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Parietal cell surface reactive autoantibody in pernicious anaemia demonstrated by indirect membrane immunofluorescence

H. J. DE AIZPURUA, B. H. TOH & B. UNGAR* *Department of Pathology and Immunology, Monash University Medical School, Prahran, Victoria and *Department of Haematology, Royal Melbourne Hospital, Melbourne, Australia*

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SUMMARY

We examined, in a 'double blind' study, 60 sera from patients with pernicious anaemia for immunofluorescence reactivity with the surface membranes of viable parietal cells isolated from dog stomachs. Fifty-three sera (88%) gave an IgG autoantibody reaction with the surface membranes of parietal cells. Surface staining was also seen with parietal cells from monkey, pig, rat and mouse. The parietal cell surface reactive autoantibody was not found in any of 14 sera from patients with chronic active hepatitis, 10 from patients with systemic lupus erythematosus and 50 from healthy persons. The surface reactivity autoantibody was present in 13 of 14 sera without parietal cell microsomal antibody, 28 of 31 sera without intrinsic factor antibody and in four of four sera without microsomal and intrinsic factor antibodies. Absorption with parietal cell enriched gastric mucosal cells neutralized the activity of the surface reactive but not the microsomal antibody and cross absorption with gastric microsomes neutralized the activity of the microsomal but not the surface reactive antibody. Surface staining of parietal cells was not abolished by absorption with dog or rat hepatocytes, dog or rat kidney cells, human fibroblasts or human AB red blood cells. The results suggest that the parietal cell surface reactive antibody is probably different from the microsomal antibody. Immune reactions of the cell surface reactive antibody with parietal cell surface antigens may play a role in the pathogenesis of the gastric lesion in pernicious anaemia.

INTRODUCTION

Pernicious anaemia (PA) is a putative organ specific autoimmune disease of the gastric mucosa characterized by chronic atrophic gastritis, achlorhydria, lack of intrinsic factor production, vitamin B_{12} malabsorption and parietal cell microsomal antibody (PCMA) (Taylor *et al.*, 1962) and intrinsic factor antibody (IFA) (Jeffries, Hosking & Sleisenger, 1962). The gastric lesion, confined mainly to the fundus and body of the stomach, shows mucosal atrophy with loss of parietal cells and chief cells and a submucosal infiltrate of lymphocytes and plasma cells (Strickland & Mackay, 1973). Humoral and cellular immune mechanisms have been implicated in the immunopathogenesis of the gastric lesion but a definitive initiating role for either has not been established. While autoantibodies to intrinsic factor may impair vitamin B_{12} absorption, a pathogenic role for PCMA remains unproven (Thomas & Jewell, 1979).

Correspondence: Dr B.H. Toh, Department of Pathology & Immunology, Monash University Medical School, Commercial Road, Prahran, Melbourne 3181, Australia.

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Masala *et al.* (1980) showed that sera containing PCMA also gave immunofluorescence reactions with the surface membranes of gastric parietal cells, and suggested that the parietal cell surface antigen is probably immunologically identical with the intracytoplasmic 'microsomal' antigen. We show here, in a 'double blind' study of sera from 60 Caucasian patients with adult PA, the presence of a circulating parietal cell surface reactive autoantibody (PCSA) which seems distinct from PCMA.

MATERIALS AND METHODS

Sera. Sera from 60 Caucasian patients with PA attending the Royal Melbourne Hospital were examined by immunofluorescence for surface membrane reactivity with parietal cell enriched mucosal cell suspensions. The patients, comprising 39 women and 21 men, aged 30–89 years (mean age 66 years) satisfied standard diagnostic criteria for PA (Ungar *et al.*, 1968). All had impaired vitamin B_{12} absorption, shown by a Schilling test result of 0–5%. Forty-six patients had PCMA and 29 patients IFA. Of the 60 patients with PA, 45 (29 women and 16 men) were free of endocrine disease and 15 (10 women and five men) had associated endocrine disease. Four patients had myxoedema, four thyrotoxicosis, three insulin-dependent diabetes mellitus, one insulin-independent diabetes mellitus and one each with myasthenia gravis, insulin-dependent diabetes mellitus with thyrotoxicosis, and insulin-dependent diabetes mellitus with Addison's disease. All sera from patients with PA were coded by one of us (B. Ungar) and tested 'blind' for cell surface membrane reactivity at the Monash Department of Pathology and Immunology. At completion of the tests, the results of parietal cell surface immunofluorescence were correlated with the presence or absence of PCMA and IFA. Control sera from 14 patients with chronic active hepatitis, 10 with systemic lupus erythematosus and 50 from healthy persons were also tested for surface membrane reactivity with gastric mucosal cells.

