

ORIGINAL ARTICLE

Identification of a Novel Antibody Associated with Autoimmune Pancreatitis

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ABSTRACT

BACKGROUND

Autoimmune pancreatitis is characterized by an inflammatory process that leads to organ dysfunction. The cause of the disease is unknown. Its autoimmune origin has been suggested but never proved, and little is known about the pathogenesis of this condition.

METHODS

To identify pathogenetically relevant autoantigen targets, we screened a random peptide library with pooled IgG obtained from 20 patients with autoimmune pancreatitis. Peptide-specific antibodies were detected in serum specimens obtained from the patients.

RESULTS

Among the detected peptides, peptide AIP₁₋₇ was recognized by the serum specimens from 18 of 20 patients with autoimmune pancreatitis and by serum specimens from 4 of 40 patients with pancreatic cancer, but not by serum specimens from healthy controls. The peptide showed homology with an amino acid sequence of plasminogen-binding protein (PBP) of *Helicobacter pylori* and with ubiquitin-protein ligase E3 component n-recognin 2 (UBR2), an enzyme highly expressed in acinar cells of the pancreas. Antibodies against the PBP peptide were detected in 19 of 20 patients with autoimmune pancreatitis (95%) and in 4 of 40 patients with pancreatic cancer (10%). Such reactivity was not detected in patients with alcohol-induced chronic pancreatitis or intraductal papillary mucinous neoplasm. The results were validated in another series of patients with autoimmune pancreatitis or pancreatic cancer: 14 of 15 patients with autoimmune pancreatitis (93%) and 1 of 70 patients with pancreatic cancer (1%) had a positive test for anti-PBP peptide antibodies. When the training and validation groups were combined, the test was positive in 33 of 35 patients with autoimmune pancreatitis (94%) and in 5 of 110 patients with pancreatic cancer (5%).

CONCLUSIONS

The antibody that we identified was detected in most patients with autoimmune pancreatitis but also in some patients with pancreatic cancer, making it an imperfect test to distinguish between these two conditions.

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AUTOIMMUNE PANCREATITIS IS AN INflammatory disease of the pancreas with unique clinical and histologic features.^{1,2} Imaging studies show that the involvement of the pancreas in autoimmune pancreatitis may be diffuse or focal.^{1,3}

Clinically, autoimmune pancreatitis may mimic pancreatic carcinoma, and the differential diagnosis may be difficult. Several diagnostic criteria have been proposed,⁴⁻⁶ but their usefulness is still under debate. Serologic markers of the disease include autoantibodies that are not organ-specific⁷ and organ-specific autoantibodies such as those against carbonic anhydrase,⁷⁻⁹ lactoferrin,⁷ and pancreatic secretory trypsin inhibitor.¹⁰ These organ-specific autoantibodies have been detected in patients with autoimmune pancreatitis, but none of them have provided satisfactory specificity and sensitivity. IgG4 has been proposed as a possible diagnostic marker,¹¹ but more recent studies have not confirmed this previous report.^{12,13} Thus, the identification of a serologic marker for autoimmune pancreatitis remains a major goal in clinical research.

The aim of our study was to identify a potential serologic marker with the ability to discriminate autoimmune pancreatitis from pancreatic adenocarcinoma. For this purpose, we used a molecular biologic approach that has already been successfully applied to other autoimmune diseases.¹⁴⁻¹⁷

METHODS

PATIENTS

Between January 2002 and November 2008, we obtained serum samples from patients and healthy controls. All serum samples were stored at -20°C . Blood samples were obtained after all the subjects provided written informed consent, and the local ethics committee approved the study.

