et al: unpublished observation). Further phenotypic analysis of basophils from patients with autoimmune pancreatitis and healthy control subjects may provide the mechanisms accounting for differences in TLR and NLR ligands that drive the production of this immunoglobulin subclass. In any case, these data suggest that activation of both NLRs and TLRs is involved in the development of enhanced IgG4 production in patients with autoimmune pancreatitis.

TLR activation enhances the production of BAFF rather than APRIL in patients with IgG4-related autoimmune pancreatitis. Thus, BAFF plays an important role in T cell–independent IgG4 production. Similarly, Alsaleh et al observed that TLR activation in synovial fibroblasts from patients with rheumatoid arthritis cause T cell–independent IgG secretion via BAFF-mediated but not APRIL-mediated signaling pathways (27). Thus, the immunopathogenesis of rheumatoid arthritis and autoimmune pancreatitis may be similar, in that T cell–independent and BAFF-dependent immunoglobulin secretion is seen in both diseases. Although BAFF binds to the receptors BCMA, TACI, and BAFF-R expressed on the cell surface of B cells (21,22), TACI delivers innate immunoglobulin-inducing signals to B cells (28). In the current study, TACI expression in B cells was up-regulated in PBMCs stimulated with MDP. Therefore, the interaction between TACI and BAFF may be involved in T cell–independent IgG4 production. Blockade of TACI-specific signaling pathways may provide new insights into the immunopathogenesis of IgG4-related disease.

The role of an IgG4 antibody response in the immunopathogenesis of IgG4-related disease is poorly understood. Several lines of evidence suggest an anti-inflammatory rather than an inflammatory role of this immunoglobulin subclass. First, the interaction between IgG4 and the Fcγ receptor or C1q is weaker than that of other IgG subclasses (20). Second, IgG4 antibodies can exchange Fab arms by swapping a heavy chain and an attached light chain (half-molecule) with a heavy–light chain pair from another molecule, which results in bispecific antibodies (29). This bispecific property of IgG4 antibodies protects against the autoimmune disease myasthenia gravis by competing pathogenic IgG1 antibodies against acetylcholine receptor (29). Third, elevated IgG4 levels are considered to be a marker of tolerance induction in IgE-related allergic disorders (20,30). These unique properties of IgG4 prompt us to speculate that enhanced IgG4 antibody responses may be an epiphenomenon associated with inflammatory reactions. In this study, we identified BAFF as a critical player in IgG4 production. Thus, it is an interesting question whether or not BAFF inhibition influences the clinical course of IgG4-related disease and IgG4-positive multiorgan lymphoproliferative syndrome.

Our findings have certain implications with respect to possible mechanisms of IgG4-related autoimmune pancreatitis. Activation of NOD-2 and TLR ligands enhances IgG4 responses by PBMCs from patients with IgG4-related autoimmune pancreatitis, which suggests the possible involvement of abnormal innate immune responses against intestinal microflora in the development of IgG4-related autoimmune pancreatitis. Alternatively, microbial infections trigger the development of IgG4-related autoimmune pancreatitis. In conclusion, abnormal innate immune responses via NLRs

and TLRs may underlie the immunopathogenesis of IgG4-related disease and IgG4-positive multiorgan lymphoproliferative syndrome.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Watanabe had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Watanabe, Yamashita.

Acquisition of data. Watanabe, Yamashita, Fujikawa, Sakurai, Kudo, Shiokawa, Kodama, Uchida, Okazaki, Chiba.

Analysis and interpretation of data. Watanabe, Yamashita, Fujikawa, Sakurai, Kudo, Shiokawa, Kodama, Uchida, Okazaki, Chiba.