Sera were stored at −20°C for a few weeks up to 4 years before rapid thawing for tests.

Tests for antibody to parietal cell microsomes and intrinsic factor. These tests were carried out at the Royal Melbourne Hospital. PCMA was demonstrated in all cases by standard indirect cytoplasmic immunofluorescence tests using snap frozen blocks of human stomach and mouse stomach (Ungar *et al.*, 1968). Bound immunoglobulin was traced with a fluorescein labelled sheep anti-human immunoglobulin active against IgG, IgA and IgM (Wellcome). Intrinsic factor blocking antibody was detected by a method as described previously (Ungar *et al.*, 1968).

Preparation of parietal cell enriched cell suspensions. The methods were modified from Romrell *et al.* (1975). Briefly, stomachs from dogs starved overnight were washed in cold (4°C) 0·85% saline and in Hank's balanced salt solution (HBSS), pH 7·2 (Commonwealth Serum Laboratories, Melbourne). The mucosa of the fundus and body of the stomach, dissected from the muscularis and submucosa, was minced and incubated for 15 min in 25 ml of Medium 199 (M199, Commonwealth Serum Laboratories, Melbourne) containing 0·75 mg/ml Type IV collagenase (Sigma, USA). Undissociated fragments, rinsed in fresh M199, were incubated for 10 min with 25 ml M199 containing 2 mM EDTA (Ajax Chemicals, Sydney). The residual fragments were treated twice for 30 min with fresh M199 containing 0·75 mg/ml collagenase, and the crude cell suspensions from these pooled. All procedures were carried out at 37°C in flasks gassed continuously with 5% CO_2/95% air in a shaking (120 cycles min) water bath. Mucosal cell suspensions were also similarly prepared from stomachs of monkey, pig, rat and mouse.

The cell suspension was filtered through a 20 ml plastic syringe packed with nylon wool and enriched for parietal cells by repeated 45 s centrifugation at 100 ***g*** max in HBSS (Soumarmon, Cheret & Lewin, 1977). Cell viability was assessed by 0·025% trypan blue dye exclusion. Cell suspensions were adjusted to a concentration of 1×10^6 cells/ml. Cell smears were prepared by placing a drop of cell suspension on a clean glass slide followed by aspiration of the drop and rapid air drying. Cell smears and intact as well as collagenase treated fragments of gastric musoca were fixed in 10% buffered formalin and stained with haematoxylin and eosin (H & E) and with a modified Zimmerman's stain for parietal cells (Ceredig & Toh, 1978). Cell smears were also fixed in ethanol:ether (1:1) for 30 s. for indirect immunofluorescence staining (Nairn, 1976) with PCMA or

with an anti-ribosomal autoantibody which stains chief cells (Mathy, Baum & Toh, 1980). Cell suspensions, mounted on glass slides with coverslips, were examined by phase contrast and Nomarski optics with a Reichert–Biovert inverted light microscope. Control cell suspensions were also fixed for electron microscopy according to Lewis & Knight (1977) and viewed on a Joel Jem 100s transmission electron microscope.

Control cell suspensions of dog or rat hepatocytes (Hopf, zum Buschenfelde & Arnold, 1976), dog or rat kidney cells and human fibroblasts (Toh & Hard, 1977) were also prepared and tested for reactivity with human sera.

