

Antibodies to a trypsin sensitive pancreatic antigen in chronic inflammatory bowel disease: specific markers for a subgroup of patients with Crohn's disease

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Abstract

The presence of antibodies against pancreatic juice (PAB) in patients with Crohn's disease has recently been reported. In our study sera from 273 patients with inflammatory bowel disease (222 with Crohn's disease, 51 with ulcerative colitis) have been examined for PAB and also for antibodies against gut tissues by means of indirect immunofluorescence. PAB were found in 68 of the 222 patients with Crohn's disease (31%), with titres ranging from 1/10 to 1/1280, and in only two patients with ulcerative colitis (4%), with titres of 1/20. None were found in 198 patients with various chronic inflammatory diseases and healthy control subjects. No differences were found between the PAB positive and negative patients when the following parameters were compared: disease activity (Crohn's disease activity index), involvement of bowel segments, incidence of extraintestinal disease, or treatment with anti-inflammatory drugs. Only seven of the patients with Crohn's disease had a history of pancreatic disease and of these, four had detectable pancreatic antibodies. Longitudinal observations of 40 patients with Crohn's disease showed a stable pattern for PAB, independent of disease activity and treatment. Partial characterisation of the PAB antigen, isolated from pancreatic juice, showed a trypsin sensitive macromolecular protein of more than 10⁶ daltons not identical with a panel of defined exocrine pancreatic proteins. By contrast, antibodies against goblet cells (GAB) were found in 13 of 51 patients with ulcerative colitis (29%) and in none of the patients with Crohn's disease or control subjects. PAB were found as a highly specific serological marker for Crohn's disease and GAB for ulcerative colitis, but the relevance of PAB and GAB in the pathogenesis in Crohn's disease remains unclear.

The aetiology and pathogenesis of chronic inflammatory bowel disease has not yet been resolved. Recently, many studies have focused on immunopathogenetic mechanisms. Disturbed functions in both the cellular and the humoral aspects of the immune system have been shown. The current thinking favours an inappropriate immune response to luminal antigens, caused by a failure of adequate suppressor T cell function, leading to a chronic immune activation of immune cells in the intestinal tract.^{1,2}

Unlike in classical autoimmune diseases, the various antibodies described in both ulcerative

colitis and Crohn's disease are not considered to have pathogenic and diagnostic importance.² In ulcerative colitis Broberger and Perlmann reported on antibodies against an extract of colon mucosa in 1959,³ and later on antibodies against intestinal goblet cells were reported.^{4,5} More likely to be important in ulcerative colitis are autoantibodies to colonic mucosa antigens which have been found using isolated colon epithelial cells.⁶

Stöcker *et al*⁷ recently showed in 39% of patients with Crohn's disease specific autoantibodies against an antigen in pancreatic juice. These findings seem to be specific for this group of inflammatory bowel diseases. The clinical importance of these antibodies remains unclear. Furthermore, preliminary analysis of the antigen showed a macromolecule in pancreatic juice that did not crossreact with amylase, lipase, trypsin, and chymotrypsin, but no further characterisation has been reported.

The aim of our study was therefore to show the disease specificity and relevance of those antibodies in patients with inflammatory bowel disease in respect of the clinical features, such as the course of the disease, extraintestinal disease, and involvement of bowel inflammation, in order to evaluate whether these antibodies describe a subgroup of patients with Crohn's disease. Also, further characterisation of the pancreatic antigen was performed.

Patients and methods

Sera from 273 patients with inflammatory bowel disease were included in this study. In 222 patients (107 women, 115 men) a diagnosis of Crohn's disease was established on endoscopic, histological, and clinical criteria, according to the European Cooperative Crohn's disease study.⁸ The ages of these 222 patients ranged from 17-74 years, mean age 28 years. The mean duration of disease was nine years (range 4 weeks-28 years). Eighty six (39%) of the patients were being treated with corticosteroids at the time of blood sampling. In 142 (64%) patients the ileum and colon were affected, in 38 (17%) the small bowel, and in 42 (19%) the colon only. In 32 patients the duodenum or stomach, or both, was also affected. Seven patients had a history of pancreatitis. Extraintestinal disease was present in 25 (11%) patients at the time of blood sampling: erythema nodosum (10), iridocyclitis (four), oligoarthritis (four), sacroiliitis (six), and primary sclerosing cholangitis (one). Fistulas were found in 15 patients. Disease activity was determined using the Crohn's

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disease activity index,⁹ which was over 150 in 65 patients and under 150 in 157 cases at the time of blood sampling.