REFERENCES

1. Finkelberg DL, Sahani D, Deshpande V, Brugge WR. Autoimmune pancreatitis. N Engl J Med 2006;355:2670–6.
2. Okazaki K, Chiba T. Autoimmune related pancreatitis. Gut 2002; 51:1–4.
3. Okazaki K, Uchida K, Fukui T. Recent advances in autoimmune pancreatitis: concept, diagnosis, and pathogenesis. J Gastroenterol 2008;43:409–18.
4. Kamisawa T, Okamoto A. Autoimmune pancreatitis: proposal of IgG4-related sclerosing disease. J Gastroenterol 2006;41:613–25.
5. Hamano H, Kawa S, Horiuchi A, Unno H, Furuya N, Akamatsu T, et al. High serum IgG4 concentrations in patients with sclerosing pancreatitis. N Engl J Med 2001;344:732–8.
6. Khosroshahi A, Stone JH. A clinical overview of IgG4-related systemic disease. Curr Opin Rheumatol 2010;23:57–66.
7. Masaki Y, Dong L, Kurose N, Kitagawa K, Morikawa Y, Yamamoto M, et al. Proposal for a new clinical entity, IgG4-positive multiorgan lymphoproliferative syndrome: analysis of 64 cases of IgG4-related disorders. Ann Rheum Dis 2009;68:1310–5.
8. Ravi K, Chari ST, Vege SS, Sandborn WJ, Smyrk TC, Loftus EV Jr. Inflammatory bowel disease in the setting of autoimmune pancreatitis. Inflamm Bowel Dis 2009;15:1326–30.
9. Zamboni G, Luttges J, Capelli P, Frulloni L, Cavallini G, Pederzoli P, et al. Histopathological features of diagnostic and clinical relevance in autoimmune pancreatitis: a study on 53 resection specimens and 9 biopsy specimens. Virchows Arch 2004;445: 552–63.
10. Strober W, Murray PJ, Kitani A, Watanabe T. Signalling pathways and molecular interactions of NOD1 and NOD2. Nat Rev Immunol 2006;6:9–20.
11. Deheragoda MG, Church NI, Rodriguez-Justo M, Munson P, Sandanayake N, Seward EW, et al. The use of immunoglobulin G4 immunostaining in diagnosing pancreatic and extrapancreatic involvement in autoimmune pancreatitis. Clin Gastroenterol Hepatol 2007;5:1229–34.
12. Akitake R, Watanabe T, Zaima C, Uza N, Ida H, Tada S, et al. Possible involvement of T helper type 2 responses to Toll-like receptor ligands in IgG4-related sclerosing disease. Gut 2010;59: 542–5.
13. Ueno K, Watanabe T, Kawata Y, Gotoh T, Tsuji Y, Ida H, et al. IgG4-related autoimmune pancreatitis involving the colonic mucosa. Eur J Gastroenterol Hepatol 2008;20:1118–21.
14. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol 2004;4:499–511.
15. Litinskiy MB, Nardelli B, Hilbert DM, He B, Schaffer A, Casali P, et al. DCs induce CD40-independent immunoglobulin class switching through BLyS and APRIL. Nat Immunol 2002;3:822–9.
16. He B, Qiao X, Cerutti A. CpG DNA induces IgG class switch DNA recombination by activating human B cells through an innate pathway that requires TLR9 and cooperates with IL-10. J Immunol 2004;173:4479–91.
17. Watanabe T, Asano N, Murray PJ, Ozato K, Tailor P, Fuss IJ, et al. Muramyl dipeptide activation of nucleotide-binding oligomerization domain 2 protects mice from experimental colitis. J Clin Invest 2008;118:545–59.
18. Watanabe T, Asano N, Fichtner-Feigl S, Gorelick PL, Tsuji Y, Matsumoto Y, et al. NOD1 contributes to mouse host defense against Helicobacter pylori via induction of type I IFN and activation of the ISGF3 signaling pathway. J Clin Invest 2010;120: 1645–62.
19. Chung H, Watanabe T, Kudo M, Chiba T. Hepatitis C virus core protein induces homotolerance and cross-tolerance to Toll-like receptor ligands by activation of Toll-like receptor 2. J Infect Dis 2010;202:853–61.
20. Aalberse RC, Stapel SO, Schuurman J, Rispens T. Immunoglobulin G4: an odd antibody. Clin Exp Allergy 2009;39:469–77.
21. Mackay F, Schneider P. Cracking the BAFF code. Nat Rev Immunol 2009;9:491–502.
22. Mackay F, Silveira PA, Brink R. B cells and the BAFF/APRIL axis: fast-forward on autoimmunity and signaling. Curr Opin Immunol 2007;19:327–36.
23. Okazaki K, Kawa S, Kamisawa T, Ito T, Inui K, Irie H, et al. Japanese clinical guidelines for autoimmune pancreatitis. Pancreas 2009;38:849–66.
24. Okazaki K, Kawa S, Kamisawa T, Shimosegawa T, Tanaka M, Research Committee for Intractable Pancreatic Disease and Japan Pancreas Society. Japanese consensus guidelines for management of autoimmune pancreatitis: I. Concept and diagnosis of autoimmune pancreatitis. J Gastroenterol 2010;45:249–65.
25. He B, Raab-Traub N, Casali P, Cerutti A. EBV-encoded latent membrane protein 1 cooperates with BAFF/BLyS and APRIL to induce T cell-independent Ig heavy chain class switching. J Immunol 2003;171:5215–24.
26. Wood N, Bourque K, Donaldson DD, Collins M, Vercelli D, Goldman SJ, et al. IL-21 effects on human IgE production in response to IL-4 or IL-13. Cell Immunol 2004;231:133–45.
27. Alsaleh G, Francois A, Knapp AM, Schickel JN, Sibilia J, Pasquali JL, et al. Synovial fibroblasts promote immunoglobulin class switching by a mechanism involving BAFF. Eur J Immunol 2011;41:2113–22.
28. Cerutti A, Puga I, Cols M. Innate control of B cell responses. Trends Immunol 2011;32:202–11.
29. Van der Neut Kolfschoten M, Schuurman J, Losen M, Bleeker WK, Martinez-Martinez P, Vermeulen E, et al. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. Science 2007;317:1554–7.
30. Savilahti EM, Rantanen V, Lin JS, Karinen S, Saarinen KM, Goldis M, et al. Early recovery from cow's milk allergy is associated with decreasing IgE and increasing IgG4 binding to cow's milk epitopes. J Allergy Clin Immunol 2010;125:1315–21.