Indirect membrane immunofluorescence tests. The method followed was essentially as described by Nairn (1976). One million musocal cells were pelleted in 0·8 ml polystyrene tubes by centrifugation at 150*g* max at 4°C for 5 min and incubated for 20 min at room temperature (20–24°C) with 25 μl of complement-inactivated serum at a dilution of 1:5 in phosphate-buffered saline (PBS). The cells were washed twice in PBS and the cell pellets reacted at room temperature with 25 μl of a 1:8 dilution of fluorescein isothiocyanate (FITC) labelled sheep anti-human gammaglobulin (Wellcome). Control cell suspensions were treated with normal human serum (NHS) of PBS. The cells were washed in PBS and in veronal-buffered glycerol, pH 8·6 and mounted on glass slides with cover slips. Cell viability after completion of the tests was assessed with 1% propidium iodide, a DNA intercalcating dye (Nairn & Rolland, 1980) which stains the nuclei of dead cells. The cell preparations were viewed with a Leitz–Diavert epi-illumination fluorescence microscope equipped with phase contrast optics. Titres of PCSA positive sera were obtained by tests with doubling dilutions of sera in PBS. The immunoglobulin class of PCSA positive sera was determined by tests with FITC labelled affinity purified anti-human IgG, IgM or IgA (Wellcome).

The presence of PCSA was compared by means of a student's *t*-test with the presence or absence of PCMA and IFA.

Serum absorptions. One millilitre of 1:5 dilution of each of 15 randomly selected PA sera containing PCSA and PCMA was absorbed for 2 h at room temperature with 12×10^6 mucosal cells, dog or rat hepatocytes, dog or rat kidney cells, human fibroblasts, human (AB) red blood cells, or with a microsomal fraction of gastric mucosa prepared by the method of Rothschild (1963). Purity of microsomal fractions was established by electron microscopy (Fig. 1). Absorption with gastric

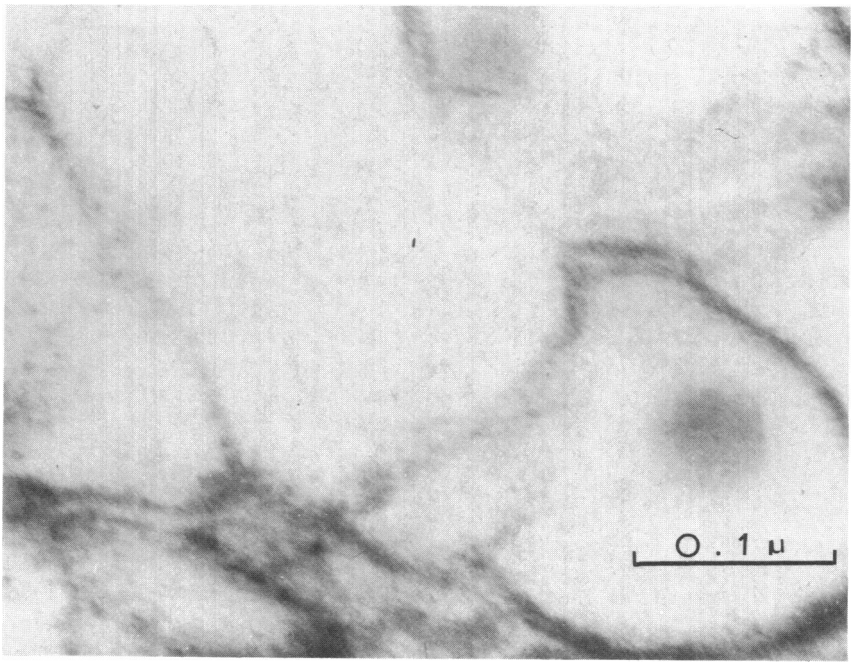

Fig. 1. Electron microscopic preparation of gastric microsomes.

microsomes was carried out in a ratio of 1 volume of serum to 2 volumes of packed microsomes. After serum absorptions, PCSA activity was assessed by using indirect membrane immunofluorescence tests while PCMA activity was assessed by using indirect cytoplasmic immunofluorescence tests on unfixed snap frozen sections of mouse stomach or gastric cell smears fixed in ethanol/ether. The test methods have been outlined above.

RESULTS

Parietal cell enriched cell suspensions

Dissociation of dog gastric mucosa by collagenase and EDTA produced a cell suspension with a viability of 90–95% as assessed by 0·025% trypan blue dye exclusion. The cell suspension contained 70–90% parietal cells and 10–30% chief cells identified by H & E and Zimmerman's histochemical stains of cell smears. Correlation between cells present in cell smears and those in tissue sections verified the two cell types found in the mucosal preparation. Examination of cell suspensions by phase contrast and Nomarski optics and of cell smears for cytoplasmic immunostaining of parietal cells by PCMA and of chief cells by anti-ribosomal antibody confirmed the cell types previously identified as parietal and chief. Phase contrast and Nomarski optics readily distinguished the large (22–28 μm) granular surfaced parietal cells (Figs. 2A & B) from the smaller (12–18 μm) smooth surfaced, granule containing chief cells.

