Plasma cell counts of human jejunal biopsy specimens examined by immunofluorescence and immunoperoxidase techniques: a comparative study

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SUMMARY Ten human jejunal biopsy specimens were examined by both immunofluorescence (IF) and immunoperoxidase (IP) methods to compare both plasma cell counts and the distribution of extracellular immunoglobulins. Each specimen was cut into at least two portions, one fixed in 5% formaldehyde in phosphate-buffered saline before being snap-frozen and sectioned on a cryostat for IF, the other being fixed in half-strength Zenker and embedded in paraffin wax by standard methods for IP. Plasma cell counts were comparable in the eight biopsy specimens for which they could be estimated, geometric mean values being IgA 22.9 (IF), 19.3 (IP) and IgM 9.5 (IF), 10.6 (IP). Two specimens showing subtotal villous atrophy had too much extracellular IgA for plasma cell counts to be feasible. For these the IF method had the advantage that the extracellular immunoglobulin was more readily distinguishable from background staining.

Immunological examination of human jejunal biopsy specimens is now frequently carried out by immunofluorescent (IF) histology. The more recently developed immunoperoxidase (IP) techniques provide an alternative with several advantages: permanent preparations can be made and counterstained, for example, with haematoxylin to show the cell nuclei, and an ordinary microscope can be used. The IP method has been used to examine human and animal intestine. A number of comparative studies have been made on various organ tissues, but we thought it desirable to make the comparison for human jejunal histology, in particular for plasma cell counts. Most plasma cell counts in jejunal tissue have been reported using IF techniques. IP techniques might give a different answer owing to the combined effects of variations in section thickness and tissue shrinkage on the number of plasma cells present in the sections. Also it might be easier to recognise plasma cells containing relatively little immunoglobulin by one method or the other, either for immunochemical reasons or because of differences in the sensitivity of the eye to weak green fluorescence and pale brown pigment.

We have therefore compared an immunofluorescent technique on formaldehyde-fixed cryostat sections with a soluble complex horseradish peroxidase technique on paraffin-embedded tissue fixed in half-strength Zenker's solution. This study is intended to establish the comparability of two techniques which we have found satisfactory rather than to study in detail the properties of the IF and IP systems.

Material and methods

BIOPSY SPECIMENS

Upper jejunal biopsy specimens were obtained with a Crosby capsule from patients referred for investigation. Part of each specimen was divided into two portions and fixed immediately. Eight biopsy specimens, five normal and three showing partial villous atrophy, are listed in Table 1 together with their plasma cell counts. A further two specimens showing subtotal villous atrophy had too much extracellular IgA for plasma cell counts to be feasible but were studied as to the distribution of the extracellular immunoglobulin; one was from a patient with severe untreated coeliac disease and one from a patient with α-chain disease.
Table 1  Counts per mucosal unit of IgA and IgM plasma cells in human jejunal biopsy specimens stained by immunofluorescence (IF) and immunoperoxidase (IP) procedures

<table>
<thead>
<tr>
<th>Biopsy no.</th>
<th>Histological classification</th>
<th>IgA</th>
<th>IgM</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>IF IP</td>
<td>IF IP</td>
</tr>
<tr>
<td>1</td>
<td>PVA</td>
<td>1-4</td>
<td>1-9</td>
</tr>
<tr>
<td>2</td>
<td>PVA</td>
<td>20-5</td>
<td>13-8</td>
</tr>
<tr>
<td>3</td>
<td>N</td>
<td>23-1</td>
<td>12-4</td>
</tr>
<tr>
<td>4</td>
<td>N</td>
<td>27-5</td>
<td>32-5</td>
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<tr>
<td>5</td>
<td>N</td>
<td>38-0</td>
<td>23-1</td>
</tr>
<tr>
<td>6</td>
<td>N</td>
<td>41-0</td>
<td>27-6</td>
</tr>
<tr>
<td>7</td>
<td>N</td>
<td>45-5</td>
<td>50-9</td>
</tr>
<tr>
<td>8</td>
<td>PVA</td>
<td>58-7</td>
<td>56-3</td>
</tr>
<tr>
<td></td>
<td>Arithmetic mean</td>
<td>32-0</td>
<td>27-3</td>
</tr>
<tr>
<td></td>
<td>Geometric mean</td>
<td>22-9</td>
<td>19-3</td>
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</tbody>
</table>

N = normal; PVA = partial villous atrophy.