In 51 patients (27 women, 24 men) ulcerative colitis was diagnosed according to the criteria of Truelove and Witts.¹⁰ The mean age was 33 years, range 20–73 years. The mean duration of the disease was 12 years (range 3 months–32 years). Endoscopic investigation showed that inflammation was limited to the left side of the colon in 28 patients and in the remainder involved the whole colon. Forty two patients were in remission, nine had active disease.

Control sera were tested from 198 patients: 12 with coeliac disease, 15 acute and chronic pancreatitis, 10 autoimmune hepatitis, 10 acute and chronic viral hepatitis, eight alcoholic hepatitis, 10 primary biliary cirrhosis, six primary sclerosing cholangitis, 20 colon cancer, 11 lupus erythematosus, 11 rheumatoid arthritis, eight Sjögren's disease, 12 thyroiditis, and 65 healthy blood donors.

TISSUES FOR IMMUNOHISTOCHEMISTRY

Specimens of human pancreas from three organ donors (blood group O) for transplantation were used to detect antibodies against pancreas (PAB). To test the organ and species specificity tissues from the normal human liver, obstructed liver with bile duct proliferation, stomach, duodenum, ileum, colon, rectum, parotid gland, and mouse and rat tissues were tested. Human colon and ileum were used to detect antibodies against intestinal goblet cells. Tissues were embedded in Tissue-tek (Miles, USA), snap frozen in 2-methyl-butane cooled with dry ice, and stored at -80°C . Sections $5\ \mu\text{m}$ thick were cut (at a temperature of -16 to -20°C on Cryocut E (Reichert-Jung, Germany)), air dried, and fixed in acetone-chloroform (1:1) for 4 minutes at 4°C .

IMMUNOFLUORESCENCE AND PEROXIDASE TECHNIQUE FOR DETECTION OF PAB AND OTHER ANTIBODIES

Sections of pancreas and control tissues were incubated for 40 minutes in a moist chamber at 20°C with $50\ \mu\text{l}$ of serum samples (diluted 1:10 with phosphate buffered saline (PBS), pH 7.4). After washing with PBS incubation followed with polyvalent fluorescein-conjugated sheep antihuman immunoglobulin (Wellcome, diluted 1:25 in PBS) for 30 minutes at ambient temperature. Subsequently, after washing the sections were embedded in Immuno-Mount (Shandon, UK) and immediately read in a Leitz fluorescence microscope (HDO 200 W mercury lamp with a filter combination I_2 , mirror RKP 510).

Alternatively, an immunoperoxidase technique was performed after serum incubation, using a peroxidase-conjugated polyvalent antihuman immunoglobulin from sheep (Wellcome, diluted 1:25 in PBS). After a second wash the peroxidase reaction was developed by incubating sections with 0.1% 3'-3'-diamino-benzidine-tetrahydrochloride and 0.003% H_2O_2 for 10 minutes. Sections were then counterstained with

Mayer's hemalum (Merck, Germany) for 30 seconds and mounted in Kaiser's glycerin-gelatin (Merck, Germany).

Antibody positive sera were diluted for reciprocal titres. Determination of immunoglobulin classes was done using monovalent antihuman immunoglobulins (Wellcome).

All slides were read under code by two different investigators. Results for PAB were considered positive if the lumen contents of pancreatic acini were labelled.