Lower Incidence of Biliary Carcinoma in Patients With Primary Sclerosing Cholangitis and High Serum Levels of Immunoglobulin E

KENJI HIRANO,* MINORU TADA,* SUGURU MIZUNO,* HIROYUKI ISAYAMA,* NAMINATSU TAKAHARA,* RIE NAGANO,* TSUYOSHI HAMADA,* KOJI MIYABAYASHI,* YUKIKO ITO,* DAI MOHRI,* KAZUMICHI KAWAKUBO,* TAKASHI SASAKI,* HIROFUMI KOGURE,* NATSUYO YAMAMOTO,* NAOKI SASAHIRA,* NORIYO YAMASHIKI,‡ YASUHIKO SUGAWARA,‡ NORIHIRO KOKUDO,‡ NOBUO TODA,§ and KAZUHIKO KOIKE*

Department of Gastroenterology, Graduate School of Medicine, ‡Artificial Organ and Transplantation Division, Department of Surgery, University of Tokyo, Tokyo; and §Department of Gastroenterology, Mitsui Memorial Hospital, Tokyo, Japan

This article has an accompanying continuing medical education activity on page e7. Learning Objectives—At the end of this activity, the successful learner will know about the importance of serum IgE levels in patients with primary sclerosing cholangitis.

BACKGROUND & AIMS: High serum levels of immunoglobulin (Ig)E often are detected in patients with primary sclerosing cholangitis (PSC), but the clinical significance is not known. **METHODS:** We analyzed data from 44 patients with PSC and known serum levels of IgE. They were divided into groups called high IgE (>170 IU/mL; n = 17) or normal IgE (n = 27). We compared occurrence of biliary carcinoma including cholangiocellular and gallbladder carcinoma, liver transplantation, and death between groups. **RESULTS:** The high IgE group had a later age of onset of PSC than the normal IgE group (54 ± 20 y vs 39 ± 16 y; $P = .010$); they also had a higher serum level of IgG (2078 ± 638 vs 1517 ± 475 mg/dL; $P = .002$) and IgG4 (104 ± 102 vs 38 ± 16 mg/dL; $P = .002$). Association with inflammatory bowel disease did not differ significantly between groups (5 of 17 vs 11 of 27; $P = .661$). No patient had biliary carcinoma in the high IgE group, but biliary carcinoma was observed during the follow-up period in 8 patients in the normal IgE group (0 of 17 vs 8 of 27; $P = .016$). The occurrence of biliary carcinoma, liver transplantation, or death did not differ between groups (4 of 17 vs 13 of 27; $P = .124$). **CONCLUSIONS: High serum levels of IgE often are observed in older patients with PSC and are associated with a reduced incidence of biliary carcinoma.**

Keywords: Prognosis; Ulcerative Colitis; Allergy; Malignancy.

