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CHARACTERIZATION OF HUMAN AUTOANTIBODIES REACTIVE TO GASTRIC PARIETAL CELLS

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SUMMARY: Human sera from autoimmune gastritis patients containing autoantibodies to gastric parietal cells were analyzed by immunological methods. Enzyme linked immuno-sorbent assay demonstrated that all nine sera reacted with pig gastric vesicles enriched in H^+/K^+-ATPase (gastric proton pump). Immunoblotting experiments indicated that the α subunit of the H^+/K^+-ATPase was the major antigen in the vesicles with two of the sera reacting strongly. We further characterized the specificitiy of the antibodies using partial sequences of the pig α subunit fused with truncated TrpE (anthranilate synthase). The antibodies from autoimmune gastritis patients reacted differently to the two fusion proteins (Met-1 to Ala-79, and Arg-606 to Ile-964), indicating that each patient sera contains a mixture of autoantibodies recognizing different epitopes with variable contents.

The gastric H^+/K^+-ATPase (gastric proton pump) is responsible for acid secretion into the lumen of the stomach (1). The ATPase is composed of the catalytic α subunit and the heavily glycosylated β subunit (2,3). It belongs to the family of cation transporters known as the P-ATPases (4). The primary structures of α subunits from several mammals are highly homologous: 98% of the residues are identical among human (5,6), pig (7), rat (8,9) and rabbit (10) sequences. Conservation of β subunits is slightly less: 80% among the sequences from human (11), pig (3), rat (12-15), mouse (16,17) and rabbit (18). The H^+/K^+-ATPase is specifically expressed in gastric parietal cells (19).

Abbreviations: BBS, borate buffered saline [30 mM sodium borate buffer (pH 8.3), 150 mM NaCl]; BBS(+), BBS containing 1% bovine serum albumin; ELISA, enzyme linked immuno-sorbent assay; PBS, phosphate buffered saline [10 mM sodium phosphate buffer (pH 7.2), 137 mM NaCl, 3 mM KCl]; SDS, sodium dodecylsulphate.

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Autoimmune gastritis was recognized in humans as an organ-specific disease which is characterized by chronic atrophic gastritis and circulating autoantibodies specific for gastric parietal cells (20). In BALB/c strain of mouse, neonatal thymectomy induces similar autoimmune diseases (21). Recently, the H^+/K^+-ATPase has become a focal point in the etiology of the disease because the antibodies from autoimmune gastritis patients were found to recognize the α and β subunit polypeptides of the ATPase (3,22).

Defining the ATPase-specific autoantibodies biochemically should yield a great deal of information on the presentation of the antigen to the immune system and therefore yield information on the pathology of the disease. Towards this end, we constructed recombinant plasmids carrying different segments of the pig gastric H^+/K^+-ATPase α subunit cDNA (7) fused in frame to the *trpE* (anthranilate synthase) gene (23). The resultant fusion proteins, used to asses the distribution of epitopes, indicated considerable variation in the specificity of the autoantibodies from gastritis patients against the α subunit of the H^+/K^+-ATPase.

MATERIALS AND METHODS

ELISA -- Pig gastric vesicles enriched in H^+/K^+-ATPase were prepared as described previously (24) and used as the antigen in ELISA assays (25). 96-well microtiter plates (Sumitomo type E, Tokyo, Japan) were coated with gastric vesicles (1 µg in 100 µl of 50 mM Tris-HCl, pH 9.5, in each well) for 30 min at 37 °C. The plates were blocked with BBS(+) for 1 hour at 37 °C and washed three times with BBS. The sera obtained from gastritis patients seen at Osaka University Hospital were analyzed. Sera were serially diluted in BBS(+), added in 50 µl total volume to the coated microtiter plate wells and incubated for 1 hour at 37 °C. After washing with BBS three times, 100 µl of horse radish peroxidase-conjugated goat anti-human IgG (Cappel, Durham, NC), diluted 1:20,000 in BBS, was added and incubated for 30 min at 37 °C. After washing three times with BBS, tetramethylbenzidine and H_2O_2 (Kpl, Gaithersburg, MA) was added. The peroxidase reaction was terminated by adding 100 µl of 1 M phosphoric acid. Absorbance at 450 nm was determined in a Bio-Rad (Richmond, CA) Model 450 Microplate Reader.

