Distribution of immunoglobulin producing cells is different in normal human appendix and colon mucosa

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SUMMARY The densities of IgG-, IgA-, IgM- and IgD-producing immunocytes were determined by paired immunofluorescence staining and morphometric analysis in the lamina propria of normal appendix specimens. Normal colon specimens were used as reference material, mostly paired from individual subjects. The density (median of cells/mm² lamina propria area) of IgA immunocytes tended to be slightly higher in the appendix than in the colon (1259 vs 962) and the same held true for IgM cells (71 vs 55). Conversely, the overall density of IgG immunocytes was much higher in the appendix than in the colon (95 vs 38). A striking feature was the fact that almost 50% of all immunocytes were of the IgG isotype adjacent to lymphoid follicles. It seemed justified to conclude, therefore, that the abundance of such follicles explains the overall enrichment of IgG-producing cells in normal appendix mucosa. These immunocytes most likely represent follicle derived B cells that have reached terminal maturation locally, whereas precursors generated from less mature memory clones probably emigrate and home ubiquitously to distant sites of the gut lamina propria where they develop into IgA-producing immunocytes.

Since the pioneering work of Crabbé and Heremans in the 1960s, several immunohistochemical studies have been published about the immunoglobulin (lg)-producing immunocytes in normal adult large bowel mucosa (Table 1). All of the reports agree with the remarkable normal preponderance of IgA immunocytes, but large variations appear with regard to the proportions of cells producing the IgM and IgG isotypes (3%–34% and 2%–35%, respectively). We have discussed elsewhere some of the technical problems that may explain these excessive discrepancies. The variations are apparently independent of whether immunofluorescence or immunoperoxidase methods have been used and seem to be unrelated to the bowel segment studied (Table 1). When attempts have been made to estimate mucosal densities of immunocytes (cells/mm² lamina propria area), the discrepancies are likewise unacceptably large, even for IgA-producing cells (Table 1).

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The human appendix has been subjected to surprisingly few immunohistochemical studies despite the fact that its abundant content of organised lymphoid tissue suggests an active immunological state. Indiveri et al examined appendectomy specimens and reported equal numbers of IgA and IgG immunocytes. Chen found that the numbers of IgG-producing cells depended on the inflammatory stage; the lowest number (13%) appeared in specimens defined histologically as 'early stage'. Data for normal appendix mucosa have apparently not been published previously.

Our previous quantitative studies have been based on paired immunofluorescence staining recorded on double exposed colour slides. We have now adopted mounting of tissue sections with medium containing paraphenylenediamine (PPD), a procedure that makes it possible to carry out prolonged and repeated observations directly in the fluorescence microscope without significant loss of staining intensity. The aim of this study was to see partly whether it is possible to reproduce our previous quantitative reference data on immunocytes in normal large bowel mucosa by doing direct
cell counting combined with histomorphometry. The latter approach was in fact required for the major purpose of this study, namely to examine whether the presence of organised lymphoid tissue would influence the composition of the adjacent mucosal immunocyte population. For this purpose we decided to carry out a comparative investigation of normal large bowel and appendix mucosa.

**Methods**

**MUCOSAL SPECIMENS**
Tissue specimens were obtained from patients operated for cancer of the large bowel (at least 6 cm away from the tumour) and were considered normal according to histological features. In addition, we obtained normal samples from four kidney donors in whom peripheral circulation was artificially maintained. Paired samples from colon and appendix were obtained in nine cases. Altogether the study was based on specimens from seven women and eight men with a median age of 48 years (range 7–79 years).

**IMMUNOHISTOCHEMICAL PROCEDURES**
Thin tissue slices (2–3 mm thick) were excised from gross specimens as soon as possible after their removal, oriented on filter paper, extracted in cold 0.01 M phosphate buffer (pH 7-6) containing 0.15 M NaCl for 48 hours to remove diffusible extracellular Ig components, fixed in cold ethanol, and processed for paraffin embedding.

Serial sections were cut at 6 μm perpendicular to the mucosal surface and were evaluated by paired direct immunofluorescence staining with rabbit IgG conjugates monospecific for human IgA, IgG, IgM, or IgD. The reagents were prepared in our laboratory by labelling with fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC); their characteristics have been reported elsewhere. The contrasting pairs of conjugates, applied for 20 hours at room temperature on adjacent sections, appear in Table 2.

