Reported series of H pylori infection in patients with Barrett's oesophagus

Author (ref)	No of patients	H pylori positive		Diagnostic method
		Stomach	Barrett's	
Talley ²	23		12 (52%)	Warthin-Starry
Paull ¹	26	10 (38.4%)	4 (15.4%)	Giemsa
Houck ⁴	34		0	Giemsa
Fallingborg ⁵	46 ^a		12 (26%)	Culture
	133 ^b		6 (4.5%)	Culture
Walker ⁶	7		2 (29%)	Culture
Francoual ⁷	11	4 (44.4%)	0	Culture
GOSPE ⁸	100		19 (19%)	Warthin-Starry, Giemsa
Present series	73	26 (35.6%)	11 (15%)	Immunohistochemistry

^a Biopsy specimens after gastroscopy.

^b Biopsy specimens before gastroscopy.

specimens with and without *H pylori*, both for chronic (81% v 79%) and acute (9% v 10%) infiltrates.

Discussion

H pylori infection plays an important role in the aetiopathogenesis of chronic gastritis, duodenal and gastric ulcers, and gastric carcinoma. However, its potential role in Barrett's oesophagus has not been completely defined. The detection rate of the bacteria using different techniques has been reported at between 0% and 52% in cases of Barrett's oesophagus,²⁻⁸ while its presence in the stomach has been reported at between 38% and 44% in the same patients (table).³⁷ In the present study the frequency of *H pylori* was low in oesophageal biopsy specimens (15%) as well as in gastric biopsy specimens (35.6%). Specialised metaplastic epithelium was not colonised by the bacteria

and the presence of *H pylori* in the oesophagus was always associated with gastric infection. This finding has been reported previously in retrospective studies^{7,8,13} indicating that *H pylori* colonisation of Barrett's mucosa is probably a consequence of gastric infection. We did not find any differences regarding the inflammatory changes in oesophageal biopsy specimens of patients either with or without *H pylori*. We conclude from our results that *H pylori* does not play an important role in Barrett's oesophagus; its presence in the metaplastic oesophageal mucosa needs the presence of a gastric-type mucosa in the oesophagus, together with a gastric infection.

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Use of buffered formaldehyde in the enzymatic digestion of inflamed mucosa

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Abstract

Mucosal inflammation is associated with altered expression of cell membrane molecules. Disaggregation of tissue for flow cytometry may introduce artefactual changes. In an attempt to prevent the induction of artefacts, cells were fixed prior to isolation. The addition of 0.1% buffered formaldehyde to the collagenase/dispase digestion of mucosal biopsy specimens from patients with inflammatory bowel disease enhances detection of CD3, CD11b, CD16, CD63, and CD14. No significant effect was noted for CD19, CD67 or CD45. The expression of CD3, CD11b and CD45 correlated with the degree of endoscopic inflammation. Dilute buffered

formaldehyde may be a useful adjunct to the enzymatic isolation of cells from mucosal specimens, by protecting surface antigens from digestion or alterations in expression.

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Keywords: paraformaldehyde, flow cytometry, inflammation.

Flow cytometry is more flexible than standard immunohistochemistry, both in terms of cell numbers analysed and the combination of antigens examined. However, prior to analysis, tissue samples must be disaggregated by mechanical, ion chelation or enzymatic methods.^{1,2}

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Density of expression of surface antigens

Antigen	Medium only	BFA treated	p value
Control	3.2 (1.5–7.5)	4.3 (1.8–7.8)	0.02
CD11b	2.2 (0.0–9.1)	8.4 (1.2–65.4)	0.02
CD54	6.2 (0.4–16.9)	3.8 (0.0–21.0)	0.58
CD45	3.3 (0.0–801.0)	4.4 (0.2–1070.0)	0.39
CD3	14.2 (1.8–23.0)	30.7 (2.6–52.3)	0.02
CD19	2.4 (0.2–25.3)	3.4 (0.5–19.1)	0.24
CD14	1.45 (0.0–3.75)	4.25 (0.75–10.55)	0.04
CD63	36.8 (1.1–80.7)	55.9 (0.71–136.8)	0.06
CD67	3.3 (0.0–43.3)	1.7 (0–55.6)	0.06
CD16	0.41 (0.0–2.65)	1.86 (0.0–12.0)	0.04

Results are expressed as the median (range) increment in the median logarithmic fluorescence intensity (dMFI) of cells stained with specific antibody compared with the same cells stained with control antibody. Significance was set at the 5% level.