Mean differences (standard deviations in parentheses)

<table>
<thead>
<tr>
<th>Anti-log mean (log IF-log IP)</th>
<th>Anti-log mean (log IF-log IP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-19 (1-42)</td>
<td>0-90 (1-55)</td>
</tr>
<tr>
<td>1-03 (1-51)</td>
<td>1-19 (1-42)</td>
</tr>
</tbody>
</table>

IMMUNOFLUORESCENT PROCEDURE

The biopsy tissue was fixed in 5% formaldehyde in phosphate-buffered saline (PBS) for 4 hours at room temperature, washed in PBS, and allowed to stand in sucrose solution (30 g in 100 ml distilled water) at +4°C overnight. The tissue was drained, embedded in Tissue-Tek II OCT (Miles Laboratories, Inc.) on a piece of cork, orientated so as to give sections perpendicular to the muscularis mucosae, snap-frozen, and stored at −80°C in a small polystyrene vial. The 6 µm cryostat sections were placed on multispot slides (CA Hendley & Co) and air-dried for at least 15 minutes. Where the tissue tended to wash off the slides the wells were coated with chrome gelatin (0.5 g gelatin, 0.05 g chrome alum in 100 ml water). The slides were washed in PBS with gentle stirring for 5 minutes, treated with the conjugates, appropriately diluted in PBS for 30 minutes, rinsed with PBS, and washed with gentle stirring for 30 minutes. Rabbit anti-human fluorescein conjugates (Dakopatts) were used at the following dilutions: IgA 1/60, IgG 1/60, IgM 1/30, C3 1/10. The sections were mounted in glycerol buffered to pH 8.5 and examined with a Leitz orthoplan fluorescence microscope. This IF procedure was used by us in a previous study,2 the use of controlled formaldehyde fixation being derived from the method of Eidelman and Berschauer.16

IMMUNOPEROXIDASE

The biopsy tissue was fixed for 2 to 4 hours according to size in half-strength Zenker’s solution (stock: mercuric chloride 5 g, potassium dichromate 2.5 g, sodium sulphate anhydrous 1 g, distilled water 100 ml; for use: 25 ml stock, 25 ml distilled water, 1:25 ml glacial acetic acid). The Zenker-fixed tissue was processed by standard histological methods to give paraffin blocks, without treatment for possible mercury pigment. Where the specimen could not be processed immediately after fixation it was washed free of fixative and stored in 70% alcohol for a limited period. The blocks and cut sections appeared to be stable at room temperature, but slides were normally stored at −20°C till used.

The 4 µm sections were dewaxed in xylene overnight or for at least half an hour, and then transferred for 5 minutes each into fresh xylene and two changes of absolute alcohol. The slides were placed in 0.3% hydrogen peroxide in methanol (1 ml 100 vol hydrogen peroxide in 99 ml methanol) for 5 minutes to inactivate endogenous peroxidase followed by a 5-minute wash in 70% alcohol and in PBS. The sections were pretreated for 10 minutes with 1/10 or 1/20 normal swine serum in PBS, then for 30 minutes with the serological reagents in order, with 10-minute washes in PBS between layers and a final 30-minute wash. The reagents (Dakopatts) are shown in Table 2. The dilutions

<table>
<thead>
<tr>
<th>First layer</th>
<th>Rabbit anti human IgG IgM</th>
<th>Dilution 1/50 1/50 1/50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Second layer</td>
<td>Swine anti rabbit IgG (heavy and light chains)</td>
<td>Dilution 1/40</td>
</tr>
<tr>
<td>Third layer</td>
<td>Rabbit anti-HRP-HRP soluble complex (PAP)</td>
<td>Dilution 1/50</td>
</tr>
</tbody>
</table>

All dilutions were in 1/10 or 1/20 normal swine serum of the reagent, as supplied by Dakopatts. HRP = horseradish peroxidase.