The characteristics of the patients are summarized in Table 1. Twenty patients had focal autoimmune pancreatitis. The diagnosis of autoimmune pancreatitis was made in two patients on the basis of histologic examination of pancreatic specimens obtained during surgery. In the 18 patients who had not undergone surgery, the diagnosis was based on at least three of the four following diagnostic criteria: fine-needle aspirates with characteristic cytologic findings (in 6 patients) or histologic findings (in 7), other autoimmune dis-

eases (in 10), typical findings on computed tomography (CT) or magnetic resonance imaging (MRI) (in 18), or clinical, laboratory, and radiologic evidence of a response to corticosteroid therapy (in 18).³ Serum samples from patients with autoimmune pancreatitis were collected within 1 month after the clinical onset of the disease and before the initiation of corticosteroid treatment. Ten patients had extrapancreatic manifestations (ulcerative colitis in five patients, sclerosing cholangitis in three, interstitial nephritis in two, and retroperitoneal fibrosis in one).

Serum specimens were collected within 1 month after the onset of symptoms from 40 patients who had histologically confirmed pancreatic adenocarcinoma. Furthermore, serum specimens from 21 patients with alcohol-induced chronic pancreatitis and from 18 patients with intraductal papillary mucinous neoplasms were analyzed. Finally, serum specimens from patients with two unrelated autoimmune diseases (systemic sclerosis and rheumatoid arthritis) were studied.

Forty age-matched and sex-matched healthy controls recruited from university and hospital workers without a history of alcohol intake or of autoimmunity and with normal findings on abdominal ultrasonography were also included in the analysis.

To validate the results obtained, we analyzed serum specimens from an additional 15 patients with autoimmune pancreatitis and 70 patients with histologically confirmed pancreatic cancer. In the group of patients with autoimmune pancreatitis, the diagnosis was made on the basis of pancreatic specimens in one patient. In the 14 patients who had not undergone surgery, the diagnosis was based on fine-needle aspirates with characteristic cytologic findings (in 4 patients) or histologic findings (in 6), other autoimmune diseases (in 6), typical on CT or MRI (in 14), and clinical, laboratory, and radiologic evidence of a response to corticosteroid therapy (in 14).

Ten of the 15 patients with autoimmune pancreatitis had the diffuse form and 5 had the focal form of the disease. Extrapancreatic manifestations were observed in six patients (ulcerative colitis in four patients, sclerosing cholangitis in one patient, and retroperitoneal fibrosis in one patient).

All patients with autoimmune pancreatitis and pancreatic cancer were tested for overall IgG4 levels. In addition, all patients included in the study

Table 1. Baseline Characteristics of the Patients in the Training and Validation Groups.

Diagnosis	No. of Patients	Mean Age (Range)	Sex		<i>Helicobacter pylori</i> -Positive Serologic Status
			Male	Female	
		yr	no.		%
Training group					
Autoimmune pancreatitis	20	45 (25–75)	12	8	83
Alcohol-induced chronic pancreatitis	21	54 (31–70)	16	5	44
Intraductal papillary mucinous neoplasms	18	62 (40–75)	12	6	44
Pancreatic cancer	40	63 (40–84)	20	20	54
Rheumatoid arthritis	20	55 (30–75)	6	14	40
Systemic sclerosis	17	48 (24–70)	3	14	45
Validation group					
Autoimmune pancreatitis	15	44 (22–75)	13	2	81
Pancreatic cancer	70	62 (40–82)	36	34	55

were tested for the presence of anti-*Helicobacter pylori* antibodies.

DETECTION OF IgG4 AND ANTI-*H. PYLORI* ANTIBODIES

Serum IgG4 levels were determined with the use of a commercially available immunonephelometric kit (BNII Nephelometer, Dade Behring). The upper limit of the normal range of serum IgG4 levels was considered to be 135 mg per deciliter, as reported by Hamano et al.¹¹ IgG antibodies to *H. pylori* were detected with the use of a commercially available kit (Enzygnost, Siemens).

PEPTIDE LIBRARY

A random dodecamer peptide library that shows peptides on the surface of *Escherichia coli* was purchased from Invitrogen and screened with pooled immunoglobulins purified from the serum samples from the 20 patients with autoimmune pancreatitis, according to the manufacturer's instructions (FlitRx Panning Kit, Invitrogen). After five rounds of biopanning experiments, the enriched library was grown, and single colonies were induced with tryptophan to express the fusion peptides. Bacteria were lysed in sample buffer and tested by means of Western blotting with the pooled immunoglobulin fraction from the patients with autoimmune pancreatitis in order to check for positive clones. DNA was extracted from positive

clones and sequenced. A set of 15 of the 30 peptides obtained from the last biopanning round was synthesized and used in a dissociation-enhanced lanthanide fluorescence immunoassay (DELFA) to test serum specimens from individual patients.