Primary sclerosing cholangitis (PSC) is a chronic cholestatic disease of unknown etiology characterized by fibrosing inflammatory destruction of intrahepatic and/or extrahepatic bile ducts. PSC is generally progressive, and usually leads to the development of cirrhosis. There is no medical therapy that can dramatically disrupt the disease progression, and liver transplantation remains the only option for patients with end-stage PSC.[1] Mean age at diagnosis is 40 years, and men are about 2 times more likely to be affected than women in Europe and the United States, whereas in Japan there are 2 age distribution peaks, in the 20s and 60s, even after immunoglobulin (Ig)G4-related sclerosing cholangitis (IgG4-SC) is strictly removed.[2] In this regard, we previously reported that high serum IgE was characteristic of PSC patients with onset age older than 50 years.[3]

The IgE level often is found to be increased in allergic diseases. Interestingly, it has been reported that an inverse association exists between allergy and malignancy such as pancreatic cancer and glioma.[4] As for IgE, an odds ratio of glioma risk in those with increased IgE levels compared with normal levels of 0.3 was reported by Wiemels et al.[5] They also showed that glioblastoma patients with increased IgE levels had longer survival times than those with normal or borderline IgE levels.[6] Thus, increased IgE levels may work in a protective manner against certain kinds of malignancies.

The most feared complication of PSC is biliary carcinoma, including cholangiocarcinoma (CCA) and gallbladder carcinoma.[1,7–10] Various studies have tried to clarify risk factors of biliary carcinoma in PSC, but conclusive factors have yet to be found. To date, it is not known whether either high or normal IgE levels in PSC might be a predictive factor for biliary carcinoma. Thus, in the present study, we examined clinical differences between PSC patients with and without high IgE levels, focusing especially on the possible association with biliary carcinoma.

Patients and Methods

Sixty-six patients were diagnosed with PSC at our institutes (64 at the University of Tokyo and 2 at Mitsui Memorial Hospital) between January 1982 and February 2011. Among them, serum IgE level was measured in 44 patients (42 patients at the University of Tokyo and 2 patients at Mitsui Memorial Hospital). These 44 patients were followed up at our outpatient department and were enrolled in the present study. In principle, laboratory data were checked every 3 months and imaging studies such as abdominal ultrasonography was performed at least once a year even when the condition of the disease was stable. We used diagnostic criteria of the Mayo Clinic (published in 2003) for the diagnosis of PSC, but excluded IgG4-SC.[3,11] Even if the serum IgE level was measured several times, the initial value was adopted. The duration of PSC was defined as the time elapsed since the first clinical, biochemical, or

Abbreviations used in this paper: CCA, cholangiocarcinoma; FEIA, fluorenzymeimmunoassay; IBD, inflammatory bowel disease; Ig, immunoglobulin; PSC, primary sclerosing cholangitis; SC, sclerosing cholangitis.