(all in 1/10 or 1/20 normal swine serum) are approximate and need adjustment from time to time. In a block titration IgA was put on at 1/25 and 1/50, the anti-rabbit IgG at 1/25 and 1/50, and the peroxidase immune complex at 1/30 and 1/60. The crucial adjustment was to reduce the dilution of the anti-IgA from 1/25 to 1/50. Irrespective of variations in the concentrations of the other two layers, strongly differentiated plasma cells were found with the anti-IgA diluted 1/50. Thus the highest dilutions compatible with strong positive staining in a known positive control should be used. The first and third layers may often be used at 1/100. If the reagents are too concentrated (especially in the first layer) all the tissues stain and may greatly reduce the contrast with positively staining plasma cells. The colour was developed in a freshly prepared solution of DAB (3,4,3',4'tetraaminobiphenyl hydrochloride—diaminobenzidine) (BDH Chemicals Ltd) with 0.01% hydrogen peroxide added immediately

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before use (15 mg DAB dissolved in 50 ml PBS with 0·17 ml of 10 vol hydrogen peroxide). A slide was gently agitated in the DAB reagent for 15 seconds, removed, and immediately rinsed in tap water and examined under a microscope. This process was continued in 5-second steps until good positive plasma cell staining, with minimum background staining, was obtained. The total time in the DAB reagent was usually about 20 seconds. The other slides were then immersed with gentle agitation for the same total period of time. The slides were rinsed in tap water, counterstained with Harris's haematoxylin, differentiated in acid alcohol (70% ethanol with 1 ml conc HCl per 100 ml), dehydrated, cleared, and mounted in DPX in the usual way.

COUNTING PLASMA CELLS
The procedure used was described by Maffei et al., modified from Brandtzæg and Baklien. A × 25 objective was used and ×10 eyepieces with a 5 mm × 5 mm grid of 100 subdivisions inserted in one. The cells were counted per 'mucosal unit', defined by us as a column the full width of the grid and the full height of the mucosa. The grid was lined up on the muscularis mucosae of vertically cut sections and moved up to the tips of the villi; each plasma cell, at least half of which fell within this column, was counted. The width of this mucosal unit on a slide micrometer was 232 μm (not the 500 μm column of Brandtzæg). Whenever possible, eight to 15 consecutive units were counted in each of two different sections for each antiserum. Owing to the subdivision of the biopsy tissue, however, in three of the IP preparations fewer than 10 mucosal units could be counted. Granular cells (probably eosinophils), which tended to take up immunofluorescent reagents weakly, were excluded by their ability to scatter light, the granules appearing red when a K530 secondary filter was used.

Results

PLASMA CELL COUNTS
These were compared for eight biopsy specimens (Table 1). While there is some variation for individual specimens (see Discussion) the mean values for all eight biopsy specimens are very similar. Owing to the presence of one set of high counts in the IgM columns it is better to compare the geometric means. The IF and IP counts were plotted against each other, and there appeared to be a random scatter about the equivalence line. The differences (IF-IP) are the distances of each point from the equivalence line measured parallel to the IF (or IP) axis, and the mean of these differences indicates the extent to which the relation between IF and IP counts differs from 1:1. Student's t test (two-tailed) was used to see if the means of the differences were significantly different from zero, considering IgA, IgM, arithmetic and logarithmic values separately. The highest t value found was 1·64, which with 7 degrees of freedom gives P = 0·14. Thus, though there were substantial differences for individual biopsy specimens, overall IF and IP values are not significantly different. Taking IgA and IgM counts together, the mean of the differences was 1·03 (anti-log mean differences of logs) with 95% confidence limits of 1·29 and 0·83. Thus the results of Table 1 would be compatible with the IF counts being from 129% to 83% of the IP counts.

EXTRACELLULAR IMMUNOGLOBULIN
The slides were examined to compare the staining of extracellular immunoglobulins by the IF and IP methods. For this, staining for IgG and C₃ was included, and also the two additional biopsy specimens. We found that considerable retraining of the eye was necessary before the less familiar peroxidase staining could be interpreted with confidence. Weak general peroxidase staining due to the presence of extracellular IgA in the lamina propria was not easily distinguished from normal tissue colour. Tissue colour may readily be made a little more pronounced by very slight overdevelopment in the DAB. By contrast, the fluorescein conjugates gave negligible non-specific staining (except sometimes of eosinophils). Thus, though strongly stained plasma cells were readily detected by either method, it was found helpful to examine the highly abnormal biopsy specimens (severe coeliac, α-chain disease) by both methods. The ability to examine the nuclei in the peroxidase preparations gave helpful additional information. When sections were stained for IgG (IF and IP) or C₃ (IF only) both methods gave staining on surfaces throughout the lamina propria, presumably being the site of precipitation of the extravascular protein by the fixatives used in both techniques.

