

# A quantitative study of the influence of fixation on immunoperoxidase staining of rectal mucosal plasma cells

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**SUMMARY** A quantitative morphometric study of the immunoglobulin-containing plasma cells in rectal biopsies from nine patients with ulcerative colitis and three patients with the irritable colon syndrome is presented. The results show that fixation of the biopsy specimens with formol-sublimate, a mercuric fixative, resulted in better staining with the immunoperoxidase method and that, by comparison with formol saline, higher cell counts were always obtained.

It has been known for some time that the results obtained with the immunoperoxidase method are dependent to some extent on the type of fixative used before processing the biopsy specimens.<sup>1</sup> Our preliminary results (Piris and Thomas, unpublished observation), carried out before the report by Bosman *et al.*<sup>1</sup> was published, coincided with theirs in that we also believe that sublimate-formaldehyde is superior to ordinary formalin and Zenker's fluid fixative. We are concerned that these differences in the consistency with which adequate positive results are obtained, the intensity of the specific peroxidase reaction, and the undesirable background non-specific staining may result in a quantitative difference brought about by the inability to detect weakly stained positive cells in a not too clear background.

The present paper deals with the results of a morphometric quantitative study of mucosal immunocytes in rectal biopsy specimens, fixed with either formol saline or sublimate-formaldehyde, and stained with an immunoperoxidase method.

## Material and methods

Two biopsy specimens from adjacent areas of the anterior rectal wall were obtained from nine patients with ulcerative colitis who were in remission. The specimens were randomly fixed in either formol saline or sublimate-formaldehyde (a 9:1 mixture by volume of saturated mercuric chloride and formalin).

Three more biopsy specimens, obtained from three patients with the irritable colon syndrome,

were immediately bisected, and each half was fixed in one of the two fixatives. After routine processing 4 $\mu$  thick sections were cut and stained with haematoxylin and eosin. All specimens included the muscularis mucosae and were free from ulceration. There was no obvious subjective difference in the degree of inflammatory infiltration in the lamina propria of the corresponding two halves of each specimen.

The specimens were then stained with the indirect ('sandwich') peroxidase method of Taylor and Burns.<sup>2</sup> After dewaxing, 2 $\mu$  sections, whether fixed in formalin or sublimate, were treated with iodine and hypochloride to remove the mercury and then washed well in water; the endogenous peroxidase activity is blocked with a fresh solution of 0.5% hydrogen peroxidase in methanol for 30 minutes; after washing in phosphate buffered saline (PBS) the sections are exposed to a 1 in 5 solution of normal swine serum for 10 minutes to reduce the non-specific background staining. The first specific antibody, a mixture of rabbit anti-human  $\kappa$  (kappa) and rabbit anti-human  $\lambda$  (lambda) light chain antisera purchased from DAKO-Immunoglobulins Ltd is then used at a dilution of 1:20 in 1:20 normal swine serum in PBS for 30 minutes. This is followed, after a wash in PBS, by incubation with peroxidase conjugated swine anti-rabbit IgG serum at 1:20. The peroxidase activity is demonstrated by adding the substrate, diaminobenzidine tetrahydrochloride and H<sub>2</sub>O<sub>2</sub>, which results in a visible brown precipitate being deposited at the site where the peroxidase is available. The slides were counterstained with Harris' haematoxylin and mounted. Two control sections were included; in one of them the first anti-

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body (rabbit anti-human  $\lambda$  and  $\kappa$  light chains) was omitted. In the other, the second antibody (swine anti-rabbit IgG) was omitted. Both control sections show no staining of plasma cells.

#### QUANTITATIVE PROCEDURE

The method was similar to that described by Skinner and Whitehead.<sup>3</sup> A Leitz microprojector fitted with a  $\times 40$  lens was used to project the image on to a grid in which points were marked 2 cm apart at the corners of equilateral triangles. By means of the mechanical stage of the microscope, the histological sections were screened, and the number of points falling on the lamina propria, as well as the number of positive staining plasma cells, were recorded. This was done ensuring that the fields counted included all possible areas of the mucosa, that is, under the surface epithelium, in the mid-zone, and at the base of the mucosa above the muscularis mucosae.

By means of a summation average graph,<sup>4</sup> it was concluded that, at the chosen magnification, the area of lamina propria examined by counting one thousand points constituted a representative sample of the whole biopsy specimen since the ratio plasma

cells: points in the lamina propria was stable in all cases, after about 700 points (Fig. 1).

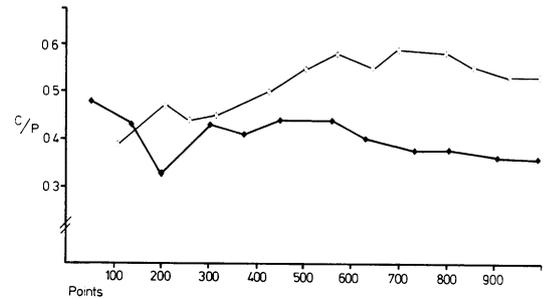


Fig. 1 Summation average graph plotted for the ratio of plasma cells to points in lamina propria (C/P). After counting 600 points the mean varies very little.