PEPTIDE SYNTHESIS

All the synthetic peptides, including the autoimmune pancreatitis (AIP) peptide (SKDERRFEQPRV), the AIP₁₋₇ peptide (SKDERRF), the AIP₆₋₁₂ peptide (RFEQPRV), the plasminogen-binding protein (PBP) peptide (AKEERRY), the ubiquitin-protein ligase E3 component n-recogin 2 (UBR2) peptide (AKEQRRQ), and the irrelevant control peptide (VTLPKDSDVLP), were manually synthesized by means of the standard method of solid-phase peptide synthesis; this method uses the 9-fluorenylmethoxycarbonyl strategy with minor modifications.¹⁸

ASSESSMENT OF ANTIBODY BINDING

The DELFIA is a time-resolved fluorescence method that can be used to study antibody binding to solid-phase proteins or peptides. The peptides were used at a concentration of 20 µg per milliliter in phosphate-buffered saline to coat DELFIA plates (PerkinElmer). Plates were then blocked for 1 hour with a blocking reagent (PerkinElmer). Serum samples were diluted in a 1:50 solution in phosphate-buffered saline plus 1% bovine serum albumin

Table 2. Peptides Used in the Study and Sequence Homologies.*

Synthetic peptides												
S	K	D	E	R	R	F	E	Q	P	R	V	AIP peptide ₁₋₁₂
S	K	D	E	R	R	F						AIP peptide ₁₋₇
R	F	E	Q	P	R	V						AIP peptide ₆₋₁₂
A	K	E	E	R	R	Y						<i>Helicobacter pylori</i> PBP (298-304) O25249
Sequence homology between AIP ₁₋₇ and <i>H. pylori</i> PBP												
S	K	D	E	R	R	F						AIP peptide ₁₋₇
:		:				:						
A	K	E	E	R	R	Y						<i>H. pylori</i> PBP (298-304) O25249
Sequence homology between <i>H. pylori</i> PBP and UBR2												
A	K	E	E	R	R	Y						<i>H. pylori</i> PBP (298-304) O25249
			:									
A	K	E	Q	R	R	Q						UBR2 (1186-1192) Q8I WV8

* PBP denotes plasminogen-binding protein, and UBR2 ubiquitin-protein ligase E3 component n-recognin 2. Identity is indicated by a vertical line, and a conservative substitution is indicated by a colon.

(Sigma) and incubated on the plates overnight at 4 to 8°C. Plates were then washed 10 times with washing buffer (PerkinElmer). Bound antibodies were detected with europium-labeled antihuman IgG antiserum (1:500 in diluting buffer, PerkinElmer). Plates were read on a Victor3 instrument (PerkinElmer), and the data were analyzed with software supplied with the DELFIA instrument. Absorbance values higher than the mean (+3 SD) for each serum dilution in the control group were considered to be positive.

RABBIT ANTISERUM PRODUCTION

Polyclonal antibodies against a peptide corresponding to *H. pylori* PBP, amino acid residues 298 to 309 (AKEERRYLRDER) were generated in New Zealand white rabbits with the use of standard techniques.¹⁵

WESTERN BLOTTING

An *H. pylori* extract (Virion) was used to detect the *H. pylori* PBP by means of an immunoblot assay. The extract was enriched for the PBP by affinity chromatography with the use of a Sepharose column coupled to a rabbit anti-PBP peptide antibody (see above). Blots were probed with primary antibodies followed by either peroxidase-linked antihuman immunoglobulin antibodies or antirabbit IgG antibodies (Sigma). HeLa cell lysates were prepared with the use of a commercially available kit (Nuclear Extract Kit, Active Motif). Cell lysates were

immunoprecipitated with mouse polyclonal anti-human recombinant UBR2 (amino acids 1 through 439) antibodies (Abnova) cross-linked to Sepharose. Eluted proteins were resolved by means of sodium dodecyl sulfate–polyacrylamide-gel electrophoresis and transferred onto a nitrocellulose membrane (Amersham Biosciences). Blots were incubated with mouse anti-UBR2 antibodies, purified human antibodies (10 µg per milliliter), or serum specimens (at a 1:50 dilution in buffer). The Renaissance chemiluminescence kit (NEN Life Science Products) was used for detection.