1542-3565/$36.00
doi:10.1016/j.cgh.2011.09.015

Table 1. Clinical Features of Patients With PSC

	High IgE group (n = 17, IgE > 170 IU/mL)	Normal IgE group (n = 27)	*P* value
Sex, M:F	8:9	16:11	.631
Onset age of PSC	54 ± 20 (11–79)	39 ± 16 (16–75)	.006
Follow-up period[a]	69 ± 66	71 ± 58	.888
Associated diseases			
Bronchial asthma	2	1	.386
Atopic dermatitis	1	0	.386
Drug allergy	4	5	.716
Inflammatory bowel disease	5	11	.661
Variceal bleeding	1	1	.999
Laboratory data			
IgE level ≤170 IU/mL	770 ± 486	69 ± 52	<.001
IgG level 800–1700 mg/dL	2078 ± 638	1517 ± 475	.002
IgG4 level <135 mg/dL	104 ± 102 (n = 17)	38 ± 16 (n = 21)	.020
IgM level 35–220 mg/dL	144 ± 76	141 ± 73	.875
Positive ANA, ≥×80	0/16	2/24	.508
Eosinophilia >1000 μL	2/16	2/26	.999
Total bilirubin level	0.91 ± 0.60 (n = 17)	0.91 ± 0.36 (n = 24)	.967
Albumin level	4.0 ± 0.42 (n = 17)	3.9 ± 0.46 (n = 24)	.724
Model for end-stage liver disease score	7.5 ± 1.6 (n = 16)	7.3 ± 1.3 (n = 23)	.687

[a]Period from the onset of PSC to the day of biliary carcinoma, liver transplant, death, or the last visit.

cholangiographic findings leading to the diagnosis of PSC. The diagnosis of biliary carcinoma was confirmed by histology except in one patient whose diagnosis was made only by imaging findings. This patient showed irregular gallbladder wall thickening with papillary nodule on computed tomography and magnetic resonance imaging. These findings became aggravated during the follow-up period, which made us suspect gallbladder cancer, although a close examination was not performed because of advanced liver failure. Medical records were reviewed to obtain information regarding demographic variables, laboratory data, and the presence of associated diseases. Serum IgE level was measured by fluorenzymeimmunoassay (FEIA) at BML, Inc. (Saitama, Japan). Less than the mean ± standard deviation was defined as a normal value.[12] Concretely, the normal value was 170 IU/mL or less in the patients aged 7 years or older. Based on the IgE value (>170 IU/mL or not), we divided patients into 2 groups: the high IgE group (n = 17; IgE > 170 IU/mL) and the normal IgE group (n = 27; IgE ≤ 170 IU/mL). We compared the 2 groups in terms of associated diseases, laboratory data (mainly immunologic parameters), and prognosis. In the present study, associated diseases included inflammatory bowel disease (IBD) and allergic diseases such as bronchial asthma and atopic dermatitis. History of variceal bleeding also was examined. Laboratory parameters included IgG, IgG4, IgM, and the presence of antinuclear antibody (γ ≥ ×80). For prognosis, 3 analyses were performed. In the first analysis, only biliary carcinoma was regarded as an end point, and the frequency of biliary carcinoma was compared between the 2 groups. In the second analysis, biliary carcinoma and death by other causes were regarded as end points, and the frequency of these end points was compared. In the third analysis, biliary carcinoma, liver transplantation, and death by other causes were regarded as end points, and the frequency of these end points was compared.

Continuous variables were reported as the mean ± standard deviation. Differences were evaluated by the Student *t* test, the Welch *t* test, the chi-square test with Yates' correction, or the Fisher exact test. Cumulative event occurrence rate curves were analyzed using the Kaplan-Meier technique with the log-rank test. A *P* value less than .05 was considered statistically significant. This retrospective study was approved by the review board of our institute.

Results

Forty-four patients (24 men and 20 women) were enrolled, and their mean age at onset of PSC was 45 ± 19 years. The clinical features of the high IgE group and the normal IgE group are summarized in Table 1. The mean onset age was significantly

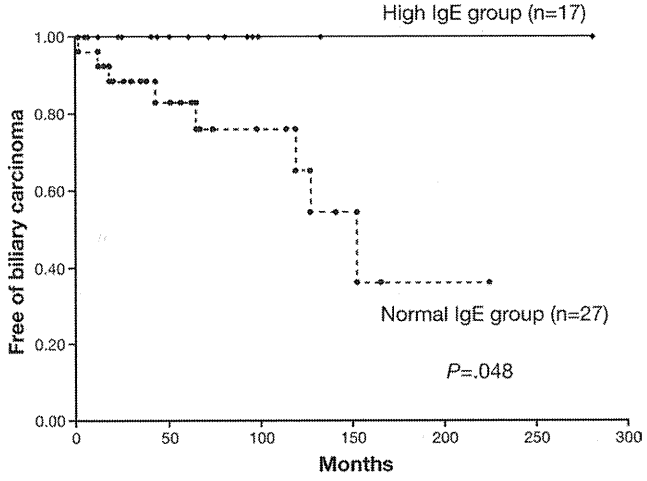

Figure 1. Kaplan–Meier curves comparing the nonoccurrence rate of biliary carcinoma between the high IgE group and the normal IgE group. Patients with liver transplantation or death from causes other than biliary carcinoma were treated as censored cases in this analysis.