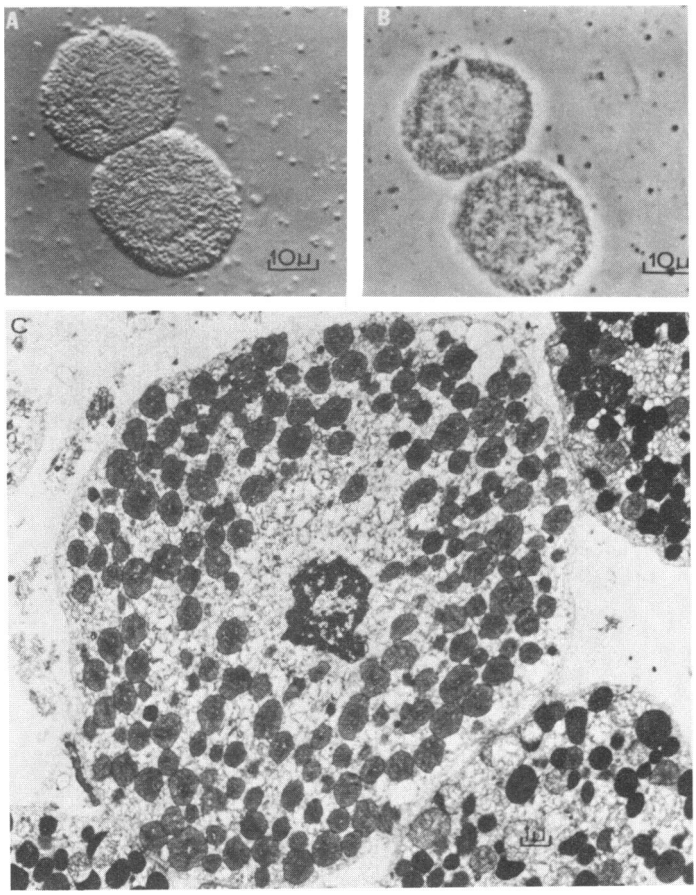

Fig. 2. Morphology of isolated dog parietal cells. Nomarski (A) and phase contrast (B) optics show large, pear shaped parietal cells with granular surfaces. Electron microscopy (C) shows numerous large mitochondria and profiles of tubulo-vesicular membranes.

Ultrastructural examination of cell suspensions provided further confirmation of the two cell types. The large parietal cells contained numerous large mitochondria, sparse endoplasmic reticulum, profiles of tubulovesicular membranes and characteristic microvilli lined canaliculi (Fig. 2C). In contrast, the smaller chief cells contained numerous large zymogen granules, and abundance of rough endoplasmic reticulum and an extensive Golgi complex.

Parietal cell surface immunofluorescence

Indirect immunofluorescent tests of canine gastric cell suspensions showed parietal cell surface staining with sera from 53 of 60 (88%) patients with PA. Surface staining appeared as a ring or as fine to coarse granules on the surface of parietal cells (Fig. 3B & D), identified by companion phase contrast optics (Fig. 3A & C). Viability of stained suspensions, assessed by 1% propidium iodide exclusion, was more than 90%. The occasional dead cell gave a diffuse, uniform dull green fluorescence of the whole cell and the nuclei of these cells stained with propidium iodide. Chief cells, identified by phase contrast optics, did not stain (Fig. 3G & H). Similar parietal cell surface staining was also seen with gastric mucosal cells from monkey, pig, rat and mouse. The 13 sera reactive with the surface membranes of canine parietal cells but negative for PCMA also reacted with the surface membranes of viable parietal cells from monkey stomachs. Conversely, the seven sera unreactive

