Clin. exp. Immunol. (1983) **54,** 405–410.

Flow microfluorimetric analysis of autoantibody reactions with parietal cell surface membranes in pernicious anaemia

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

(Accepted for publication 10 June 1983)

SUMMARY

Using flow microfluorimetry (FMF), 60 sera from patients with pernicious anaemia (PA) were examined for immunoreactivity with the surface membranes of viable canine parietal cells. FMF analyses showed that the percentage of parietal cells giving a surface staining reaction with a fluorescence intensity > 50 arbitrary units was $44{\cdot}5 \pm 17{\cdot}5\%$ for sera from 60 patients with PA compared to $13{\cdot}7 \pm 2{\cdot}7\%$ for sera from 14 patients with chronic active hepatitis, $10{\cdot}7 \pm 6{\cdot}7\%$ for sera from 10 patients with systemic lupus erythematosus and $16{\cdot}5 \pm 4{\cdot}4\%$ for sera from 50 healthy persons. Surface staining detected by FMF was restricted to parietal cells and abolished by absorption with parietal cell enriched preparations but not by absorption with dog or rat hepatocytes, dog or rat kidney cells, human fibroblasts, human AB red blood cells or dog gastric microsomes. The intensity of the parietal cell surface staining reactions correlated with the presence of antibody reactions with parietal cell surfaces previously demonstrated by immunofluorescence microscopy but did not correlate with the presence of microsomal or intrinsic factor autoantibodies. The results provide further support for the presence of a parietal cell surface reactive autoantibody distinct from the conventional parietal cell microsomal autoantibody.

Keywords parietal cell surface antibodies pernicious anaemia flow microfluorimetry

INTRODUCTION

Pernicious anaemia (PA) is a putative organ specific autoimmune disease of the stomach, characterized by lack of intrinsic factor production, vitamin B_{12} malabsorption, parietal cell microsomal antibody (PCMA) and instrinsic factor antibody (IFA) (Thomas & Jewell, 1979). The gastric lesion, confined mainly to the fundus and body of the stomach, is associated with mucosal atrophy, loss of parietal and chief cells and submucosal lymphocytic infiltration (Strickland & Mackay, 1973).

Using indirect membrane immunofluorescence, we recently reported the presence of a parietal cell surface reactive autoantibody (PCSA) which seems distinct from the conventional parietal cell microsomal autoantibody (De Aizpurua, Toh & Ungar, 1983). In the present study we used flow microfluorimetry to examine quantitatively the reactions of sera from patients with PA with the cell surface membranes of viable parietal cells isolated from dog stomachs.

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

MATERIALS AND METHODS

Sera. Sera from 60 patients with PA with or without associated endocrine disease, 14 from chronic active hepatitis (CAH), 10 from systemic lupus erythematosus (SLE) and 50 from healthy persons (NHS) were examined by flow microfluorimetry for immunofluorescence reactivity with the surface membranes of parietal cell enriched gastric mucosal cells. Details of the sera used were as described in our previous study (De Aizpurua *et al.*, 1983).

Parietal cell enriched preparations. The method for preparing parietal cell enriched preparations have been reported (De Aizpurua *et al.*, 1983). Briefly, stomachs from dogs starved overnight were opened and washed in 0·85% saline at 4°C. The mucosa from the fundus and body was dissected free of muscularis and submucosa, loosely minced and subjected to the following treatments. (i) Fifteen minutes in 25 ml M199 containing 0·75 mg/ml type IV collagenase (Sigma, USA). (ii) Ten minutes in 25 ml of fresh M199 with 2mM EDTA (Ajax chemicals, Sydney). (iii) Thirty minutes in M199 containing 0·75 mg/ml type IV collagenase. The cell suspensions were harvested, spun at 200*g* for 5 min and enriched for parietal cells by reiterative centrifugation at 100*g* for 45 s. 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. Cell viability was assessed by 0·025% trypan blue dye exclusion. Cell types were identified by haematoxylin & eosin staining of cell smears, Normarski and phase contrast optics and by electron microscopy.