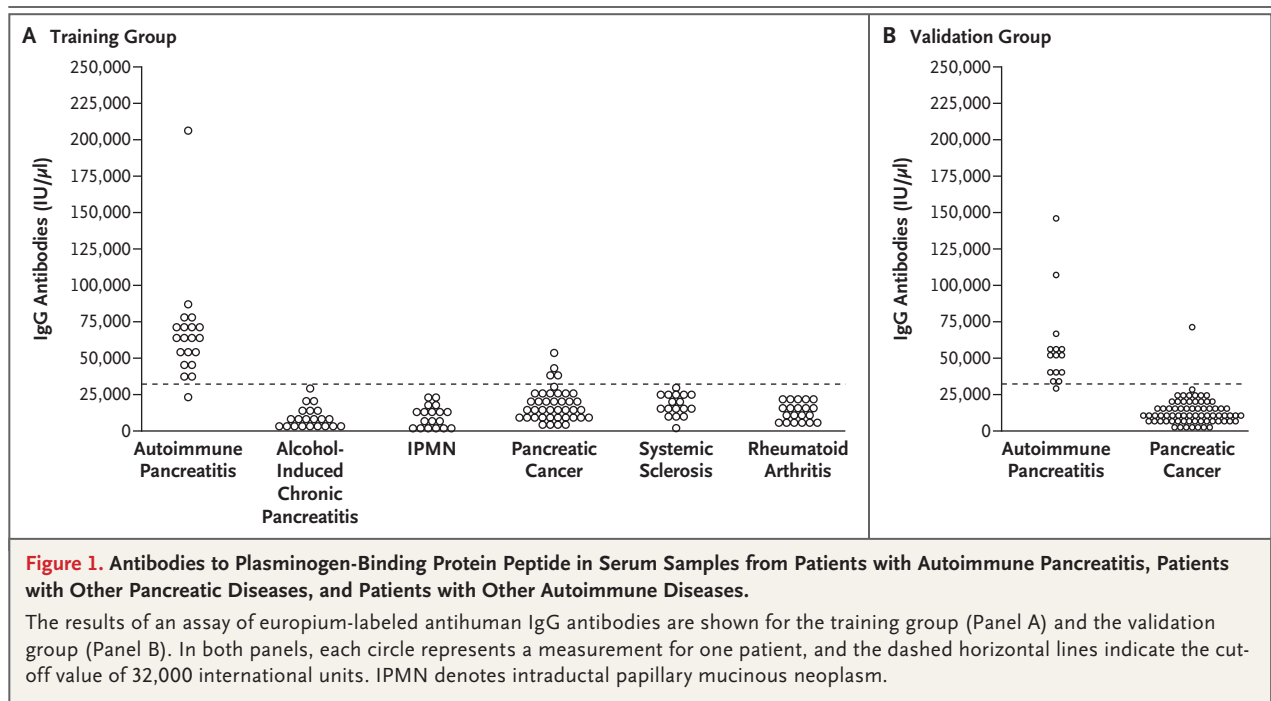
STATISTICAL ANALYSIS

We evaluated the sensitivity and specificity of the test with the use of receiver-operating-characteristic (ROC) curve analysis, estimating the area under the curve (AUC) with 95% confidence intervals. Statistical analysis was carried out with the use of SPSS software, version 13 (SPSS).