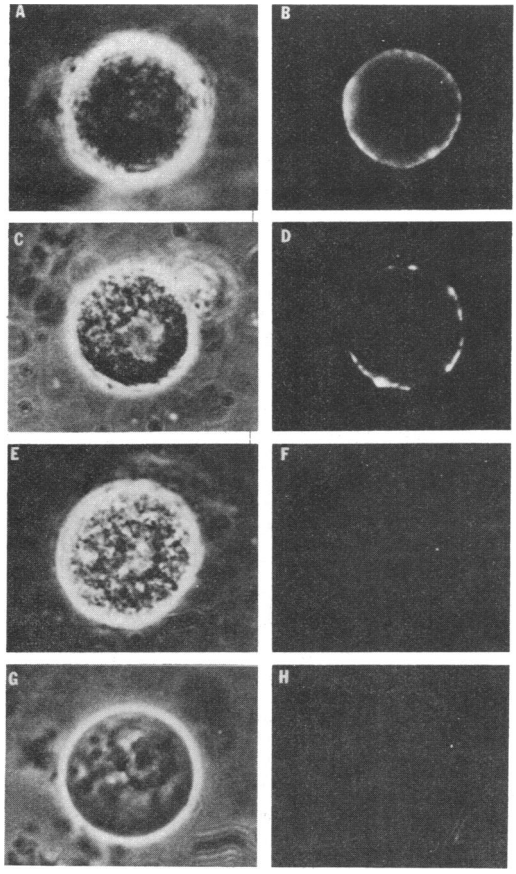

Fig. 3. Microscopic visualization of immunofluorescent surface staining reactions of isolated canine parietal cells. Pareital cells, reacted with serum from patient with pernicious anaemia, and identified by companion phase contrast optics (A, C) shows uniform ring surface staining in B and surface staining of fine to coarse granules in D. Parietal cells (E) reacted with control serum (NHS) gave no surface staining reactions (F). Chief cells (G) did not stain (H). Indirect membrane immunofluorescence and phase contrast and fluorescence microscopy (× 500).

Table 1. Results of immunoabsorptions studies on 15 sera from patients with pernicious anaemia containing parietal cell surface and microsomal autoantibodies

	Parietal cell immunofluorescence	
Absorptions	Cell surface	Cytoplasmic
Nil	+	+
Parietal cell enriched preparations	−	+
Gastric microsomes	+	−
Kidney cells	+	+
Liver cells	+	+
Fibroblast	+	+
AB red blood cells	+	+

with the surface membranes of canine parietal cells did not give surface staining reactions with monkey parietal cells. The immunoglobulin class of the surface reactive antibody was IgG. Serum titrations gave antibody titres ranging from 5 to 40. Surface staining of gastric parietal cells was not seen with any of 14 sera from patients with chronic active hetatitis, 10 from patients with systemic lupus erythematosus or 50 from healthy persons (Fig. 3E & F). Human fibroblasts, dog or rat hepatocytes and dog or rat kidney cells did not give cell surface staining with the PCSA positive sera.