Cell analysis by flow microfluorometry. Mucosal cell suspensions were reacted by indirect membrane immunofluoresence tests with patient's sera, NHS or phosphate-buffered saline (PBS) controls as described previously (De Aizpurua *et al.*, 1983). Ten micrograms per millilitre of bovine type 1 DNAase (Sigma) was added to the cell suspensions to prevent cell clumping. The cell preparations were initially analysed for 0° and 90° scatter profiles, parameters based mainly on cell size (Loken & Herzenberg, 1975). Cell viability of stained preparations was assessed with 0·25 μg/ml fluorescein diacetate (Nairn & Rolland, 1980) and surface fluorescence of cell suspensions, gated at 90° scatter peaks, examined. For quantitation of test results, the percentage of cells with a fluorescence intensity greater than 50 arbitrary units was calculated because this represented the best cut-off between results obtained with patients' sera and NHS (see x-axis Fig. 2 a–d). The results, expressed as mean ± s.d., were compared by means of a Student's *t*-test. To identify the cell type present in the 90° scatter peaks (Fig. 1), a cell sort was carried out by gating for cells in the first peak (channels 13–69) and in the second peak (channels 85–170). The sorted cells were fixed for electron microscopic examination. Cell analysis and cell sorting was carried out on a fluorescence activated cell sorter, (FACS-II, Becton Dickinson) equipped with an argon laser oprated at 488 nm wavelength and 400 mW for fluorescein excitation. Fluorescence was detected with a S-11 response photomultiplier tube at 550 V with a 520 nm barrier filter.

Serum absorptions. Absorptions of each of 12 randomly selected parietal cell surface positive sera were carried out at room temperature for 2 h with either 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 from gastric mucosal prepared according to Rothschild (1963). Purity of the microsomal fractions was established by electron microscopy.

Absorbed sera were analysed for immunofluorescence reactivity with parietal cell enriched preparations by flow microfluorimetry as described above.

Tests for parietal cell microsomal antibody and intrinsic factor antibody. These tests were carried out by methods as described previously (De Aizpurua *et al.*, 1983).

RESULTS

Parietal cell suspensions

Collagenase-mediated dissociation of dog gastric mucosa produced cell suspensions with viability of 90–95% as assessed by 0·025% trypan blue dye exclusion. Reiterative centrifugation at 4°C gave populations of > 75% parietal cells and < 25% chief cells with no change in cell viability. Cells were

identified by haematoxylin & eosin staining of cell smears, Normarski and phase contrast optics and by electron microscopy.

Selection and analysis of cells by flow microfluorimetry

Criteria for cell selection and analysis were based on (i) cell size, obtained by diffraction of the laser beam by cells (0° or low angle light scatter); (ii) properties of cytoplasmic and nuclear components detected by light refracted at right angles (90° or high angle light scatter) and (iii) viability assessed with fluorescein diacetate.

Using these three parameters, chief and parietal cells can be easily distinguished from one another, allowing ready analysis of each population.

Fluorescence histogram analysis

Analysis of the 0° and 90° scatter profiles of the gastric cell suspensions showed two peaks which were virtually identical for test and control experiments (Fig. 1). Electron microscopic examination of cells sorted at the gated 90° scatter peak showed that the first peak consisted of chief cells (Fig. 2a) and the second peak of parietal cells (Fig. 2b). Viability of cell suspensions as analysed by fluorescein diacetate was > 85%. Analysis by fluoresein diacetate also clearly distinguished between the two cell types.

The 53 sera which gave perietal cell surface staining on microscopic assessment as reported previously (De Aizpurua *et al.*, 1983) showed significant cell surface staining when gated for cells in the second (parietal cell) peak (Fig. 3). No fluorescence was detected on cells in the first (chief cell) peak. The percentage of cells with a cell surface fluorescence intensity registering > 50 channel units was $44{\cdot}5 \pm 17{\cdot}5\%$ for the 53 positive PA sera and $18{\cdot}1 \pm 2{\cdot}3\%$ for seven PA sera which lacked the cell surface autoantibody by fluorescence microscopy.

Specificity studies

FMF showed that the percentage of cells with a fluorescence intensity > 50 channel units was $13{\cdot}7\% \pm 2{\cdot}7\%$ for chronic active hepatitis, $10{\cdot}7 \pm 6{\cdot}7\%$ for systemic lupus erythematosus and $16{\cdot}5 \pm 4{\cdot}4\%$ for healthy persons (Fig. 4). These results were comparable with the mean of $18{\cdot}1 \pm 2{\cdot}3\%$ for the seven PCSA negative sera from patients with PA and significantly different ($P < 0{\cdot}001$) from the mean ($44{\cdot}5 \pm 17{\cdot}5\%$) for the 53 PCSA positive sera.