Immunoblotting -- Pig gastric vesicle proteins were separated by SDS polyacrylamide gel electrophoresis (25 µg protein on a 8 x 10 cm gel) (26) and electroblotted to a nitrocellulose filter (Schleicher & Schuell, Dassel, Germany) (27). The filter was blocked by incubating with 5% skim milk in PBS overnight at 4 °C. The filter was incubated with human sera (diluted 50 times with PBS) for 1 hour at 24 °C in an Immunetics (Cambridge, MA) Miniblotter MN 16 system. After washing with PBS containing 0.1% Tween-20, the filter was incubated with the peroxidase conjugated goat anti-human IgG (the same dilution as above) for 1 hour at 24 °C, the binding of which was visualized by an Amersham ECL chemiluminescence kit.

Construction of plasmids for expression of fusion proteins -- Restriction fragments of the pig gastric H^+/K^+-ATPase α subunit cDNA [for αN1, *Nco*I (base -2, numbering from the first base of the initiation codon) to *Pst*I (base 237) encoding a polypeptide

corresponding to Met-1 to Ala-79; and for αMC, *Sma*I (base 1,816) to *Bam*HI (base 2,888) corresponding to Arg-606 to Ile-964] were ligated in frame into the multicloning site of pATH10 (αN1) or pATH11 (αMC) (23). Short segments from vector plasmids [pUC18 and pBluescript SK II(+)] or synthetic DNA were used as linkers if necessary. To convert cohesive ends to blunt ends, DNA fragments were treated with Klenow enzyme. DNA sequences were confirmed using published methods (28).

The expression of the TrpE-H^+/K^+-ATPase fusion proteins in the *Escherichia coli* strain, XL1-Blue, were induced by the addition of indole acrylic acid (23). The proteins were purified by electroeluting from an SDS polyacrylamide gel, concentrating with a CENTRICUT V-10 (Kurabo, Osaka, Japan), and used in ELISA and immunoblotting assays. Protein was determined by standard procedures (29) using bovine serum albumin as a standard.

Chemicals -- Oligonucleotides were synthesized on a Pharmacia Gene Assembler Plus (Uppsala, Sweden). Restriction enzymes, T4 DNA ligase and Klenow enzyme were purchased from Takara Shuzo Co. (Kyoto, Japan) and Nippon Gene Co. (Toyama, Japan). Sequenase was from United States Biochemical Corp. (Cleveland, OH). All other chemicals were of the highest grade available.

RESULTS AND DISCUSSION

Gastric vesicles are immunoreactive to sera from autoimmune gastritis patients.

Each of the sera from nine autoimmune gastritis patients reacted positively with parietal cells assayed by immunofluorescent staining (Table I, left column). Furthermore, each of the sera reacted with pig gastric vesicles enriched in H^+/K^+-ATPase (Fig. 1). These vesicles are primarily from the tublovesicular membranes in cytoplasm or apical plasma membrane surface of the parietal cells (31) and not normally exposed to the immune system. Most of the sera (from P1-P4, P7 and P8) reacted strongly to the vesicles, whereas three of them (from P5, P6 and P9) reacted less strongly (Fig. 1, open bars). The pattern of reactivity to the vesicles paralleled that to the parietal cells, except that sera from P1 and P6 had stronger than expected reactions to the gastric vesicles (Table I, compare left and middle columns).

The H^+/K^+-ATPase α subunit is the major antigen of the gastric vesicles.

To analyze the proteins recognized by the autoantibodies, gastric vesicle proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting using each of the gastritis patient sera. As shown in Fig. 2, all the sera reacted with a band of 94k dalton protein, exactly the same mobility as the α subunit of the H^+/K^+-ATPase. Sera from P2 and P8 reacted most strongly, while those of P3, P4, P6 and P7, moderately and P1, P5 and P9, weakly. We note that the

Table I. Summary of the reactivities of autoantibodies from autoimmune gastritis patients

Serum	Parietal Cell*	Gastric Vesicle	α Subunit
P1 (F, 51)	x 80	+ + +	+
P2 (F, 65)	x 640	+ + +	+ + +
P3 (F, 27)	x 320	+ + +	+ +
P4 (M, 65)	x 160	+ + +	+ +
P5 (F, 77)	x 80	+ +	+
P6 (F, 66)	x 10	+ +	+ +
P7 (F, 61)	x 160	+ + +	+ +
P8 (F, 67)	x 320	+ + +	+ + +
P9 (F, 50)	x 80	+ +	+