Table 2. Clinical Profile of 8 Patients Associated With Biliary Carcinoma

Patient	Location of BCA	IgE level, *IU/mL*	IBD	Sex, M/F	Age at onset of BCA	Period from the onset of PSC to BCA, *mo*
1	EHBD	97	–	M	22	2
2	GB	70	+	F	48	65
3	GB	69	+	M	61	43
4	EHBD	39	–	F	52	119
5	IHBD	23	+	M	29	152
6	EHBD	22	+	F	40	12
7	IHBD	11	+	F	48	18
8	IHBD	9	+	M	39	127

BCA, biliary carcinoma; EHBD, extrahepatic bile duct; GB, gallbladder; IHBD, intrahepatic bile duct.

higher in the former group (54 ± 20 vs 39 ± 16 y; $P = .010$). Association with bronchial asthma, atopic dermatitis, drug allergy, IBD, and variceal bleeding did not differ between the 2 groups. There was no patient with allergic vasculitis or parasitic infection. The frequency of allergic rhinitis was not exactly known after reviewing medical records because allergic rhinitis caused by pollen allergy is a very common disease in Japan[13] and many patients did not seem to report their mild allergic rhinitis if they had one. Sixteen patients were associated with IBD, and all of them had ulcerative colitis. Among them, none was associated with colorectal cancer. There was no patient with anomalous pancreatobiliary ductal union, which was confirmed by endoscopic retrograde cholangiopancreatography or magnetic resonance cholangiopancreatography. As for laboratory data, there were significant differences in IgG (2078 ± 638 vs 1517 ± 475 mg/dL; $P = .002$) and IgG4 (104 ± 102 vs 38 ± 16 mg/dL; $P = .020$) levels. Total bilirubin and albumin levels at the onset of PSC showed no significant difference. There was no difference in the model for end-stage liver disease score.

The mean disease durations (from onset to the day of biliary carcinoma, liver transplantation, death, or last visit) in the 2 groups were 69 ± 66 and 71 ± 58 months ($P = .888$). The mean follow-up periods at our institute (from the first visit to our institute to the day of biliary carcinoma, liver transplantation, death, or last visit) in the 2 groups were 50 ± 43 and 34 ± 27 months ($P = .193$). There was no occurrence of biliary carcinoma in the high IgE group, but biliary carcinoma occurred in 8 patients in the normal IgE group (0 of 17 vs 8 of 27; $P = .016$). The cumulative occurrence rate of biliary carcinoma analyzed by the Kaplan–Meier method also showed a significant difference ($P = .048$) (Figure 1). There was no occurrence of carcinoma of duodenal papilla and pancreas. Clinical profiles of the 8 patients with biliary carcinoma (6 CCA and 2 gallbladder carcinoma) are summarized in Table 2. There were 2 patients whose CCA was found within 1 year after the diagnosis of PSC. Although there is no denying that occult CCA existed at the diagnosis of PSC, their onsets were not synchronous clinically. Therefore, we also included these 2 patients in the present analyses. Biliary carcinoma was diagnosed 59 months on average after PSC onset. Surgical treatment was performed in 3 patients, and 2 showed recurrence 13 and 23 months later. Chemotherapy with or without radiation was chosen in 3 patients, and the other 2 patients were followed up only with best supportive care. Serum IgE values of these 8 patients were less than 100 IU/mL.

The comparison between patients with and without biliary carcinoma was summarized in Table 3. Besides serum IgE, association with ulcerative colitis was related to the occurrence of biliary carcinoma.

When biliary carcinoma and death by other causes were regarded as end points, these end points were observed in 4 patients (death from liver failure) in the high IgE group, and in 10 patients (8 biliary carcinoma, 1 death from liver failure, and 1 suicide) in the normal IgE group (4 of 17 vs 10 of 27; $P = .509$). The cumulative occurrence rate of end points according to the Kaplan–Meier method showed no significant difference ($P = .766$) (Figure 2).