Absorption of each of 12 randomly selected PCSA positive sera with 12×10^6 viable parietal cells abolished surface fluorescence of parietal cells (Fig. 3a,b). No neutralization was observed when the

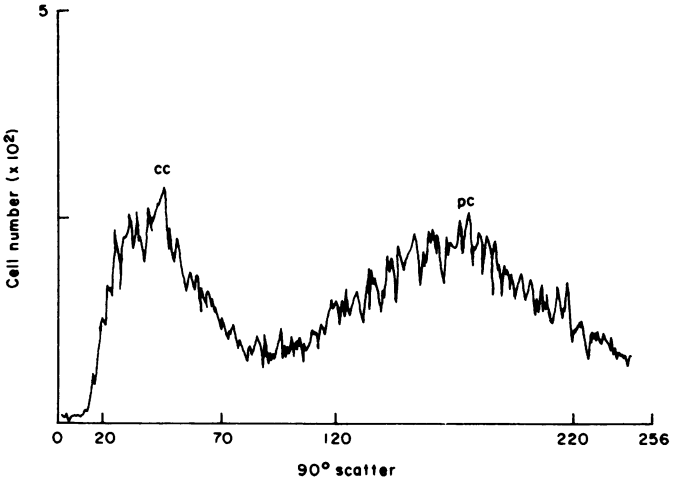

Fig. 1. Ninety degree scatter analysis of dog gastric mucosal cells by flow microfluorimetry. Ninety degree scatter profile shows chief cell (cc) and parietal cells (pc) peaks, as identified by cell sorting and electron microscopy.

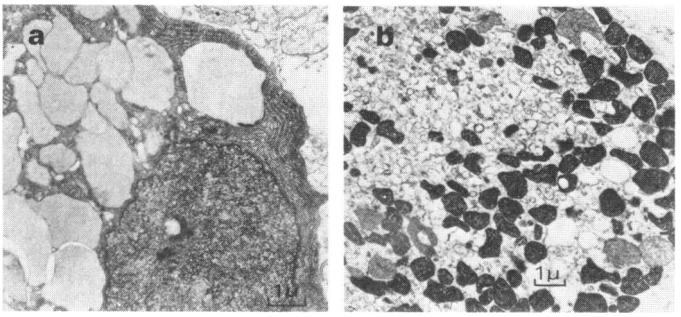

Fig. 2. Chief cells (a) were identified by the presence of numerous zymogen granules and an abundance of rough endoplasmic reticulum while parietal cells (b) were identified by numerous mitochondria, sparse endoplasmic reticulum and profiles of tubolovesicular membranes.