**FLUORESCENCE MICROSCOPY**
The microscope used was a Leitz Orthoplan equipped with an Osram HBO 200 W lamp for TRITC (red) and an XBO 150 W lamp for FITC (green)

<table>
<thead>
<tr>
<th>Serial tissue section</th>
<th>Rhodamine ('red') conjugates</th>
<th>Fluorescein ('green') conjugates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α chain (IgA)</td>
<td>γ chain (IgG)</td>
</tr>
<tr>
<td>2</td>
<td>γ chain (IgG)</td>
<td>α chain (IgA)</td>
</tr>
<tr>
<td>3</td>
<td>μ chain (IgM)</td>
<td>α chain (IgA)</td>
</tr>
<tr>
<td>4</td>
<td>δ chain (IgD)</td>
<td>μ chain (IgM)</td>
</tr>
</tbody>
</table>

![Table 1 Various reports on isotype distribution (%) and density (d) of Ig-producing cells in normal adult human large bowel mucosa](http://gut.bmj.com/)

<table>
<thead>
<tr>
<th>Bowel segment</th>
<th>IgA (%)</th>
<th>IgM (%)</th>
<th>IgG (%)</th>
<th>IgA (d)</th>
<th>IgM (d)</th>
<th>IgG (d)</th>
<th>Method</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colon</td>
<td>91-5</td>
<td>4-5</td>
<td>4-0</td>
<td></td>
<td></td>
<td></td>
<td>DIF</td>
<td>Crabbe and Heremans (1966)</td>
</tr>
<tr>
<td>Rectum</td>
<td>85-2</td>
<td>9-3</td>
<td>5-5</td>
<td></td>
<td></td>
<td></td>
<td>DIF</td>
<td>Getzay et al (1968)</td>
</tr>
<tr>
<td>Rectum</td>
<td>90-4</td>
<td>6-8</td>
<td>2-8</td>
<td>1523</td>
<td>115</td>
<td>47</td>
<td>DIF</td>
<td>Soltoft et al (1973)</td>
</tr>
<tr>
<td>Rectum</td>
<td>92-6</td>
<td>2-9</td>
<td>4-5</td>
<td></td>
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<td></td>
<td>DIF</td>
<td>Skinner and Whitehead (1974)</td>
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<tr>
<td>Colon</td>
<td>90-8</td>
<td>5-6</td>
<td>3-6</td>
<td></td>
<td></td>
<td></td>
<td>DIF</td>
<td>Bakken and Brandtzaeg (1975)</td>
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<tr>
<td>Rectum</td>
<td>89-2</td>
<td>3-3</td>
<td>5-5</td>
<td></td>
<td></td>
<td></td>
<td>DIF</td>
<td>Brown et al (1975)</td>
</tr>
<tr>
<td>Colon/ileum</td>
<td>71-3</td>
<td>19-1</td>
<td>9-6</td>
<td>1497</td>
<td>401</td>
<td>201</td>
<td>DIF</td>
<td>Green and Fox (1975)</td>
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<tr>
<td>Colon/rectum</td>
<td>84-5</td>
<td>13-6</td>
<td>1-9</td>
<td>749</td>
<td>120</td>
<td>17</td>
<td>DIF</td>
<td>Meuwissen et al (1976)</td>
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<tr>
<td>Colon</td>
<td>52-1</td>
<td>34-1</td>
<td>13-8</td>
<td>1375</td>
<td>900</td>
<td>365</td>
<td>DIF</td>
<td>O'Donoghue and Kumar (1979)</td>
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<tr>
<td>Colon</td>
<td>90-7</td>
<td>5-6</td>
<td>3-7</td>
<td>1186</td>
<td>73</td>
<td>49</td>
<td>DIF</td>
<td>Rognum et al (1979)</td>
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<tr>
<td>Colon</td>
<td>81-4</td>
<td>6-5</td>
<td>12-2</td>
<td>790</td>
<td>63</td>
<td>118</td>
<td>IPT</td>
<td>Rosekrans et al (1980)</td>
</tr>
<tr>
<td>Colon</td>
<td>80-9</td>
<td>7-0</td>
<td>12-1</td>
<td>806</td>
<td>70</td>
<td>121</td>
<td>IPT</td>
<td>Otto and Gebbers (1981)</td>
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<tr>
<td>Colon</td>
<td>81-1</td>
<td>13-2</td>
<td>5-7</td>
<td></td>
<td></td>
<td></td>
<td>PAP</td>
<td>Regadera et al (1983)</td>
</tr>
<tr>
<td>Colon</td>
<td>54-6</td>
<td>10-6</td>
<td>34-8</td>
<td>707</td>
<td>101</td>
<td>195</td>
<td>PAP</td>
<td>Present study</td>
</tr>
</tbody>
</table>