These processes require the prolonged exposure of cells to intemperate conditions. The use of stringent isolation protocols may unduly influence the expression of cellular surface antigens through regulatory changes, shedding of, or alterations to the antigen.³

Early fixation, through the incorporation of paraformaldehyde into regimens for processing whole blood preparations, has been reported to prevent altered leucocyte surface antigen expression.⁴ However, the feasibility and benefit of carrying out early antigen fixation before the disaggregation of solid tissue is unknown. The aim of this study was to determine the influence of fixative use, using flow cytometry, on the identification of surface antigens on cells isolated from endoscopic biopsy specimens of inflamed colonic mucosa.

Methods

MONOCLONAL ANTIBODIES AND REAGENTS

The following monoclonal antibodies were used: SimulTest IgG₁ and IgG₂, anti-CD3, anti-CD19, and anti-CD14 (Becton Dickinson, Mountain View, California, USA); anti-CD11b, anti-CD16, anti-CD45, anti-CD63, and anti-CD67 (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands); anti-bcl-2 and phycoerythrin conjugated F(ab')₂ immunoglobulin rabbit anti-mouse (Dako, Glostrup, Denmark); and anti-CD54 (Monosan, Uden, The Netherlands). Reagents included: collagenase type IV (Sigma, St Louis, Missouri, USA); dispase I (Boehringer-Ingelheim, Mannheim, Germany); and RPMI 1640 culture medium and fetal bovine serum (FBS) (Life Technologies, Paisley, Scotland). Buffered formaldehyde (4%) (BFA) was prepared freshly in phosphate buffered saline from paraformaldehyde.

TISSUE COLLECTION AND CELL ISOLATION

Following informed consent, six endoscopic biopsy specimens were obtained at routine sigmoidoscopy from 27 patients (14 men, 13 women; median age 38 years (range 19–64

years)) with a history of ulcerative colitis. These biopsy specimens were obtained under a protocol approved by the Medical Ethics Committee of the Academic Medical Centre, Amsterdam. The endoscopic appearance of the mucosa was scored according to a modified Baron scale⁵: grade I (n=13), normal/mild inflammation (Baron 0–1); grade II (n=14), moderate/severe inflammation (Baron 2,3). Tissue was equally divided between tubes containing RPMI 1640 medium supplemented with 10% FBS, with and without (medium only) 0.1% BFA (v/v). Within fifteen minutes, the biopsy specimens were transferred to fresh RPMI/FCS containing collagenase type IV (50 U/ml) and dispase type I (1.2 U/ml). Once again, 0.1% BFA was added to the appropriate tubes.

The tubes containing the biopsy specimens were set on an inclined rotating table (Multi-purpose rotator, Scientific Industries Inc., New York, USA) for one hour at 37°C. Cells were subsequently washed twice in fresh RPMI 1640/FBS (with or without BFA), pelleted, counted by light microscopy using eosin, and resuspended in phosphate buffered saline containing 0.1% bovine serum albumin, 0.01% sodium azide and 0.3 mM EDTA, to a concentration of 2×10^6 cells/ml.

CELL LABELLING AND ASSESSMENT

Cell suspensions were incubated with the relevant monoclonal antibody at previously determined optimal concentrations for 30 minutes at 4°C. This process was repeated with the appropriate label for unconjugated primary antibodies. Prior to flow cytometry (FACScan, Becton Dickinson, Belgium), labelled cells were washed twice and resuspended in 1% BFA and stored at 4°C.

During flow cytometry, 10^4 events were saved using Lysys II software (Becton Dickinson, Belgium). Because of overlapping cell populations based on forward and side scatter windows, total cell populations were scanned without the use of specific gates. The density of expression of surface antigens was measured as the change in the median fluorescence intensity (dMFI), using a logarithmic scale, of cells stained with specific antibody compared with the same cells stained with control antibody.