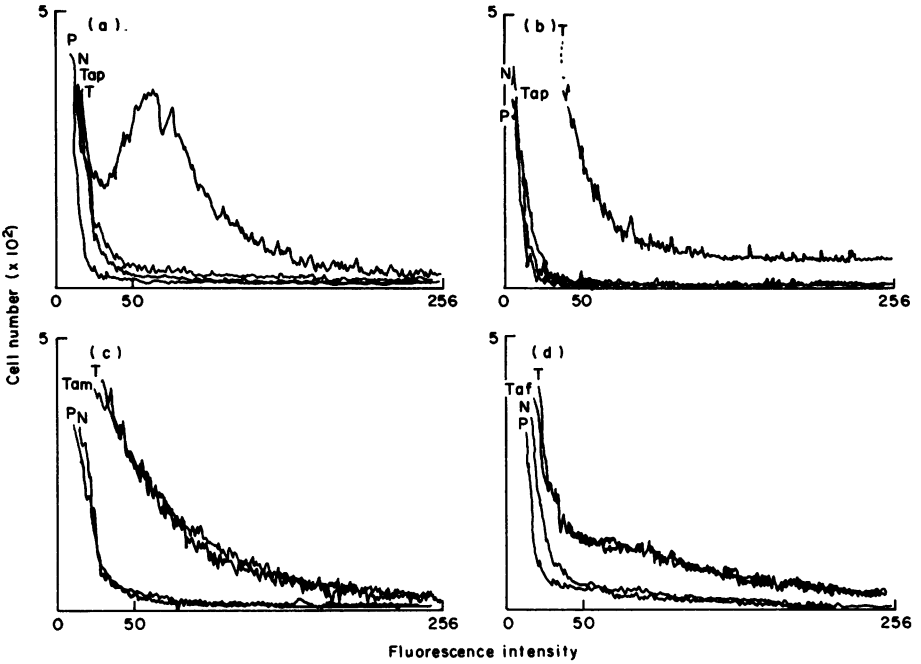

Fig. 3. Specificity of parietal cell surface fluorescence demonstrated by surface staining. immunoabsorption and flow microfluorimetry. Surface fluorescence of cells gated for the 90° scatter, pc peak (see Fig. 1) shows a distinct population of highly fluorescent parietal cells obtained with test serum (T) from a patient with pernicious anaemia compared to NHS (N) and PBS (P) controls (panel a). Parietal cell surface fluorescence was neutralized by absorption with parietal cell enriched preparations (Tap, panels a,b) but not by absorption with gastric microsomes (Tam, panel c) or by human fibroblasts (Taf, panel d). Sera in a, b, c and d were from different patients with pernicious anaemia, with test serum a giving more intense surface staining reactions with parietal cells than test sera b–d.

same sera were absorbed with viable dog or rat kidney or liver cells, human fibroblasts (Fig. 3d), human AB red blood cells or dog gastric microsomes (Fig. 3c).

Correlation of intensity of antibody reaction with parietal cell surface membranes with PCMA and IFA

Fig. 4 shows that there is no difference in the intensity of parietal cell surface staining in the presence or absence of PCMA or IFA, as indicated by the mean value for cell surface fluorescence intensity expressed as % cells with a fluorescence intensity > 50 channel units.

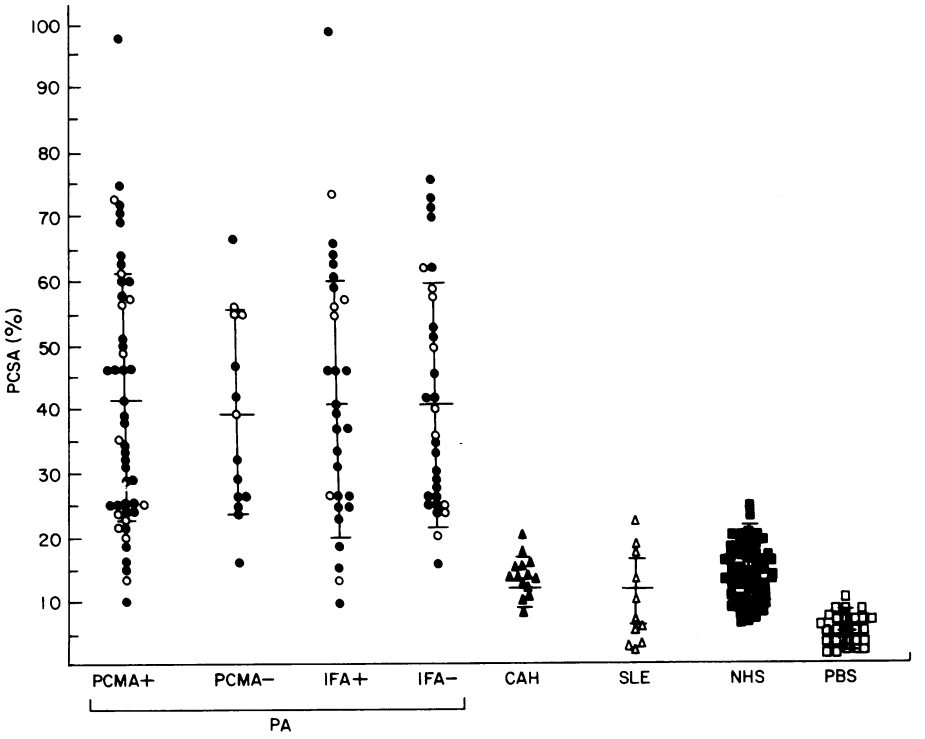

Fig. 4. Intensity of parietal cell surface fluorescence quantified by flow microfluorometry. Sera from patients with PA with (○) or without (●) associated endocrine disease were divided into $PCMA^+$ and $PCMA^-$ groups, IFA^+ and IFA^- groups and compared with NHS, PBS and sera from patients with CAH and SLE. Results expressed as % parietal cells with fluorescence intensity > 50 arbitrary units. Bars represent mean ±s.d.