ELISA TEST

PAB positive sera tested on a variety of known pancreatic antigens or partially purified pancreas antigen (see below) using an enzyme linked immunosorbent assay (ELISA). The following commercially available pancreatic proteins were used at a concentration of $5\ \mu\text{g/ml}$: amylase, lipase, phospholipase A and C, enterokinase, carboxypeptidase A and B, chymotrypsin A and B, chymotrypsinogen, elastase, trypsin, trypsin inhibitor (Böhringer, Germany), lactoferrin (Sigma), and kallikrein (Sigma). Microtitre plates were coated with antigens in bicarbonate buffer for 20 hours at 4°C . After washing, strong PAB positive sera and control sera from patients with ulcerative colitis, pancreatitis, viral hepatitis, autoimmune diseases, and healthy subjects (1:80 diluted in PBS) were applied for one hour. After washing, peroxidase conjugated antihuman immunoglobulins (1:500 diluted) (Dakopatts, Denmark) were added and developed with orthophenyldiamine 0.05% and 0.01% H_2O_2 in citrate buffer, pH 5.0. Reaction was stopped after 20 minutes with H_2SO_4 . Analysis was done by Microelisa Auto Reader, MR 580 (Dynatech, USA) at 520 nm wave length. Tested sera were considered positive when extinction was higher than double the standard deviation of the mean extinction of 45 sera from healthy subjects.

CHARACTERISATION OF PANCREATIC ANTIGEN

Pancreatic juice

Pancreatic juice was obtained from five surgical patients by draining pancreatic ducts after partial pancreas resection and stored at -20°C (after adding trypsin inhibitor to some of the samples).

Absorption study

Four different high titre sera with PAB detected by immunofluorescence, were absorbed with increasing amounts of pancreatic juice from two different patients to a final dilution of 1:30 for 30 minutes and centrifuged at $15\ 000\ \text{g}$ for 10 minutes before testing by immunofluorescence. As antigen control, liver homogenate and bile were used.

Chromatography

Fractionation of pancreatic juice was performed by gel chromatography on a Sephacryl S 300 column (Pharmacia, Sweden) with a gel-bed of $90 \times 2.5\ \text{cm}$. Fractions were eluted with 0.05



Figure 1: Immunofluorescence pattern of pancreatic antibodies (PAB) on human pancreas: positive labelling in the lumen of pancreatic acini.

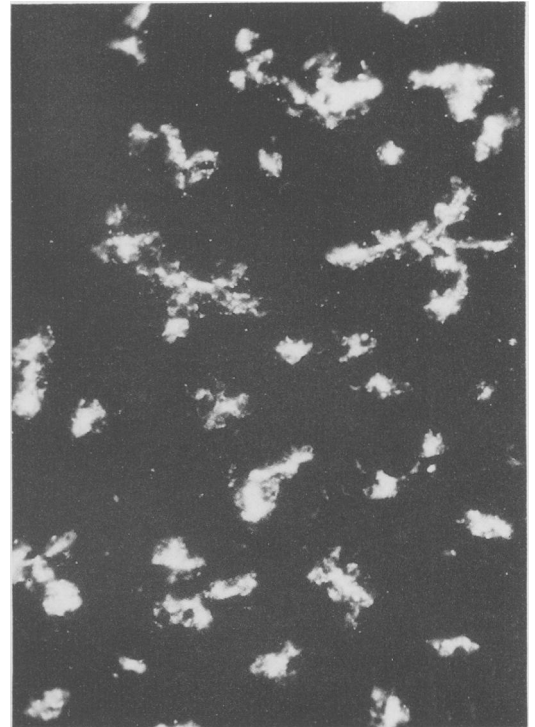


Figure 2: Immunofluorescence on mouse pancreas: strong labelling in the lumen of pancreatic acini.

mol/l Tris/HCl buffer. Antigen activity in the pooled fractions were tested in an ELISA system with three different strong positive sera. For further purification the antigen-containing peak of the Sephacryl S 300 run was submitted to ion exchange chromatography on DEAE-Sephacel (Pharmacia). Gradient elution was done by a 0.01 mol/l Tris/HCl buffer with increasing NaCl solution from 0.05 to 0.25 mol/l in five steps.

Detergents, solvents, thermostability

Cryostat sections were exposed to sodium dodecylsulphate 1% (Serva, Germany), Triton X 100 1% (Serva), Tween 20 1% (Serva) for 30 seconds and to ethanol 95% and acetone-chloroform (1:1) for 15 minutes. Thermostability of the antigen was measured by exposing to various temperatures in a water bath.