*Density of immunocytes in lamina propria (cells/mm² section area).
†Some of the data from other laboratories have been recalculated to make comparison possible.
‡DIF=direct immunofluorescence technique; PAP=peroxidase-antiperoxidase technique; IPT=indirect immunoperoxidase technique.
Italicised figures refer to studies by the authors’ laboratory.
Ig production in appendix and colon mucosa

fluorescence. A Ploem-type epi-illuminator provided narrow band excitation light and selective filtration of the contrasting emission colours. The tissue sections were mounted in polyvinyl alcohol (PVA) containing PPD (0.2-2.0 g/l). The sections were remounted in PVA alone when storage was required after the first counting (which took place within three days after the initial mounting).

All cell enumerations were carried out by the same observer throughout the study and based on the use of a x40 Leitz immersion objective. Definitive cell profiles showing bright cytoplasmic staining were counted and also more faintly fluorescent cells with purely green or red colour and a distinct nucleus.

IMMUNOCYTE ENUMERATION AND HISTOMORPHOMETRY

Cell enumerations were based on a lamina propria section area at full height (from the muscularis mucosae to the surface epithelium), limited by a distinct crypt at each side and including the domes of lymphoid follicles. A grid (Leitz code No. 519902) representing 0.032 mm² was moved systematically throughout this area which included on an average 26 grids. A sufficiently large area was always included to ascertain a stabilised mean number of IgA cells/grid (Fig. 1). The mean was usually stabilised at about 20 grids (95% confidence interval, 16–25 grids).

After completion of the cell enumerations, the cover slip was removed and the section was stained with haematoxylin and eosin (H & E). A pencil tracing of the actual lamina propria area was then made with a Leitz Orthoplan microscope equipped with a Leitz drawing device and a x40 objective. The included area was determined by means of a Kontron MOP-AMO 3 instrument (Munich, GFR).

DATA TREATMENT AND STATISTICAL METHODS

The density of IgA and IgG immunocytes was determined in two sections with reversed fluorescence colours (Table 2) and the mean values were used for these isotypes (When the counting was repeated, each mean was based on four determinations). The density of IgM immunocytes, however, was calculated by extrapolation from the IgA/IgM-cell ratio determined in the third section (Table 2).

The statistical analyses were performed as two-tailed tests. Differences were considered significant when p<0.05. For estimation of location parameters, the median with 95% confidence interval (95% CI) was used. Confidence interval was calculated using the Bernoulli-Wilcoxon procedure. For evaluation of differences between the first and the second series of cell enumerations, the Wilcoxon's signed midrank test was used. Comparisons between groups were based on the Wilcoxon's midrank sum test. For testing differences in dispersion between the first and second enumeration,

Fig. 1 Influence of the total number of grids evaluated on the estimated mean number of IgA-producing cells in three specimens from appendix and three from colon mucosa. The means are stabilised at about 20 grids.
the Sandvik's test with correction for ties was used.23

**Reproducibility**

Enumerations were repeated blindly in the same sections after four to six weeks for the first five samples from both appendix and colon. The median values for IgA cells were virtually identical whereas those for IgG cells tended to be slightly although statistically significantly (p<0.02) lower and less dispersed in the second enumeration (Table 3).

**Results**

**Overall Lamina Propria**

The median density of IgA-producing immunocytes in the lamina propria between the follicles of the appendix (1259 cells/mm²; 95% CI, 833–1545) tended to be higher than in the colon (962 cells/mm²; 95% CI, 695–1435), but there was no significant difference (Fig. 2). The same held true for IgM immunocytes (71 cells/mm²; 95% CI, 24–128 vs 55 cells/mm²; 95% CI, 33–88). IgG immunocytes, on the other hand, showed in the appendix a density (95 cells/mm²; 95% CI, 61–251) that was more than twice as high as (p<0.02) in the colon (38 cells/mm²; 95% CI, 11–61).