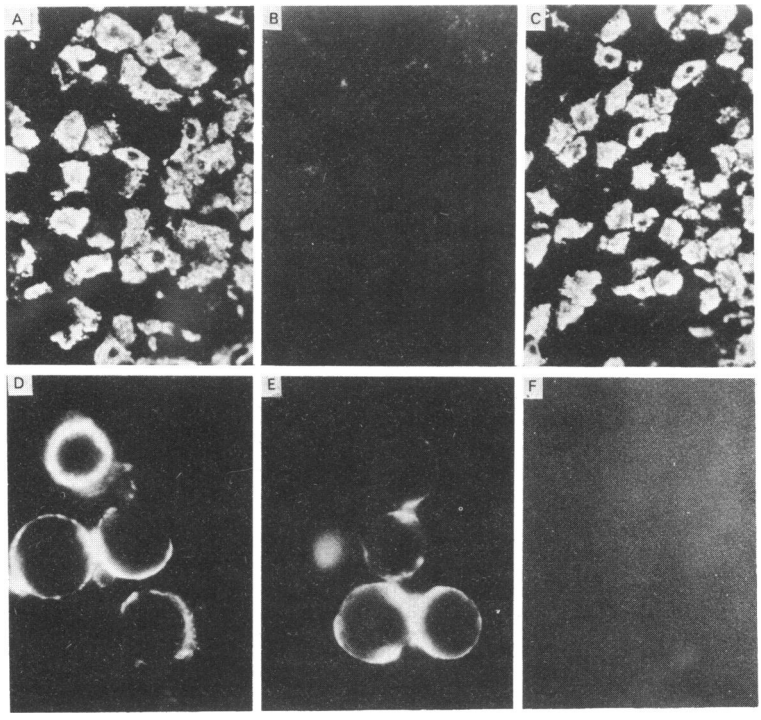

Fig. 4. Fluorescent microscopic visualisation of unfixed frozen sections of mouse stomach (A–C), and viable cell suspensions (D–F) of dog parietal cells. Cytoplasmic immunostaining of parietal cells (A) was neutralized by serum absorption with gastric microsomes (B) but not by parietal cell enriched preparations (C) while parietal cell surface membrane immunostaining (D) was neutralized by parietal cell enriched preparations (F) but not by gastric microsomes (E). Indirect immunofluorescence. (A–C) × 100, (D–F) × 200.

Serum absorption studies

Absorption of each of 15 randomly selected sera containing PCSA and PCMA with 12×10^6 viable parietal cells completely abolished cell surface but not cytoplasmic fluorescence of parietal cells, while the same test sera absorbed with viable dog or rat kidney cells, dog or rat hepatocytes, human fibroblasts or human red blood cells did not neutralize parietal cell surface or cytoplasmic staining. Conversely, sera absorbed with gastric microsomes completely abolished cytoplasmic but not cell surface staining of parietal cells (Table 1, Fig. 4).

Parietal cell surface antibody and antibody to microsomes and intrinsic factor

Table 2 compares the presence of PCSA in the presence or absence of PCMA and IFA. PCSA was present in 13 sera which were negative for PCMA (detected on mouse and human stomach sections) and in 28 sera which were negative for IFA. PCSA was also present in all four sera which were negative for both PCMA and IFA. Conversely, of seven sera negative for PCSA, six were positive for PCMA (of which three were also IFA positive) and one was positive for IFA but negative for PCMA. There was also no significant difference ($P > 0{\cdot}05$) in the occurrence of PCSA in patients with (13 of 15, 87%) or without (40 of 45, 89%) associated endocrine disease.

Table 2. Correlation of parietal cell surface autoantibody with microsomal and intrinsic factor autoantibodies

Microsomal and intrinsic factor antibodies (n = No. of sera)	Parietal cell surface antibody positive
Microsomal antibody absent and intrinsic factor antibody present ($n = 10$)	9
Microsomal and intrinsic factor antibodies absent ($n = 4$)	4
Microsomal antibodies present and intrinsic factor antibody absent ($n = 27$)	24
Microsomal and intrinsic factor antibodies present ($n = 19$)	16

DISCUSSION

The case for autoimmunity in chronic atrophic gastritis and PA is still unproven. Arguments for an autoimmune pathogenesis rest mainly on the histological presence of lymphocytes and plasma cells in the atrophic gastric mucosa in the apparent absence of any extrinsic antigenic stimulus and on the presence of PCMA in 75–90% and of IFA in 50–70% of patients with PA (Thomas & Jewell, 1979). Although pathogenic effects of PA sera on parietal cells have been demonstrated *in vivo* (Thomas & Jewell, 1979) and *in vitro*, (Loveridge *et al.*, 1980) the nature of the reactive autoantibodies involved has not been clearly defined.

In the present study we used viable, parietal cell enriched suspensions prepared by a combined collagenase–EDTA-induced dissociation of the mucosa of the fundus and body of the stomach from dog and several other mammalian species. The cell suspension contained 70–90% parietal cells and 10–30% chief cells identified by histochemical stains, phase contrast and Nomarski optics, cytoplasmic immunostaining and electron microscopy. This technique enabled us to demonstrate the presence of an IgG class PCSA in the sera of 53 of 60 (88%) patients with PA but in none of 74 control subjects. PCSA was demonstrated by microscopic visualization of immunofluorescent stained parietal cells, identified by companion phase contrast optics, and easily distinguishable from the smaller chief cells which did not stain. Viability of the cell suspensions before and after immunofluorescence staining, assessed by 0·025% trypan blue dye exclusion and 1% propidium iodide, was >90%.

Specificity of PCSA for parietal cells was supported by the absence of surface reactivity with

other cell types (dog chief cells, dog or rat hepatocytes, dog or rat kidney cells, human fibroblasts) and confirmed by complete abolition of PCSA activity by absorption with parietal cell enriched preparations. Control absorptions with dog or rat hepatocytes, dog or rat kidney cells, human fibroblasts or human AB red blood cells failed to neutralize PCSA activity. It is unlikely that PCSA is a heterophile antibody since parietal cell surface reactions similar to those obtained for dog stomachs were seen in gastric mucosal cells prepared from monkey, pig, rat and mouse.