#### Results

The appearance of the haematoxylin and eosin stained slides from the specimens fixed in sublimate is better than that of the ones fixed in formol saline;

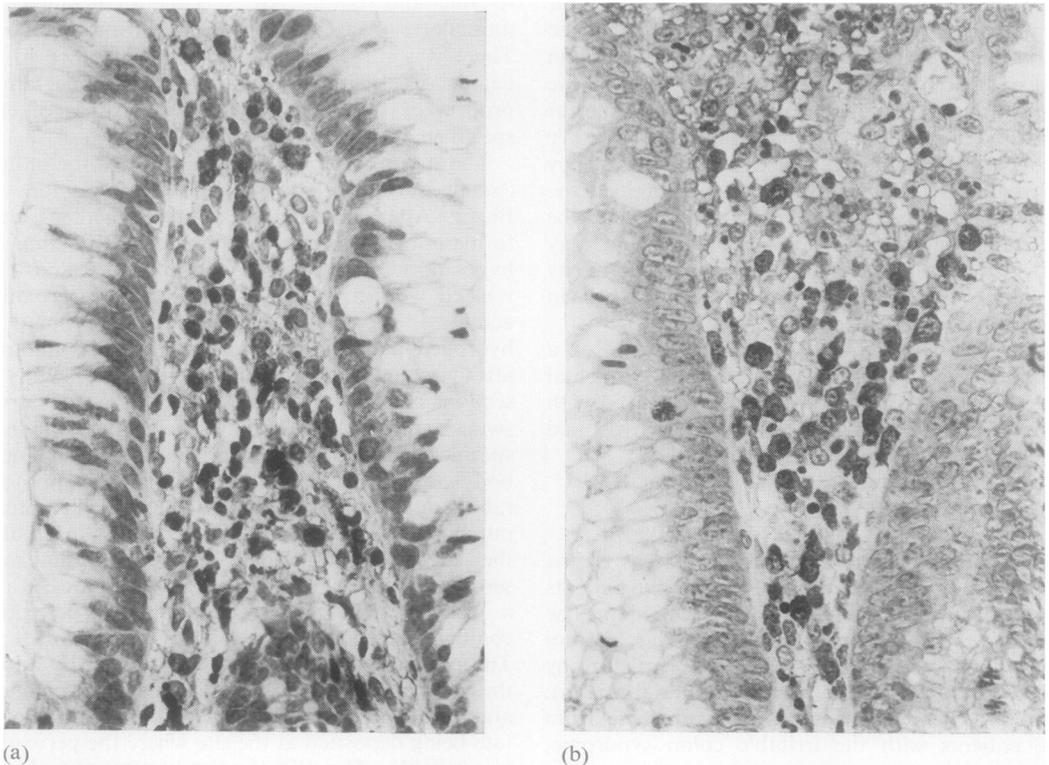


Fig. 2 Case 7. Rectal biopsy specimen fixed with (a) formol saline and (b) formol sublimate and stained with an immunoperoxidase method for the demonstration of immunoglobulin-containing plasma cells.  $\times 395$

Case	Sex	Age	Diagnosis	C/P (FS)	C/P (FHg)
1	M	56	ICS	0.15	0.62
2	F	47	ICS	0.16	0.53
3	M	55	UC	0.28	0.34
4	F	31	UC	0.26	0.34
5	F	50	UC	0.44	0.95
6	F	39	UC	0.61	1.01
7	M	31	UC	0.56	0.61
8	M	69	UC	0.57	0.72
9	F	30	UC	0.13	0.29
10	M	70	UC	0.23	0.55
11	M	36	UC	0.16	0.52
12	M	29	ICS	0.15	0.33

C/P = ratio of plasma cells to points; FS = formal saline; FHg = formal sublimate; ICS = irritable colon syndrome; UC = ulcerative colitis. Student's *t* test for paired samples: *t* = 5.46, *p* < 0.001.

in particular, the nuclear detail is enhanced. With the peroxidase method, the intensity of the reaction appears much greater, and this allows for better definition of the cell contours. Also, the staining of connective tissue fibres and ground substance is less marked (Fig. 2). As expected after blocking endogenous peroxidase the granules of polymorphonuclear neutrophils and eosinophils were not stained in the sections fixed with either fixative. The use of iodine and hypochloride in formalin fixed sections made no difference to the staining results when compared with similar specimens also fixed in formalin but not treated with these compounds.

The quantitative results of the 12 cases are summarised in the Table, in which the ratios of plasma cells to points in the lamina propria are given in the corresponding columns according to the fixative used. In all cases more immunoglobulin-containing plasma cells were seen in the specimens fixed in formal sublimate. The degree of difference was not constant, being marginal in some cases whereas in others it was threefold or fourfold.

The difference can be analysed statistically by means of Student's *t* test for paired observations, which gives a value of *t* of 5.46, highly significant at the 0.001 level.

## Discussion

Because of the undoubted role of immunological mechanisms in some forms of gastrointestinal disease, the local cellular infiltration by immunoglobulin-producing plasma cells has recently been the subject of several studies.<sup>3 5 6</sup> Many of these studies are based on immunofluorescent methods, but it is clear that the possibility of using routinely fixed paraffin-embedded tissue sections represents an enormous advantage. The immunoperoxidase technique provides a means for the study of immunocytes, and of many other types of cells containing antigenic material against which an antibody can be raised such as hormones; the biopsy specimens can be stored

permanently and studied at a convenient time; also the final preparation is stable and does not fade with time, thus obviating the need for immediate staining and photography. Although formalin-fixed material is satisfactory substrate for this immunohistochemical method, it is our belief that the final result, and the consistency of the reaction, are much improved by the use of the sublimate-formaldehyde. This fixative does not affect the staining characteristics of the tissues by the routine methods such as haematoxylin and eosin or periodic acid Schiff, and the number of cells stained by non-immunological methods—such as carbol chromotome for eosinophils—is no different from that obtained when formal saline is used as a fixative.<sup>7</sup>

Formol sublimate contains mercuric chloride, and reasonable precautions should be taken to avoid inhaling dust when preparing the mercuric chloride solution and to prevent direct contact with the skin. Because of its corrosive properties metal instruments and containers with metal caps should not be used. Excessive fixation of tissues should be avoided to prevent undue hardness.

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