The numbers of IgM immunocytes in the nine paired samples of appendix and colon were significantly correlated (r=0.71; p<0.02) whereas no such relationship was revealed for IgA (r=0.28) or IgG immunocytes (r=−0.18).

IgD immunocytes were virtually absent in both mucosal tissues. In one patient there was a density of eight IgD-producing cells/mm² in the appendix and 24 such cells/mm² in the colon, whereas in the remaining samples we found only one cell.

When ignoring IgD immunocytes, the percentages of IgA-, IgM- and IgG-producing cells in the appendix were 87.3%, 5.6% and 7.0%, respectively (Fig. 3). In the colon the same figures were 90.2%,

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Table 3  Reproducibility of immunocyte enumeration (cells/mm²) in tissue sections from the appendix (n=5) and colon (n=5)

<table>
<thead>
<tr>
<th>Immunocyte isotype</th>
<th>First enumeration</th>
<th>Second enumeration</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgA</td>
<td>Median: 1258</td>
<td>1249</td>
</tr>
<tr>
<td></td>
<td>95% confidence interval: 743–1636</td>
<td>724–1644</td>
</tr>
<tr>
<td></td>
<td>Observed range: 658–1734</td>
<td>670–1731</td>
</tr>
<tr>
<td>IgG</td>
<td>Median: 44</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>95% confidence interval: 9–247</td>
<td>8–240</td>
</tr>
<tr>
<td></td>
<td>Observed range: 0–390</td>
<td>0–365</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Density of Ig-producing cells in appendix and colon mucosa. Medians (horizontal solid lines) and 95% confidence interval (horizontal broke lines) are indicated.

**Fig. 3.** Median isotype percentages of Ig-producing cells in appendix and colon mucosa. Vertical bars indicate the 95% confidence intervals.
4-6%, and 3-0%, the proportion of IgG cells being significantly lower than in the appendix (p<0.05).

FOLLCLE ASSOCIATED LAMINA PROPRIA
In the 0.1 mm zone adjacent to lymphoid follicles in the appendix mucosa including the domes, there was an increased proportion of IgG-producing immunocytes compared with the more distant lamina propria (Fig. 4); almost 50% of the immunocytes in this zone were IgG-positive (Table 4). IgG immunocytes likewise tended to be enriched adjacent to lymphoid follicles in the colon mucosa but it contained too few such structures for performance of a systematic study.

Table 4  Isotype ratio (IgA/IgG) of Ig-producing immunocytes in appendix mucosa

<table>
<thead>
<tr>
<th>Overall lamina propria (n=12)</th>
<th>Zone (0.1 mm) adjacent to lymphoid follicles (n=11)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median ratio:</td>
<td>10.3</td>
</tr>
<tr>
<td>95% confidence interval:</td>
<td>5.3–17.4</td>
</tr>
<tr>
<td>Observed range:</td>
<td>2.8–31.3</td>
</tr>
</tbody>
</table>

*In one sample it was technically impossible to evaluate this zone.

Discussion

METHODOLOGICAL CONSIDERATIONS
This study was partly undertaken to establish a method for direct determination of mucosal immunocyte densities. Like most previous studies (Table 1), our work was based on fluorescence microscopy. Valnes et al.24 have shown that there are only minor discrepancies between immunofluorescence and immunoperoxidase staining as to the enumeration of Ig-producing cells. The unacceptably large variations appearing in Table 1 cannot, therefore, be ascribed to differences in staining methods per se. We have found that the most reliable tissue preparation method to reveal all isotypes of Ig-producing cells, is the prewashing-and ethanol fixation procedure25 used in the present and all of our previous studies. In addition, we have based our immunocyte counts on paired immunofluorescence staining that affords internal control of false-positive cells15 20 and makes it possible to examine simultaneously in the same section the
inter-relationship between different classes of immunocytes.

In our laboratory previous studies of intestinal immunocyte populations have been related to a "tissue unit" that constitutes a 6 μm thick and 500 μm wide mucosal block at full height from the muscularis mucosae to the lumen. The included area of lamina propria will therefore vary among different specimens; the total immunocyte number per "tissue unit" depends both on this variable and on the cell density. An average measure of the total local immunocyte response is thereby obtained, and in our opinion this is necessary for well founded evaluation of disease-associated changes. Comparisons based solely on cell density may provide false information to this end, a fact also pointed out by Skinner and Whitehead.