RESULTS

PEPTIDE LIBRARY

We screened a peptide library with pooled immunoglobulins derived from a panel of 20 patients with autoimmune pancreatitis to identify only those peptides that may have been relevant to the pathogenesis of the disease. Using a DELFIA, we identified a peptide (the AIP peptide SKDER-RFEQPRV) that was recognized by IgG in serum



samples from 17 of 20 patients with autoimmune pancreatitis (85%) but not by serum IgG from healthy controls. However, serum samples obtained from 22 of 40 patients with pancreatic cancer (55%) and 10 of 21 patients with alcohol-induced chronic pancreatitis (50%) reacted with this peptide. To better map the immunoglobulin reactivity toward this epitope and to better discriminate between autoimmune pancreatitis and pancreatic cancer, we decided to synthesize two peptides — AIP₁₋₇ peptide (SKDERRF) and AIP₆₋₁₂ peptide (RFEQPRV) (Table 2).

The AIP₆₋₁₂ peptide was recognized by the serum samples from 4 of 20 patients with autoimmune pancreatitis (20%) and from 20 of 40 patients with pancreatic cancer (50%). The AIP₁₋₇ peptide was recognized by the serum samples from 18 of 20 patients with autoimmune pancreatitis (90%) and from 4 of 40 patients with pancreatic cancer (10%). These data show that the AIP₁₋₇ peptide sequence contains an epitope recognized by nearly all the serum specimens from patients with autoimmune pancreatitis.

H. PYLORI AND AUTOIMMUNE PANCREATITIS

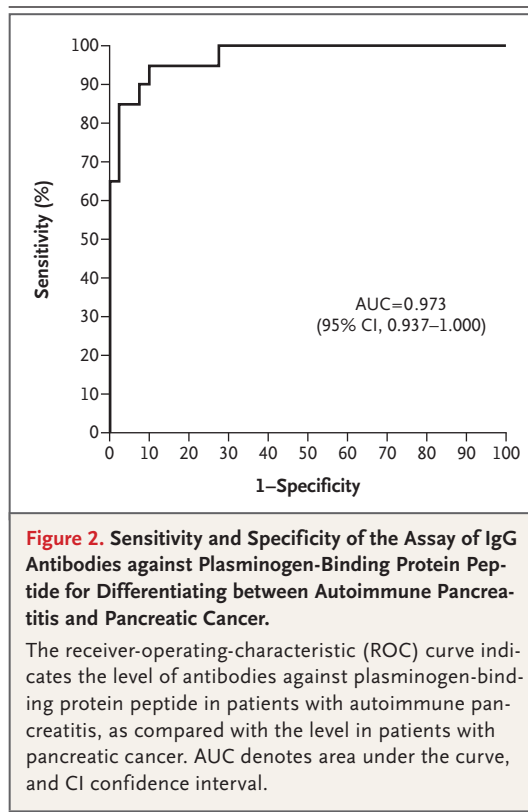
Since *H. pylori* infection has been associated with the pathogenesis of autoimmune pancreatitis,¹⁹⁻²¹ we decided to compare the AIP₁₋₇ peptide sequence with known bacterial protein sequences in a pro-

tein data bank (Swiss-Prot database), using BLASTP software from the Basic Local Alignment Search Tool (BLAST) network service of the National Center for Biotechnology Information (NCBI). We found that the peptide had a high degree of homology (four identities and three conservative substitutions) with the PBP encoded by *H. pylori* (Table 2). We then synthesized the bacterial peptide called PBP peptide (AKEERRY) and used it to test the panel of serum specimens from the patients.

We found that 19 of 20 patients with autoimmune pancreatitis (95%) had serum IgG antibodies against the peptide. The serum samples from 4 of 40 patients with pancreatic cancer (10%) recognized this sequence; the age range of these patients was 48 to 79 years. Such reactivity was not detected in healthy controls or in patients with the other pancreatic diseases studied (Fig. 1A).

Serum antibodies against the identified peptide were also not detected in serum specimens from the patients with the two systemic autoimmune diseases (rheumatoid arthritis and systemic sclerosis) that we studied (Fig. 1A).