In a control experiment to test for altered cell membrane permeability, peripheral blood lymphocytes were isolated, incubated with 0.1% BFA, 1% BFA or 0.1% saponin and labelled with a monoclonal antibody to the intracellular antigen bcl-2. Positive staining was seen only with saponin treated cells (data not shown).

STATISTICAL ANALYSIS

Data were entered onto QuattroPro for Windows 5.0 (Borland Inc., USA) and statistical analyses performed using SPSS for Windows 6.0 (SPSS Inc., USA). The dMFI of medium only and BFA treated cells for each antibody was compared using the Wilcoxon rank sum

test for paired non-parametric data. Correlation values are presented as the Pearson correlation co-efficient. For all analyses, significance was set at the 5% level.

Results

Cell yields for both medium only and BFA treated biopsy specimens were similar (3.6 ± 1.2 v 3.9 ± 0.8 million cells from three specimens). The presence of 0.1% BFA during cell isolation resulted in enhanced detection of CD45, CD11b, CD3, CD19, CD14, CD16, and CD63. However, this was at the expense of an increase in control antibody binding (table). The presence of BFA slightly diminished the intensity of fluorescence associated with both CD54 and CD67, although this did not reach statistical significance.

When compared with grade of macroscopic mucosal inflammation, several antigens correlated strongly with increasing score: CD3 ($r=0.77$, $p<0.002$); CD11b ($r=0.72$, $p<0.001$); and CD54 ($r=0.73$, $p<0.001$). Results from the most intensely staining treatment group, medium only (CD54 and CD67) or BFA (CD11b, CD45, CD3, CD19, CD14, CD63, and CD16), were used to analyse each antigen.

Discussion

Flow cytometry provides a rapid method for the identification and screening of specific cell populations in heterogeneous suspensions. Solid tissues present particular difficulties, as they require both vigorous disaggregation and the use of proteolytic enzymes. Such stringent conditions can affect surface antigens through cleavage or changes in regulation. The use of fixatives in the processing of blood for flow cytometry has been reported.⁴ The results of our study show that such an approach is feasible with solid tissues; however, this is achieved at the expense of an increase in the level of binding of non-specific antibody. The finding that low concentrations of BFA did not render cells permeable is in keeping with other reports.⁶ Localised inflammation, as seen in inflammatory bowel disease, results from the sequential adhesion, activation and transmigration of leucocytes.⁷ During this process, surface antigens are altered both by translocation (CD11b, CD63, CD67) and de novo synthesis (CD54).⁸ We have found that fixation with 0.1% BFA prior to flow cytometry enhances the detection of the neutrophil antigens CD11b, CD16 and CD63 and the monocyte/granulocyte subset marker CD14. The low

levels of CD16 detected are due to shedding of this glycosylphosphatidylinositol (GPI) linked molecule on neutrophil activation.⁹

Identification of the mixed populations present in mucosal specimens is complicated by the action of proteolytic enzymes on those antigens lacking a transmembrane domain—for example, those with GPI linkages. Several of the antigens probed in this study possess such a structure. The addition of BFA significantly enhanced the detection of both CD14 and CD16. However, this effect on GPI linked molecules was not uniform, as can be seen from results with CD67.

The close correlation between endoscopic mucosal appearance and prevalence of certain surface antigens (CD11b, CD54 and CD3) suggests that flow cytometry carried out on BFA treated cell suspensions derived from small numbers of biopsy specimens can yield useful results. The use of dual labelling would enhance the efficacy of such investigations, in particular, by enabling specific cell populations to be probed. However, the relative effect of BFA on each antigen needs to be quantified. The removal of epithelial cells would aid this process further, by reducing background autofluorescence. Importantly, the use of dilute BFA did not impair enzyme function, as measured by cell yield. This is of particular relevance to biopsy based studies as tissue quantities, and thus cell numbers, are limited.

In conclusion, the incorporation of dilute BFA during the enzymatic disaggregation of solid tissue provides a useful adjunct to the investigation of cellular surface antigens by flow cytometry. This particular application of BFA provides some degree of protection for such antigens and may prevent isolation related changes in their regulation.

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