The presence of PCSA in 13 sera which were negative for PCMA and the absence of PCSA in six sera which were positive for PCMA suggests that the antigen which reacts with PCSA is different from that which reacts with PCMA. This was supported by cross-absorption studies which showed that PCSA but not PCMA activity was neutralized by absorptions with gastric mucosal cells and that, conversely PCMA but not PCSA activity was neutralized by absorptions with gastric microsomes. PCSA was also present in 28 of 31 sera without IFA suggesting that PCSA is also not antigenically related to IFA. Furthermore, neither the microsomal nor intrinsic factor antigens are likely to be present on the surface membranes of parietal cells since the latter were obtained from unstimulated stomachs in which the parietal cell canalicular system is not likely to be open to the exterior via the microvillous apical surface. Also IF being a secretory product of parietal cells, is unlikely to remain on the surface membranes of parietal cells subjected to treatment with enzyme, EDTA and repeated washing in M199. These suggestions are supported by the observations that four sera containing IFA and six sera containing PCMA did not stain the surface membranes of isolated parietal cells. The canalicular secretory system of parietal cells has been reported to contain the cytoplasmic microsomal antigen which reacts with PCMA (Hoedemaeker & Ito, 1970). The nature of the antigenic determinants on parietal cell surfaces recognized by PCSA is not known. Immunochemical studies are currently in progress in our laboratory to identify the parietal cell surface autoantigen. We were unable to assess whether there are other surface antigens recognized by PCMA as suggested by Masala *et al.* (1980). Recent studies suggest a good correlation between surface and cytoplasmic autoreactivity in thyroid and adrenal autoimmune diseases (Khoury *et al.*, 1981a, 1981b) but not in pancreatic autoimmune disease (Lernmark *et al.*, 1978).

While PCSA reacts with the surface membranes of isolated, viable parietal cells, it gives no staining reactions with the surface membranes of non-viable parietal cells in frozen sections of stomach. Thus 13 sera with PCSA activity did not stain parietal cells *in situ*. Also, 15 sera with both PCSA and PCMA activity gave no staining of *in situ* parietal cells after neutralization of PCMA but not PCSA activity by serum absorptions with gastric microsomes. These results suggest that the parietal cell surface autoantigen is only expressed on living cells, or that the process of snap freezing employed in the preparation of frozen sections has not preserved the antigenicity of cell surface membrane antigens, or both. It may be for these reasons that it has not previously been possible to demonstrate autoantibody reactions with parietal cell surface membranes until methods of isolating viable parietal cells had been adequately described (Romrell *et al.*, 1975). The same seems to have been the case for autoantibody reactions with the surface membranes of isolated islet cells (Lernmark *et al.*, 1978) and of isolated hepatocytes (Hopf *et al.*, 1976).

The contribution of genitic factors to the genesis of the gastric lesion of PA is suggested by the results of studies of HLA patterns (Ungar *et al.*, 1981). Ungar, Francis & Cowling (1976) have suggested that PA results from the action of autoimmune and other factors on a susceptible gastric mucosa. The recognition of PCSA documented in the present study may lead to a new insight into the immunopathogenesis of the gastric lesion of PA since the corresponding surface autoantigens are readily accessible to autoimmune attack by antibodies or sensitized lymphocytes. Surface reactive autoantibodies have also been reported in other putative organ specific autoimmune diseases e.g. HBsAg negative chronic active hepatitis (Hopf *et al.*, 1976) and insulin-dependent diabetes mellitus (Lernmark *et al.*, 1978) and, in the case of the latter, the surface autoantibody has been shown to be cytotoxic to pancreatic beta cells (Eisenbabarth, Morris & Scearce, 1980). Whether PCSA is cytotoxic to parietal cells, either by complement activation or by recruitment of other cell types (e.g. K. cells), can now be directly tested in the laboratory. It should also be possible to ascertain whether PCSA acts by binding to surface receptors (Carnegie & Mackay. 1975) for gastrin or other peptide hormones which have trophic effects on the gastric mucosa (Johnson, 1976).

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