When biliary carcinoma, liver transplantation, and death by other causes were regarded as end points, these end points were observed in 4 patients (death from liver failure) in the high IgE group, and in 13 patients (8 biliary carcinoma, 3 liver transplantation, 1 death from liver failure, and 1 suicide) in the normal IgE group (4 of 17 vs 13 of 27; $P = .124$). The cumulative occurrence rate of end points according to the Kaplan–Meier method showed no significant difference ($P = .370$) (Figure 3).

Discussion

Biliary carcinoma including CCA and gallbladder carcinoma represents the most feared biliary complication occurring in patients with PSC. The reported prevalence of CCA in PSC varies from 3.6% to 36%, depending on the methods used to establish the diagnosis and the length of follow-up evaluation.[2,14–18] In this study, the incidence of biliary carcinoma during the follow-up period was 18% (8 of 44), and that of CCA was 14% (6 of 44). This may not seem so high in comparison with previous reports, but the rate was much higher than that observed in a Japanese national survey of PSC in 2003 (3.6%) despite a seemingly similar

Table 3. Comparison Between Patients With and Without Biliary Carcinoma

	Biliary carcinoma (+), n = 8	Biliary carcinoma (−), n = 36	*P* value
Sex, M:F	4:4	20:16	.999
Age at onset of PSC	37 ± 14	47 ± 19	.198
Ulcerative colitis (+)	6/8	10/36	.019
IgG level >1700 mg/dL	2/8	15/36	.455
IgG4 level >135 mg/dL	0/7	3/36	.999
IgE level >170 IU/mL	0/8	17/36	.016
Mean IgE level	43 ± 32	405 ± 481	<.001

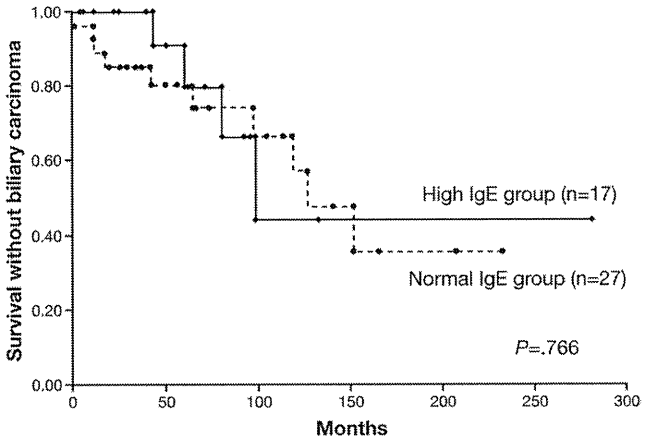

Figure 2. Kaplan–Meier curves comparing the survival rate without biliary carcinoma between the high IgE group and the normal IgE group. Patients with liver transplantation were treated as censored cases in this analysis.

follow-up period (median, about 5 y). Two PSC patients with CCA in our series were referred to our institute only after the diagnosis of CCA was made. In addition, because our institute (University of Tokyo) is a tertiary hospital in which liver transplantation is performed, our series may include patients with more severe liver dysfunction and younger age than those in the national survey. These factors may have contributed to the comparatively high rate of CCA.

In our previous study,[3] it was found that a portion of the PSC patients, most of whom were elderly, showed high serum IgE levels, but the clinical significance of IgE level in PSC was still unclear. However, in addition to allergy and parasitic infections, IgE recently was shown to possess anticancer effects.[4] It has been reported that IgE can destroy pancreatic and ovarian cancer cells through antibody-dependent cell-mediated cytotoxicity.[19-21] The serum level of IgE was increased significantly in patients with pancreatic cancer compared with controls.[20] It is also known that total IgE levels may be related to natural killer cell activity, whose low level is associated with an enhanced risk of cancer at all sites.[22,23] These findings made us suppose that IgE also might be associated with biliary carcinoma.