Enzymes

Microtitre plates were incubated with 6.5 µg/ml of partially chromatographically purified pancreatic antigen as above. Various enzymes such as bovine trypsin (Boehringer), nagarse from *Bacillus subtilis* (proteinase, Serva), enterokinase from swine intestine (Sigma), phospholipase A from bovine pancreas and C from *Clostridium perfringens* (Sigma), mixed glycosidase from *T. corneatus* (Seikagaku Kogyo, Japan), neuraminidase from *C. perfringens* type 5 (Sigma), hyaluronidase from sheep testes (Boehringer), chondroitinase ABC from *Proteus vulgaris* (Seikagaku Kogyo), amylase type VIa, elastase type I, and lipase type II from swine pancreas (Sigma), and carboxypeptidase A and B, alpha and beta chymotrypsin from bovine

pancreas (Sigma) were added at a concentration of 100 µg/mg protein in appropriate buffers and temperatures for 8 hours. After extensive washing the ELISA procedure was continued as shown above.

Alternatively, enzyme treatment was performed in Eppendorf tubes. Chromatographically purified pancreatic antigen was incubated in a time dependent manner with enzymes (100 µg/mg pancreatic antigen) from 10 minutes to 24 hours. Enzymes were inactivated by chloroform. The aqueous phase adjusted to 6.5 µg protein/ml was taken to incubate ELISA plates. Trypsin was inactivated by trypsin inhibitor (200 µg/mg). Pancreatic protein treated in an identical manner but without added enzymes was used as a control.

Results

IMMUNOCYTOCHEMISTRY PATTERN, ORGAN SPECIFICITY, AND ABSORPTION STUDIES OF PAB
Specific labelling of PAB positive sera on pancreatic tissue appeared as a dense aggregation in the lumen of pancreatic acini (Fig 1). Occasionally a weak reaction in the cytoplasm of the human acinar cells was observed as well. The pancreatic islets did not show any labelling. The same pattern was obtained using pancreatic tissue from three humans or from rats and mice (Fig 2). Human salivary gland, breast, stomach, small and large intestine, liver, adrenal cortex, and kidney showed no fluorescence with PAB positive sera. Specific labelling of PAB serum on pancreas could be abolished after absorption with human pancreatic juice, but not with liver homogenate and bile control experiments.

TABLE I Incidence of PAB (antibodies to a pancreatic antigen) and GAB (antibodies against intestinal goblet cells) in sera of patients with inflammatory bowel disease and other diseases using an immunofluorescence test on human pancreas as substrate

Patients	No	PAB positive	GAB positive
Crohn's disease	222	68 (31%)	0
Ulcerative colitis	51	2 (4%)	13 (26%)
Celiac disease	12	0	0
Pancreatitis	15	0	0
Autoimmune hepatitis	10	0	0
Chronic viral hepatitis	10	0	0
Alcoholic hepatitis	8	0	0
Primary biliary cirrhosis	10	0	0
Primary sclerosing cholangitis	6	0	0
Colon cancer	20	0	0
Lupus erythematosus	11	0	0
Rheumatoid arthritis	11	0	0
Sjögren's disease	8	0	0
Thyroiditis (autoimmune)	12	0	0
Healthy blood donors	65	0	0

All results were identical, whether immunofluorescence or immunoperoxidase testing was used.

INCIDENCE AND SPECIFICITY OF PAB

Using immunofluorescence or immunoperoxidase on human pancreatic tissues as substrate, PAB occurred in 31% of patients with Crohn's disease (Table I). Titres ranged from 1:10 to 1:1280. Thirty one (44%) patients had IgG specific PAB, 34 (51.5%) IgG and IgA specific PAB, and only one had IgA, IgM, or IgM and IgG.

In ulcerative colitis only two patients had PAB in low titres (1:20) and in other control subjects the antibody was not detected (Fig 3). In contrast, 13 (26%) of 51 patients with ulcerative colitis and none of the 222 patients with Crohn's disease or control patients with other diseases showed fluorescence of intestinal goblet cells on cryostat sections of ileum and colon (Table I).

