Related IgA1 and IgG producing cells in blood and diseased mucosa in ulcerative colitis

V C Thoree, S J C Golby, L Boursier, M Hackett, D K Dunn-Walters, J D Sanderson, J Spencer

Gut 2002;51:44–50

Ulcerative colitis (UC) is a chronic, relapsing, organ specific, inflammatory disease of the colon which tends to be restricted to the mucosa. In areas of chronic inflammation in UC, the inflammatory infiltrate includes an increase in mucosal plasma cells. Although plasma cells secreting all isotypes are increased in UC, the population with the greatest percentage increase is that secreting IgG, predominantly IgG1. In addition, the ratio of IgA1:IgA2 secreting plasma cells increases.

The higher IgA1:IgA2 ratio and increased proportion of IgG producing cells are characteristic of the peripheral humoral response. Evidence suggests that proinflammatory complement fixing IgG autoantibodies, which may arise through cross reactivity with bacterial antigens, are involved in the pathogenesis of UC.

IgG antibodies to organ specific autoantigens such as colonic epithelial cells, and mucosal production of more broadly distributed autoantigens such as antineutrophil cytoplasmic antibodies, are consistently identified in the colon in UC. It is not known whether this IgG response originates in the periphery or in the mucosa. It is possible that mucosal IgG in UC represents a local mucosal response which has switched to IgG or a peripheral response which may have been initiated by peripheral antigen which homed to the colonic mucosa. The clonal distribution of IgG secreting cells and isotype switched variants in UC is not known.

Aims: To investigate the clonal distribution of mucosal IgG in UC and to search for related IgG and IgA secreting cells in normal and diseased mucosa and blood in UC. To investigate characteristics which may discriminate between the mucosal and peripheral repertoire in the normal mucosa and in UC.

Patients: Blood and normal and diseased mucosa from two patients with UC were studied.

Methods: Immunoglobulin gene analysis and clone specific polymerase chain reaction were used to study the clonal distribution and characteristics of IgG and related IgA in the mucosa and blood of patients with UC.

Results: The IgG response in the mucosa of UC patients included widespread clones of cells that were present in both the diseased mucosa and blood but that were scarce in normal mucosa. Clonally related IgA class switch variants, all IgA1, were detected but also only in the diseased mucosa and blood. This suggests that these clones home preferentially to the diseased mucosa. We showed that J51 usage was characteristic of the peripheral repertoire, and that examples of J51 usage were observed in mucosal IgG in UC.

Conclusions: Overall, these data are consistent with a model of UC in which a peripheral response is expressed and expanded in the colonic mucosa.

Background: Ulcerative colitis (UC) is a chronic inflammatory bowel disease in which the colorectal mucosa is infiltrated with plasma cells producing IgG autoantibodies. It is not known whether this represents a local mucosal response which has switched to IgG or a peripheral response which may have been initiated by peripheral antigen which homed to the colonic mucosa. The clonal distribution of IgG secreting cells and isotype switched variants in UC is not known.

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small family with a bias towards J6 usage in UC, although earlier studies using microdissection could not determine whether this was isotype restricted. V1 and V3 were studied because of their common usage and because V3 is preferentially used by one group of autoantibodies in UC. The distribution of IgA subclasses in UC has previously been analysed with a bias towards JH6 usage in UC, although it has been shown that within this population, it is vitally important to control for the variable and constant region usage when analysing hypermutation and J region usage. Therefore, control IgM and IgA sequences using V5 from another study of mucosal plasma cells were used, which were isolated from human duodenum. IgG sequences using V5 were generated from the same tissues specifically for use in this study.

### Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from colonic mucosa or PBLs using 1 ml of TRIzol reagent (Life Technologies, Paisley, UK) according to the manufacturer’s instructions. One fifth of the total RNA yielded was used for synthesis of single strand cDNA using oligo-dT primer and M-MuLV reverse transcriptase (Promega, Southampton, UK) in a final volume of 20 μl.