Reactivities of autoantibodies to parietal cells, gastric vesicles (from Fig.1) and the H^+/K^+-ATPase α subunit (from Fig. 2) were summarized. In the parentheses, sex (F, female; M, male) and ages of the patients (P1-P9) are shown.
+ + +, strong reactivity; + +, moderate reactivity; +, weak reactivity.
* Acetone-fixed cryostat sections of mouse stomach were stained with immunofluorescent (fluorescein isothiocyanate-conjugated) goat anti-human IgG after reaction with patient serum (30). The numerical values indicate the limit dilution giving positive staining.

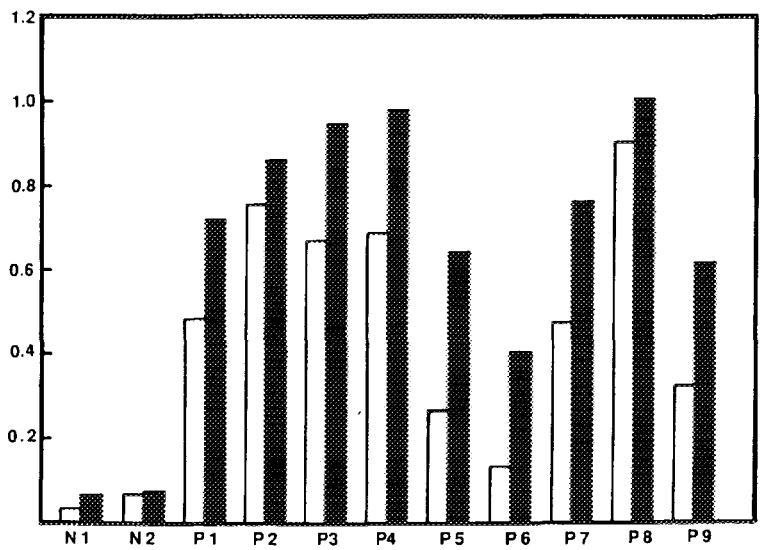

Fig. 1. Binding of human autoantibodies to pig gastric vesicles.
The binding of the autoantibodies to the vesicles was visualized by ELISA. Absorbance at 450 nm was plotted against dilution of the patients' sera (open bar, 1/400 dilution; closed bar, 1/50 dilution). N1 and N2, sera from normal individuals; P1 - P9, sera from autoimmune gastritis patients.

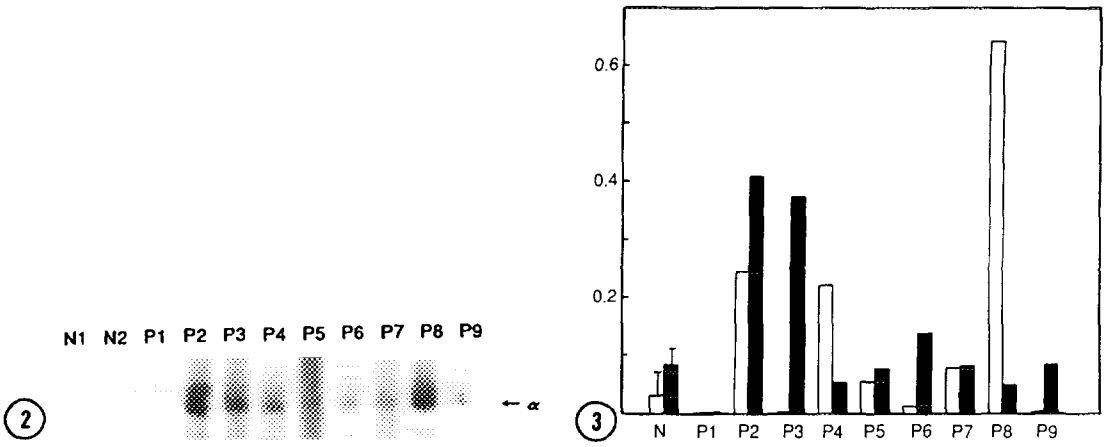

Fig. 2. Reactivity of human autoantibodies to H^+/K^+-ATPase α subunit.
Pig gastric vesicle proteins were separated by SDS polyacrylamide gel electrophoresis and subjected to immunoblotting. The patient sera were diluted to 1/50. N1 and N2, sera from normal individuals; P1 - P9, sera from autoimmune gastritis patients.