Nuclear antibodies were found in 15 of the 222

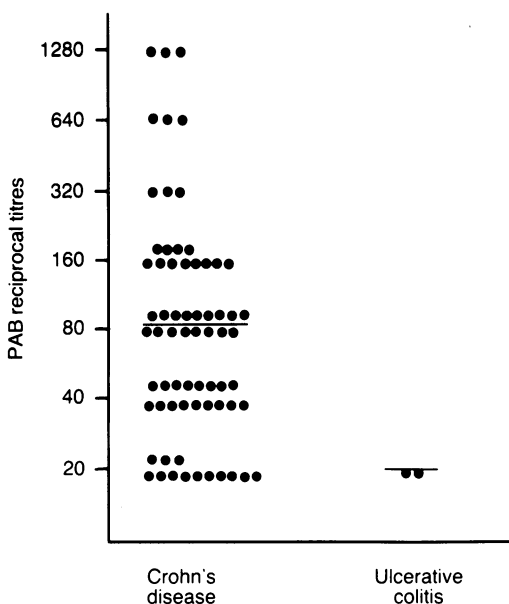


Figure 3: Titres of antibodies to a pancreatic antigen (PAB) in Crohn's disease (68 of 222 patients PAB positive) and ulcerative colitis (2 of 51 patients positive) detected by immunohistochemistry.

Crohn's disease patients (7%) and four of the 51 ulcerative colitis patients (8%). There was no correlation of the incidence of PAB with nuclear antibodies.

CORRELATION OF PAB WITH CLINICAL FEATURES

The occurrence of PAB did not correlate with clinical activity in Crohn's disease. Twenty one of 68 (31%) PAB positive patients and 44 of 154 (29%) PAB negative patients had active Crohn's disease (Crohn's disease activity index (CDAI) >150). In PAB positive sera, CDAI values did not correlate with titres of PAB (r=0.013). Furthermore, there was no correlations with the age and sex of patients, extraintestinal disease, bowel involvement, and treatment. Raised serum lipase and amylase activities did not differ between PAB positive and negative patients. PAB were found in four of seven patients with Crohn's disease and a coincidental pancreatitis.

LONGTERM OBSERVATIONS

Forty patients with Crohn's disease (20 PAB positive, 20 PAB negative) were followed up for 18 months. Fluctuations of antibody titres ranged within two steps and no significant influence of treatment (steroids, sulphasalazine) on PAB could be evaluated. Only one patient had a decrease in titre from 1:1280 to 1:20 on treatment with steroids (Fig 4). None of 20 patients negative for PAB in the initial investigation developed a new antibody formation, although relapse occurred in five during the observation period (increase of median CDAI from 40 to 180).

CHARACTERISTICS OF PANCREATIC ANTIGEN

Chromatography

Antigenic activity was eluted in the void volume when pancreatic juice was submitted to gel chromatography on Sephacryl S 300, indicating a molecular weight of more than 1.3x10⁶ daltons. Applying this peak to ion exchange chromatography, the antigenic activity was obtained in peaks eluted by a low ionic concentration of 0.1 mol/l NaCl.

Physicochemical properties

The chromatographically purified antigen

TABLE II Enzyme digestion of pancreatic antigen

Enzyme digestion with:	Reactivity in ELISA
Trypsin	++
Nagarse	++
Enterokinase	--
Glycosidase	--
Neuraminidase	--
Phospholipase A and C	--
Hyaluronidase	--
Chondroitinase ABC	--
Amylase	--
Carboxypeptidase	--
Elastase type I	--
Lipase type II	--
Chymotrypsin	--

++ = antigen destroyed; -- = not destroyed.

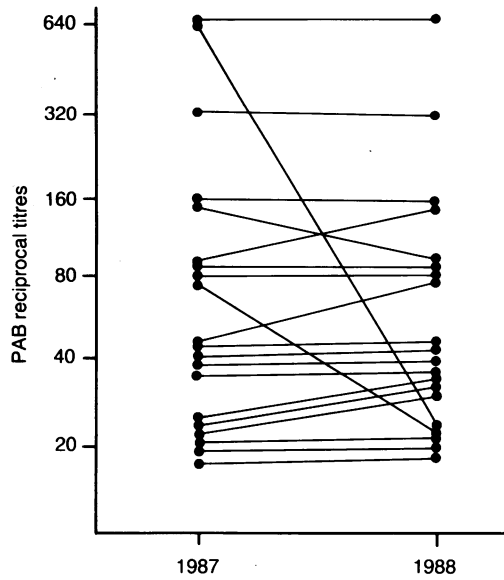


Figure 4: Antibodies to a pancreatic antigen (PAB) in 20 patients with Crohn's disease: titres during follow up of over one year.

showed thermolability when exposed to 56°C for 30 minutes. Detergents (sodium dodecylsulphate, Triton × 100, and Tween 20 concentrations of 1%) and ethanol 95% and chloroform acetone (1:2) had no effect on antigenic activity.