The sensitivity and specificity of the quantitative analysis of our assay, with a cutoff value of 32,000 international units, were 95% and 97%, respectively. The AUC in the ROC analysis was 0.988 (95% confidence interval [CI], 0.970 to 1.000; *P*<0.001). By comparing only patients with



autoimmune pancreatitis with patients with pancreatic cancer, the sensitivity and specificity were 95% and 90%, respectively, with an AUC in the ROC analysis of 0.973 (95% CI, 0.937 to 1.000; $P < 0.001$) (Fig. 2).

To validate these data, a second series of patients was evaluated for the presence of anti-PBP peptide antibodies with the use of the same cut-off value. Fourteen of 15 patients with autoimmune pancreatitis (93%) and 1 patient (60 years of age) of 70 with pancreatic cancer (1%) had a positive test (Fig. 1B), with an AUC in the ROC analysis of 0.988 (95% CI, 0.963 to 1.000; $P < 0.001$).

With the training and validation groups combined, 33 of 35 patients with autoimmune pancreatitis (94%) and 5 of 110 patients with pancreatic cancer (5%) had a positive test for antibodies, with a sensitivity of 94% and a specificity of 95%.

A total of 19 of 35 patients with autoimmune pancreatitis (54%) had a positive test for IgG4, with a cutoff of 135 mg per deciliter.¹¹ The 2 patients who had a negative test for the anti-PBP peptide antibodies were IgG4-positive, whereas all the remaining 16 IgG4-negative patients had a

positive test for anti-PBP peptide antibodies. Eleven of the 110 patients with pancreatic cancer (10%) were positive for IgG4. The results of the *H. pylori* testing are shown in Table 1.

The results of the enzyme-linked immunosorbent assay that were obtained with the PBP peptide were confirmed by Western blot analysis with the use of an *H. pylori*-derived protein extract; 32 of the 35 serum samples from patients with autoimmune pancreatitis showed reactivity against a protein band corresponding to the PBP. Representative examples are shown in Figure 3. Antibodies against the *H. pylori* PBP were not detected in healthy controls, patients with intraductal papillary mucinous neoplasms, or patients with alcohol-induced chronic pancreatitis; these antibodies were detected in 4 of 110 patients with pancreatic cancer.

AUTOANTIGEN TARGETS IN AUTOIMMUNE PANCREATITIS

Since autoimmune pancreatitis is characterized by chronic pancreatic tissue damage, we next compared the PBP peptide with human pancreatic proteins in a protein data bank (Swiss-Prot database of known human sequences), using BLASTP software from the NCBI BLAST network service, and we found that the peptide was homologous (five identities and one conservative substitution) to UBR2, which is highly expressed in acinar cells of the pancreas (Table 2). Affinity-purified antibodies against the PBP peptide detected in serum samples from patients with autoimmune pancreatitis recognized the UBR2 protein on Western blotting (data not shown). Reactivity against such protein was also detected in 29 of 35 serum specimens from patients with autoimmune pancreatitis, but not in serum specimens from patients with alcohol-induced chronic pancreatitis. Moreover, UBR2 was detected on Western blotting in only 2 of 40 patients with pancreatic cancer. IgG antibodies against the UBR2 peptide were detectable in serum samples from 22 of 35 patients with autoimmune pancreatitis. Such reactivity was not detected in serum specimens from patients with other pancreatic diseases.

Finally, in the patients with autoimmune pancreatitis, the binding of serum antibodies to the purified *H. pylori* PBP was inhibited by the protein itself, the PBP peptide, and the UBR2 peptide but not by an irrelevant control peptide. Similarly, in

these patients, the binding of serum antibodies to UBR2 peptide was inhibited by the homologous peptide, the *H. pylori* protein, and the PBP peptide but not by the irrelevant peptide (see the Supplementary Appendix, available with the full text of this article at NEJM.org).

DISCUSSION

We report on a serologic marker that is present in most patients with autoimmune pancreatitis. In clinical practice, the differential diagnosis between autoimmune pancreatitis and pancreatic cancer is difficult. It has been reported that up to 10% of patients who undergo pancreatic resection for a suspected pancreatic cancer have a final diagnosis of pancreatitis.²² The criteria for the diagnosis of autoimmune pancreatitis are not yet completely defined; therefore, the identification of serologic markers is relevant in clinical practice.