Discussion
The tissue used for IF histology and IP histology in this study had to be separately fixed. Thus it was not possible to compare counts on serial sections, and the comparison was on different pieces of tissue taken from the same jejunal biopsy. Counts vary widely, even between different areas on the same section, and the 20 mucosal units suggested for counting were not available for all of the preparations. These limitations probably explain the scatter about the equivalence line. Our results suggest that plasma cell counts would be the same by the two
methods, but a much larger sample would be needed to show small differences.

An important variable which has received little attention is the effect of tissue shrinkage. Here the rather thicker sections used for IF (6 μm as against 4 μm) seem to have compensated for the increased shrinkage found in wax-embedded sections, assuming that the plasma cells were equally detectable by the two methods.

Fixation methods greatly affect the results of immunohistochemical staining apart from any effects of tissue shrinkage. In IF, our experience has been that plasma cells stand out far less well in unfixed biopsy tissue, presumably because of loss of immunoglobulin during staining. Fixation also retains extracellular immunoglobulins and other extracellular proteins such as C3. Alcohol fixation of the tissue and, in our experience, of cut sections, gives an appearance of a continuous film of the extracellular protein, which masks the structures in the lamina propria and causes plasma cells to stand out much less clearly. By contrast, in formaldehyde-fixed tissue the protein appears to become attached to the surfaces of plasma cells and connective tissue components. Where the concentration of extracellular immunoglobulin is high it becomes difficult to distinguish positive from negative plasma cells, and there is intense connective tissue staining. Thus considerable care is needed in interpreting extracellular staining when fixed biopsy specimens are examined.

In IP, the use of routine histological blocks, as suggested by Taylor and Burns, gave variable results in our hands. Experience in several laboratories has been that consistent results are found only when the biopsy specimens are fixed under controlled conditions. In ordinary histological practice, the length of time in fixative and the pH of the formol saline may vary. (Other variables, for example, the temperature of the wax bath, could conceivably have an effect in some circumstances.) A number of studies have shown that treatment of sections with trypsin may greatly improve the staining (IF and IP) of sections from wax blocks fixed in formol saline. This is probably due in part to allowing readier diffusion of the antisera to the antigen. An alternative is to use a different fixative. We have found fixation in half-strength Zenker very satisfactory for IP without the need for treating the sections with proteolytic enzymes. Other workers recommend formol-sublimate.

Variations in the serological procedures would also be likely to affect the sensitivity of the system. We have here compared a direct IF procedure with a three-layer IP procedure, and our results might not apply to IF or IP staining carried out in other ways.

In our experience, the IP method is more sensitive than the IF to changes in the titre of the serological reagents. Thus, in IP, doubling the concentration of the first layer increased the level of background staining to the point where plasma cells stood out much less clearly. In IF, background staining was always slight, and wide variations of dilutions could be used without the plasma cell staining being affected. This might make IP inconvenient for sporadic use but would probably not be so important where the procedure was used regularly. The greatly increased time for staining in IP (three layers as against one) was also sometimes inconvenient.

In view of these complications it may seem surprising that we have obtained comparable counts by two very different methods. There is no reason, however, why two different techniques should not give the same result provided that: (1) tissue shrinkage effects are corrected; (2) the serological reagents can reach the antigen freely; (3) the configuration of the antigen has not been seriously altered; and (4) the serological reagents are used under optimum conditions. It seems that these prerequisites were met in this comparison.

We conclude that these IP and IF methods give very similar plasma cell counts in human jejunal biopsy specimens. The IP method of well-preserved, permanent, counterstained preparations was a great advantage, but the IF method had the advantage of speed and of making the distinction between background staining and staining of extracellular immunoglobulin easier.

We are grateful to Dr M Shiner for allowing us access to her biopsy material, and to the Department of Surgical Histology, Northwick Park Hospital, for processing the Zenker-fixed biopsy specimens and for providing helpful technical advice. Dr ID Hill, Division of Computing and Statistics, calculated the confidence limits and gave helpful statistical advice. FJP acknowledges with gratitude the receipt of a scholarship from the Conselho Nacional de Desenvolvimento Cientifico e Tecnologico (of Brasil).

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