Fig. 3. Reactivity of human autoantibodies to partial sequence of the α subunit.
Partial sequences of pig gastric H^+/K^+-ATPasep α subunit were expressed in *Escherichia coli* as fusion proteins with truncated TrpE. Approximately 10 pmols of fusion protein/well were used for ELISA. The patient sera and goat anti-human IgG were diluted to 1/50 and $1/10^4$, respectively. Absorbance (450 nm) with truncated TrpE protein was subtracted from the data. The bars indicated the absorbancies with αN (open bar) and αMC (closed bar). P1 - P9, sera from autoimmune gastritis patients. Average values and standard deviations were shown for seven normal individuals (N).

α subunit band was the only distinct band detected in the immunoblots developed by either the chemiluminescence technique or by using [^{125}I]-labeled Protein A (data not shown).

As summarized in Table I, the reactivities of the antibodies to the α subunit band (right column) were well correlated with the reactivities against the parietal cells (left column). The exception was the serum from P1 which reacted strongly with gastric vesicles but not with the parietal cells or the α subunit. In this case, the weaker reaction to the immunoblotted protein, may be due to the treatment of the sample before exposing to the antibodies. The serum from P6 reacted similarly to gastric vesicles and α subunit.

It was demonstrated previously that all sera from thymectomized mice showing atrophic gastrsis have antibodies for the α subunit but rarely for the β subunit (32). This observation from a model animal system may be related to our similar finding that antibodies against the β subunit could not be detected in our collection of gastritis patient sera.

Autoantibodies recognize different epitopes of the α subunit.

Because of the differing reactivities of the autoantibodies to the H^+/K^+-ATPase α subunit, we suspected that the epitopes recognized may also vary. To address this question, fusion proteins containing segments of the α subunit were expressed in *E. coli*, purified and used in ELISA assays. Two regions of the α subunit were fused to TrpE: in protein αN1, the amino terminal 79 amino acids (Met-1 to Ala-79), and for αMC, the carboxyl-terminal 359 amino acids (Arg-606 to Ile-964). Fig. 3 shows that most of the patient sera reacted with one or both of the fusion proteins demonstrating that the H^+/K^+-ATPase is, in fact, the antigen recognized by the autoantibodies.

Interestingly, the various autoantibodies reacted with the fusion proteins in different patterns. Sera from P2 and P8 which had the strongest reaction to the α subunit, both recognized αN1. P2 also reacted with αMC, but P8 in addition to serum from P4 reacted only with αN1. Sera from P3 and P6 were reactive to αMC only, while those from P1, P5, P7 and P9 did not show strong reactivity to either fusion protein. In these cases, the epitopes recognized are likely in the parts of the α subunit not represented, or that the fusion proteins presented the epitopes differently from that recognized by the antibodies. We confirmed the reactivities of the P2, P5, P6 and P8 sera in immunoblotting experiments (data not shown).

The sera from seven normal individuals including N1 and N2 (Figs. 1 and 2) did not react with αN1 or αMC (average values and standard deviations shown in Fig. 3). These observations clearly demonstrate that the human autoantibodies recognize many epitopes, and the amounts and composition of the antibodies are different among patients.

Hydropathy analysis of the H^+/K^+-ATPase α subunit (8,33) suggests that the amino terminal region (including all of the protein represented in αN1) and 60-75% of the protein represented in αMC, are found on the cytoplasmic surface of the plasma membrane. The presence of antibodies recognizing cytoplasmic epitopes suggests that the autoantibodies may be selected at a later stage of the disease after the parietal cells have been damaged. Only then could the cytoplasmic domains become exposed to the immune system and autoantibodies against such epitopes

produced. Such a pathway of disease progress is not contradictory to the observation that we could not detect autoantibodies against the β subuint whose cytoplasmic domain is limited to a very short amino terminal sequence (3,11-18). We suggest that the levels of the autoantibodies may be an indicator of the stage of autoimmune gastritis, and the fusion proteins, such as the ones constructed in this study, may be used for classification of the antibodies and the progress of the disease.

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