DISCUSSION

The application of flow microfluorimetry to the analysis of the reactivity of PA sera with the cell surface membranes of isolated viable canine parietal cells has permitted a rapid, accurate, comprehensive and objective evaluation of cell surface fluorescence staining reactions in this disease. The ability of an advanced microfluorimeter to select a cell subpopulation for scrutiny allows quantitative analysis of the cell surface staining reactions within that population. A cell viability of >85% obtained with fluorescein diacetate virtually excludes serum reactivity with intracellular antigens since autoantibodies do not normally penetrate the cell membranes of living cells (Theofilopoulos & Dixon, 1982).

Histogram analysis of our results showed that the cell surface staining reactions obtained with sera from patients with PA were restricted to the parietal cell peak, identified by cell sorting and electron microscopy. The intensity of the parietal cell surface staining reactions, expressed as % cells with a fluorescence intensity > 50 channel units, was significantly higher in sera from the 60 patients with PA than in the 74 control sera. Specificity of the surface staining reactions for parietal cells was supported by the abolition of surface activity by serum absorption with parietal cell enriched preparations. In contrast, control absorptions with dog or rat hepatocytes, dog or rat kidney cells, human fibroblasts or human AB red blood cells failed to neutralize the parietal cell surface staining reactions.

The intensity of the parietal cell surface staining reactions correlated with the presence of PCSA previously identified by immunofluorescence microscopy (De Aizpurua *et al.*, 1983). The absence of a correlation between surface staining intensity with PCMA or IFA and the failure to neutralize

PCSA activity by serum absorption with gastric microsomes provides further support for the suggestion that PCSA is different from PCMA or IFA (De Aizpurua *et al.*, 1983). We suggest that this technique of flow microfluorimetry may also be used for the quantitative analysis of cell surface staining reactions in other autoimmune diseases. In this connection, the fluorescence activated cell sorter has also been used to detect islet cell surface antibodies in sera obtained from diabetic patients (Van de Winkel *et al.*, 1982). Furthermore, these authors showed that the cell sorter may also be used to purify autoantibody positive cells for subsequent examination by electron microscopy.

REFERENCES

De Aizpurua, H.J., Toh, B.H. & Ungar, B. (1983) Parietal cell surface reactive autoantibody in pernicious anaemia demonstrated by indirect membrane immunofluorescence. *Clin. exp. Immunol.* **52,** 341.

Loken, M.R. & Herzenberg, (1975) Analysis of cell populations with a fluorescence-activated cell sorter. *Ann. N.Y. Acad. Sci.* **254,** 163.

Nairn, R.C. & Rolland, J.M. (1980) Fluorescent probes to detect lymphocyte activation. *Clin. exp. Immunol.* **39,** 1.

Rothschild, J. (1963) The isolation of microsomal membranes. In *The structure and function of the membranes and surfaces of cells (Biochemical Society Symposium No. 22)* (ed. by D.J. Bell & J.K. Grant) p. 4. Cambridge University Press, Cambridge.

Strickland, R.G. & MacKay, I.R. (1973) A reappraisal of the nature and significance of chronic atrophic gastritis. *Am. J. Dig. Dis.* **18,** 426.

Theofilopoulos, A.N. & Dixon, F.J. (1982) Autoimmune diseases. Immunopathology and etiopathogenesis. *Am. J. Path.* **103,** 321.

Thomas, H.C. & Jewell, D.P. (1979) Gastritis. In *Clinical gastrointestinal Immunology*. p. 83. Blackwell Scientific Publications, Oxford.

Van de Winkel, M., Smets, G., Gepts, W. & Pipeleers, D. (1982) Islet cell surface antibodies from insulin-dependent diabetics bind specifically to pancreatic B cells. *J. clin. Invest.* **70,** 41.