In the present study, there were 8 patients whose serum IgE level ranged between 100 and 170 IU/mL, and none of them had biliary carcinoma. Thus, in referring to a risk factor for biliary carcinoma in PSC, it may be more exact to use the term *lower IgE* instead of *normal IgE*. By using receiver operating characteristic curve analysis to show the ideal cut-off IgE value to predict the occurrence of biliary carcinoma in patients with PSC, the cut-off value turned out to be 100 IU/mL. Sensitivity, specificity, and accuracy were 100%, 69%, and 77%, respectively. The area under the curve was 0.84.

In one patient with PSC and CCA (patient 4 in Table 2), IgE level was measured repeatedly before and after the diagnosis of CCA, but there was no significant change in its level (20–53 IU/mL). Its variability did not seem to be related to CCA at least as far as this patient is concerned.

Increased IgE was strongly associated with old-age onset of PSC. However, in general, the serum IgE level does not change with age.[24] Even in patients with increased serum IgE levels, the association with bronchial asthma or atopic dermatitis was not observed frequently. Thus, we speculate that the increase of IgE level is derived from PSC itself. However, it seems difficult to conclude that PSC with high IgE level is quite different from PSC with normal IgE level. Irrespective of the IgE levels, the association of IBD was observed, and liver dysfunction was generally progressive. Although the high IgE group showed higher IgG and IgG4 level than the normal IgE group (Table 1), we never considered that PSC with high IgE level in the present series was equal to IgG4-SC. Obvious jaundice at onset (total bilirubin level, >3 mg/dL), which often is recognized in IgG4-SC, was not observed in the present series. Even in the high IgE group, only 3 patients had IgG4 levels higher than 135 mg/dL, a commonly used cut-off value for the diagnosis of IgG4-related diseases.[25] Two of them had ulcerative colitis, which is infrequent in IgG4-related diseases.[25] Moreover, 3 patients with increased IgE level received steroid treatment, but remarkable improvement was not observed.[3] Because both IgE and IgG4 productions are Th2-dependent in general, IgE-inducing antigens are also efficient IgG4 inducers.[26] In a sense, it may not be unusual that patients with high IgE level show a somewhat high IgG4 level. Considering these data, PSC with increased IgE should be differentiated from IgG4-SC.

Various studies have attempted to clarify the risk factors of CCA in PSC. One previous study reported that the presence of IBD was associated with an increased risk of CCA, but this was not confirmed by other studies.[16,27] Two previous studies have identified abdominal pain as a predictor of CCA in PSC patients.[16,28] On the other hand, absence of symptoms was a predicator of CCA according to the study of Burak et al.[15] They suggested that variceal bleeding was a major risk factor for the later development of CCA, but in our study there were no patients with biliary carcinoma and a history of variceal bleeding. Among the 8 patients with biliary carcinoma in our series, one was a smoker and none was a habitual drinker, although studies have reported their association with CCA.[28,29] Taken together, definitive factors still have to be found. The relation between IgE and biliary carcinoma has not been discussed fully. We believe our finding is very simple, but also very innovative. Because ulcerative colitis seemed to be related to biliary carcinoma in our study

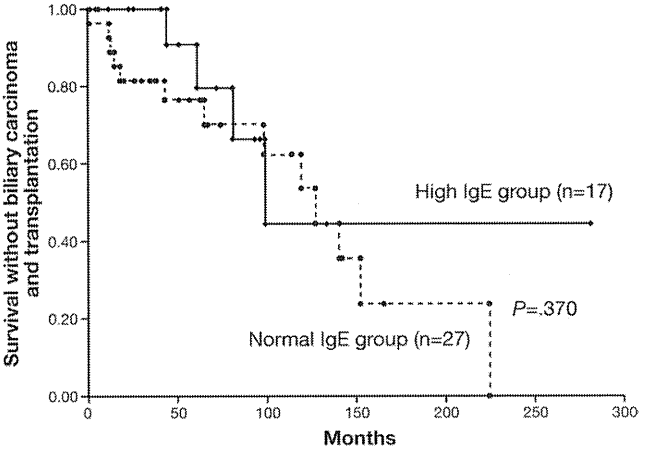

Figure 3. Kaplan–Meier curves comparing the survival rate without biliary carcinoma and liver transplantation between the high IgE group and the normal IgE group.