"Tissue unit" recording was difficult to apply on the appendix mucosa, however, because of its content of numerous lymphoid follicles. We therefore developed a method to determine the immunocyte density of the lamina propria in a representative manner, including all layers of the mucosa. The data obtained were remarkably similar to those reported previously by our laboratory for normal large bowel mucosa, both with regard to cell densities and isotype proportions (Table 1).

The direct counting method is less time consuming than photographic recording of fluorescent cells. Nevertheless, it is a subjective choice at what brightness a cell should be considered an Ig-producing immunocyte. This difficulty, in addition to other obvious variables, must be taken into account when comparing the results of various authors. The largest variations appearing in Table 1, however, remain unexplained. It is obvious that workers in this field should pay more attention to the methodological problems inherent in immunohistochemical enumeration of Ig-producing cells.

BIOLICAL CONSIDERATIONS

Studies of the rabbit appendix have suggested that it plays an immunological role in the mucosal defence system. Lymphoid follicles are absent at birth and fail to develop after early ligation of the appendix. Elimination of the flora by appendicostomy and sterilisation of the lumen causes rapid degeneration of the follicles.

It seems justified to assume that the lymphoid follicles of the appendix are analogous to the Peyer's patches in having the capacity to generate IgA-cell precursors that migrate via lymph and blood to the distant gastrointestinal lamina propria. There is evidence to suggest that gastrointestinal immunocytes of other isotypes than IgA likewise originate in lymphoepithelial structures. The preferential accumulation of IgG-producing cells adjacent to the lymphoid follicles, as shown in this study, indicates that most of these immunocytes represent clones that tend to undergo terminal maturation locally rather than after emigration. Parrot has suggested that some migration of blasts takes place directly from Peyer's patches to nearby crypt regions; these blasts may mainly end up as IgG immunocytes in the follicle associated mucosal zone.

Local production of IgG has been demonstrated in Peyer's patches of rats and mice, and we have recently shown that there is a remarkably high proportion (38%) of IgG immunocytes among the Ig-producing cells found in the dome area of human Peyer's patches. It is worth noting that these IgG immunocytes show much poorer J-chain expression than their counterparts in distant lamina propria (37% vs 82%), and a similar trend is seen for the IgA-producing cells present at these two sites (85% vs 97% J-chain positivity). Relatively decreased expression of J chain is likewise a characteristic of the IgG-producing cells that accumulate preferentially adjacent to solitary follicles elsewhere in the gut and particularly in the appendix. Accumulation of J chain-negative IgG- and IgA-producing cell is still more striking in the tonsils, however.

Altogether, it seems that stimulated B cells with down-regulated J chain-expressing potential tend to remain locally. They apparently belong to mature memory clones and therefore express mainly IgG, whereas most members of relatively early memory clones probably emigrate from the lymphoepithelial structures after stimulation and differentiate to J chain-positive immunocytes at distant mucosal sites.

We found that the proportion of IgM immunocytes was similar in colon and appendix mucosa. Söltof et al. reported 6-5% IgD immunocytes in the rectum, but like Crabbé and Heremans and Brown et al. we found almost no IgD-producing cells in the intestinal lamina propria. By contrast, salivary, nasal, and lacrimal glands contain a significant proportion of such cells, both normally and especially in selective IgA deficiency. We have proposed that the tonsils and other lymphoepithelial structures of the upper aeroalimentary tract may supply glandular sites of this region with immunocyte precursors. There is no evidence to suggest whether the appendix preferentially supply a certain gut segment with such precursors, but experiments in rats have indicated that lodging of IgA immunocytes takes place mainly within the segment where they have been generated—that is, in the duodenum or in the colon.

We have found that normal human appendix
mucosa contains relatively more IgG-producing cells than the colonic counterpart. This difference can be ascribed to preferential accumulation of IgG immunocytes adjacent to the numerous lymphoid follicles in the appendix. We propose that this accumulation reflects local terminal maturation of B cells belonging to relatively mature memory clones. Generation of such clones in lymphoepithelial structures may depend on the magnitude of topical immunological stimulation and apparently increases in the order Peyer’s patches, appendix, and tonsils. The potency of a lymphoid tissue to function as precursor source of relatively immature B-cell clones that can emigrate and seed secretory sites may hence be reversely related to the local IgG response.

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References