Effect of enzyme treatment on antigenic activity

The reactivity of 208 sera from patients with Crohn's disease and 135 sera from patients with other diseases (ulcerative colitis, acute and viral hepatitis, autoimmune diseases, pancreatitis) and healthy controls were tested in an ELISA system using chromatographically purified pancreatic antigen which was either native or had been treated with various enzymes (Table II) before coating the microtitre plates.

When native antigen was used for testing, 54 (26%) of the patients with Crohn's disease showed a positive reaction but also two of 25 (8%) patients with ulcerative colitis, three of nine (33%) patients with acute and chronic viral hepatitis, and two of 53 (4%) control patients. The relation of the results of ELISA and immunocytochemistry is shown in Table III.

The ELISA using the chromatographically purified antigen is less sensitive for PAB detection in Crohn's disease than the immunofluorescence test. Furthermore, ELISA also detected 'PAB reactivity' in some sera from patients with ulcerative colitis, pancreatitis, viral hepatitis, and healthy controls, although previous immunofluorescence showed no reactivity.

When microtitre plates that had been coated with pancreatic antigen after digestion with

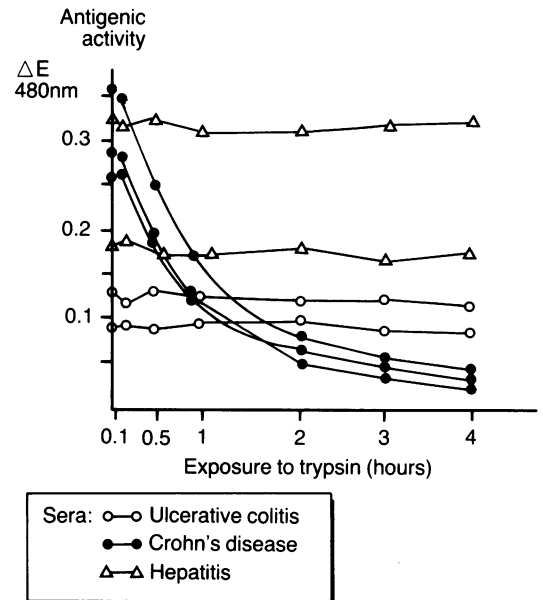


Figure 5: Detection of antibodies to a pancreatic antigen (PAB) using an ELISA system. Microtitre plates were coated with chromatographically purified antigen, either native or treated with trypsin. Using the trypsin treated antigen only PAB in sera from patients with Crohn's disease lose their reactivity, not PAB in sera from patients with hepatitis or ulcerative colitis.

various enzymes were used, after trypsin treatment the positive reactivity of sera containing PAB from patients with Crohn's disease was abolished, but not when sera containing PAB from other patients were tested (Fig 5). Treatment with all other enzymes did not influence the reactivity of PAB positive sera from patients with any of the diseases tested (Table II).

Reactivity of PAB with defined pancreatic proteins

PAB positive sera did not react with any of the following pancreatic antigens: kallikrein, lactoferrin, trypsin, chymotrypsinogen, chymotrypsin A and B, amylase, elastase, lipase, phospholipase A and C, enterokinase, carboxypeptidase, and bovine trypsin inhibitor, which have been used as antigen in an ELISA system.

Discussion

In the present study we detected antibodies against a pancreatic antigen (PAB) in about one third of patients with Crohn's disease using an immunofluorescence test as reported by Stöcker *et al.*⁷ Although the sensitivity was low, the antibody pattern was highly specific for this disease and therefore of diagnostic value. This antibody pattern was not observed in any of the sera from patients with various other chronic inflammatory disorders (including ulcerative colitis and pancreatitis) – except in two of 51 patients with ulcerative colitis and then only in very low titres. Although the differential diagnosis between ulcerative colitis and Crohn's disease is sometimes difficult to make, no evidence of features of Crohn's disease have appeared thus far in those two patients during a 15 year follow up.