### PCR amplification of IgV,D,Jg,Cg, transcript

All reactions were performed using a Hybaid Omnigene PCR machine. The sequences of all PCR primers used for the amplification of Ig heavy gene segments are shown in table 1. IgV,D,Jg,Cg sequences were amplified using a semi-nested PCR strategy. A 5 μl sample of the 20 μl reverse transcription product was amplified using 0.5 units of Taq DNA polymerase (Promega) in a 50 μl PCR reaction using 100 ng of each primer, 200 μM of each dNTP and 1.5 mM MgCl2 in 1×Taq DNA polymerase reaction buffer. A hot start of 94°C for seven minutes was performed before addition of the Taq DNA polymerase. For the first round of PCR, 30 cycles of 40 seconds at 94°C, 45 seconds at 60°C, and two minutes 40 seconds at 72°C were performed, followed by an additional five minute extension of the PCR products at 72°C. For the second round of PCR, 30 cycles of 40 seconds at 94°C, 45 seconds at 55°C, and two minutes 40 seconds at 72°C were performed, followed by an additional five minute extension of the PCR products at 72°C. The second round PCR product was used as template DNA. PCR primers were manufactured by Genset SA (Paris, France) or Interactiva Biotechnologie GmbH (Ulm, Germany).

### Cloning and nucleotide sequencing

PCR products were analysed on a 3.5% NuSieve 3:1 agarose gel (Flowgen, Lichfield, UK) and stained with ethidium bromide. PCR products were cloned into the pGEM-T Vector (Promega), sequenced on both strands using the dye terminator cycle sequencing kit (PE Applied Biosystems, Warrington, UK), and analysed with an ABI 377 automated DNA sequencer (PE Applied Biosystems). Some of the Ig genes were sequenced by Qiagen GmbH (Hilden, Germany) or MWGAG Biotech (Ebersberg, Germany). Vg and J gene segment sequences were analysed using GeneJockey II software and the V BASE Sequence Directory which contains all known human germline heavy and light chain gene segments. J gene segments were assigned manually. An Ig gene rearrangement was considered productive if the V(D)J junction maintained the reading frame into the salivary gland.

### MATERIALS AND METHODS

#### Human tissues used

Samples of colonic mucosa from two patients with UC undergoing total colectomy were received in the laboratory within an hour of surgery. Patient No 1 was a male aged 23 years and patient No 2 a female aged 64 years. The samples were immediately snap frozen in liquid nitrogen and stored until required. In addition, a peripheral blood sample was obtained from each patient on the day of surgery. Peripheral blood lymphocytes (PBLs) were isolated using Ficoll-paque research grade (Amersham Pharmacia Biotech Ltd, Little Chalfont, UK). Some of the Ig genes were sequenced by GeneJockey II software and the V BASE Sequence Directory which contains all known human germline heavy and light chain gene segments. J gene segments were assigned manually. An Ig gene rearrangement was considered productive if the V(D)J junction maintained the reading frame into the salivary gland.

### Table 1

<table>
<thead>
<tr>
<th>Oligonucleotide primers</th>
<th>Sequence (5’ to 3’)</th>
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<tr>
<td>External 5’ primers*</td>
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<td>V1 leader</td>
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</tr>
<tr>
<td>V3 leader</td>
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</tr>
<tr>
<td>V5 leader</td>
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<tr>
<td>Internal 5’ primers†</td>
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<tr>
<td>V1-FR1</td>
<td>CTTCAGTGAGTTGTTCTGCAAGG</td>
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<tr>
<td>V3-FR1</td>
<td>GTGTCCTCGAGCTCTCTGTCAG</td>
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<td>V5-FR1</td>
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<td>3’ Cµ primers</td>
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<tr>
<td>Cµ3</td>
<td>CACCCTGAGGGGAA</td>
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</table>

*External 5’ primers hybridise to the 5’ leader sequence. †Internal 5’ primers hybridise to the framework region 1.