Our assay was able to discriminate between autoimmune pancreatitis and pancreatic cancer in the majority of the patients. A total of 5% of patients with pancreatic cancer were positive for anti-PBP peptide antibodies with our assay, suggesting that it is an imperfect test to rule out pancreatic cancer. The mean age of the patients with autoimmune pancreatitis was lower than that of the patients with pancreatic cancer; however, we do not believe that this difference influenced the test. Indeed, the test was negative in other groups of patients with a mean age that was similar to that of the patients with autoimmune pancreatitis.

High IgG4 levels are helpful in the diagnosis of autoimmune pancreatitis, although the level chosen and the frequency reported vary among studies.¹¹⁻¹³ Therefore, the use of the IgG4 level to discriminate autoimmune pancreatitis from pancreatic cancer is still under debate. We detected high IgG4 levels in 54% of the patients with autoimmune pancreatitis and in 10% of the patients with pancreatic cancer in our study.

The assay for anti-PBP peptide antibodies that we describe here was not positive in healthy controls or in patients with systemic sclerosis or rheumatoid arthritis, autoimmune diseases that are unrelated to autoimmune pancreatitis. It has yet to be determined whether the assay can be of value in identifying the subgroup of patients with auto-

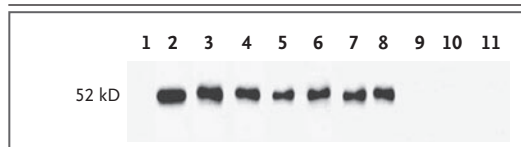


Figure 3. Binding of Serum IgG Antibodies to *Helicobacter pylori*-Derived Protein in Patients with Autoimmune Pancreatitis.

An *H. pylori* bacterial lysate was probed with normal pooled human serum IgG (lane 1), rabbit antiserum raised against the peptide of the *H. pylori* plasminogen-binding protein (PBP) (lane 2), affinity-purified antibodies against the peptide of *H. pylori* PBP in serum specimens from patients with autoimmune pancreatitis (lane 3), serum specimens from five different patients with autoimmune pancreatitis (lanes 4 through 8), affinity-purified antibodies against an irrelevant control peptide in serum specimens from patients with autoimmune pancreatitis (lane 9), and serum specimens from two healthy subjects (lanes 10 and 11). A peroxidase-labeled antihuman IgG antibody and an antirabbit IgG antibody (lane 2) were used for detection on Western blotting.

immune pancreatitis among patients who have particular autoimmune diseases, such as ulcerative colitis, Crohn's disease, and Sjögren's syndrome, that are known to be associated with autoimmune pancreatitis.

A role of *H. pylori* infection in the pathogenesis of autoimmune pancreatitis has been suggested.^{19-21,23,24} In this study, we found that the peptide AIP₁₋₇, which we identified by screening a peptide library of serum specimens from patients with autoimmune pancreatitis, is homologous to an amino acid sequence of PBP of *H. pylori*. The PBP peptide is also homologous to the human protein UBR2, which is highly expressed in pancreatic acinar cells. Therefore, it seems likely that pancreatic acinar cells may be the target of autoimmune activity in patients with autoimmune pancreatitis.

In conclusion, we describe the identification of a novel antibody that we detected in most of the patients with autoimmune pancreatitis in our study. However, this antibody was also present in a few patients with pancreatic cancer and therefore cannot be used alone to distinguish autoimmune pancreatitis from pancreatic cancer.

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The International Committee of Medical Journal Editors (ICMJE) is seeking two new member journals to be represented by their editors-in-chief. Information about the ICMJE is available at www.icmje.org. Given the small number of applicants and the ICMJE's recent awareness that many journals interested in applying had not seen the initial announcement posted in late June, the ICMJE has decided to extend the deadline for applications to November 30, 2009. Candidate journals should meet the following criteria:

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- statement on why the journal/editor-in-chief wants to be an ICMJE member (should not exceed 1000 words in length)
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