Antibodies against pancreatic tissue, as previously described in about 30% of patients with

TABLE III Number of PAB positive sera in immunofluorescence (IFL) compared with ELISA using chromatographically purified antigen and number of sera positive for both IFL and ELISA

	No of sera tested	Positive sera:		
		IFL	ELISA	IFL + ELISA
Crohn's disease	208	62	54	51
Ulcerative colitis	25	2	2	2
Pancreatitis	10	0	4	0
Viral hepatitis	9	0	3	0
Autoimmune disease	38	0	0	0
Healthy control subjects	53	0	2	0

acute pancreatitis, showed mainly acinar cell cytoplasmic fluorescence.^{11,12} Sera from patients with Crohn's disease, however, showed a completely different pattern, with strong fluorescence in the lumen of pancreatic acini. In addition, antibodies in sera from patients with acute pancreatitis were mainly in the IgA subclass, only temporarily detectable, and of low titre (1:1 to 1:10), whereas PAB were mainly in the IgG subclass and showed a fairly stable antibody pattern over a long observation period with high titres (1:10 to 1:1280), only slightly influenced by treatment. Therefore, it is questionable whether PAB in Crohn's disease and antibodies against pancreatic tissue in pancreatitis have the same antibody specificity.

The unique appearance of PAB in patients with Crohn's disease raises the question of whether they represent a clinical subgroup of Crohn's disease patients. No clinical entity could be defined, however, as our analysis of clinical data did not show any correlation between PAB and the extent and pattern of inflammatory gut involvement, the activity of the disease, sex of the patient, or extraintestinal disease, all factors that have been associated with classical autoimmune diseases. Whether the PAB positive subgroup of patients with Crohn's disease share other immunological features of an autoimmune state has to be investigated further. This might suggest the need for immunosuppressive treatment.

Immunofluorescence shows that PAB are organ (pancreas) specific and do not react with other epithelial or glandular tissues nor with Paneth cells in the intestine, which show morphological homologies with exocrine pancreas cells.¹³ This finding suggests a connection between this antibody and pancreatic involvement in Crohn's disease. In our series only seven patients with Crohn's disease had a history of clinical or biochemical pancreatitis. Three of them had PAB. Although initially pancreatitis in Crohn's disease was regarded as a sequelae and a complication when duodenal involvement of Crohn's disease occurred,^{14,15} recent data suggest that pancreatitis may be another genuine extraintestinal manifestation of Crohn's disease.^{16,17} In necropsy studies Chapin *et al*¹⁸ found pancreatic fibrosis in 38% and dilatation of acini with acidophilic deposits in 31% of Crohn's disease patients. In another study a decrease of pancreatic function was observed in a third of patients with inflammatory bowel disease by measuring enzyme and bicarbonate secretion.¹⁹ Such data indicate subclinical pancreatitis in patients with Crohn's disease. To find out the true relation of PAB with pancreatic involvement in Crohn's disease patients it will be necessary to use sensitive pancreatic function tests.

PAB react with products of secretory cells. Why a sensitivity to this antigenic material in some patients with Crohn's disease occurs remains obscure. Analogous to GAB – also antibodies to a secretory product – which we and others²⁰⁻²² have found exclusively in ulcerative colitis, we suggest that PAB induction may be triggered by luminal antigens, such as bacterial antigens, sharing antigenic determinants with intestinal autoantigens.²³⁻²⁵

To clarify the question of why PAB occurs in Crohn's disease, it is essential to define exactly the nature of the antigenic determinants reacting with those antibodies. Our results so far show that the antigenic site of the pancreatic macromolecule, as it has been prepared from pancreatic juice, is a protein since it was completely destroyed by trypsin digestion. In contrast some PAB found in sera from patients with 'non-Crohn's disease' by ELISA reacted obviously with other determinants of this macromolecule, as trypsin treatment did not influence their reactivity. This emphasises the unique specificity for PAB in Crohn's disease. Our preliminary characterisation allows the development of a specific ELISA for PAB detection and further investigation of their role in Crohn's disease.

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