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>Patient No 1</th>
<th>Patient No 2</th>
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<td>Mucosa</td>
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<td>VH3</td>
<td>VH5</td>
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<td>IgG</td>
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<tr>
<td>Total</td>
<td>24</td>
<td>26</td>
<td>44</td>
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</tbody>
</table>

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the J segment (inframe) and there were no translational stop codons. Where the reading frame was not maintained into the J gene segment, the rearrangements were designated out of frame. Nomenclature according to the V BASE Sequence Directory has been used. When a group of related genes was found, the mean number of mutations for that group was used in the analysis of the overall frequency of somatic mutations. Because of the nature of the internal VH1 FR1, VH3 FR1, and VH5 FR1 PCR primers used for amplification, somatic mutations could not be detected in the first 70 nucleotides, 69 nucleotides, and 56 nucleotides of the FR1 regions of IgVH1, IgVH3, and IgVH5 gene segments, respectively.

**Design of B cell clone specific CDR3 5’ PCR primers**

Criteria used for designing the 5’ CDR3 primers were: PCR primer length equivalent or above 16 bp, high GC base composition (>60%), lack of homology with either the CH antisense 3’ primer or with any other mammalian sequences (as determined by searching GenBank), lack of internal repeat, and a C or a G at the 3’ end of the primer when possible. It was also essential that the clone specific sequence was present 3’ of the primer before JH to allow confirmation that the PCR was clone specific by sequencing. Negative controls were first round PCR product IgVH4DJH-CH amplified from a PBL or tonsil from at least five different patients.

**Statistical methods**

Comparisons of J gene segment usage were carried out using \( \chi^2 \) tests. All other data were tested for normality using Microstat software. As the populations compared were normally distributed, arrays were compared using the Student’s \( t \) test. Observed differences were considered to be statistically significant at \( p < 0.05 \).

**RESULTS**

A total of 230 sequences were analysed. Details of the number of different heavy chain sequences and rearrangements studied are shown in table 2. We analysed 183 different sequences from two patients with UC, 138 from the mucosa and 45 from blood. Some of these sequences shared the same CDR3 but had single base differences in the V region and could therefore be considered to be part of the same clone which had diversified by somatic hypermutation. The asterisk identifies the RNA splice site and the sequence after the asterisk represents the appropriate constant region sequence. The primers are in bold italics. The underlined bases in the constant region sequence identify the nucleotides that enable the classification of the IgA sequences as IgA1 subclass.

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**Identification of related sequences**

Within the related groups of cells sharing the same CDR3, some used different constant regions. Related IgM and IgA1 sequences from the same block of diseased mucosa from patient No 2. IgM–1 is related to IgA1–1, sharing the same CDR3, and IgA–2 is related to IgG–2, sharing the same CDR3. Each pair of sequences has been diversified by both class switch recombination and somatic hypermutation. All four sequences use VH5–1 and J4.8. Dashes indicate identity of the sequences to germline. Upper case letters in the alignments represent replacement mutations and lower case letters silent mutations from germline. The asterisk identifies the RNA splice site and the sequence after the asterisk represents the appropriate constant region sequence. The primers are in bold italics. The underlined bases in the constant region sequence identify the nucleotides that enable the classification of the IgA sequences as IgA1 subclass.

**Figure 1**

Alignment of two sets of related sequences identified by CDR3 homology from the diseased mucosa from patient No 2. IgM–1 is related to IgA1–1, sharing the same CDR3, and IgA–2 is related to IgG–2, sharing the same CDR3. Each pair of sequences has been diversified by both class switch recombination and somatic hypermutation. All four sequences use VH5–1 and J4.8. Dashes indicate identity of the sequences to germline. Upper case letters in the alignments represent replacement mutations and lower case letters silent mutations from germline. The asterisk identifies the RNA splice site and the sequence after the asterisk represents the appropriate constant region sequence. The primers are in bold italics. The underlined bases in the constant region sequence identify the nucleotides that enable the classification of the IgA sequences as IgA1 subclass.
observed in the same block of diseased mucosa from patient No 2. These sequences shared seven common mutations from the germline. In addition, IgA and IgG had acquired a further seven and 10 mutations, respectively, after the switch event (fig 1). Identical IgM sequences were observed in blood and mucosa from patient No 2, which shared 13 mutational differences from the germline VH5 sequence from which they originated.

The frequency of V region hypermutation in the VH5 sequences of each isotype in mucosa in UC (data from patient Nos 1 and 2 pooled) and in control mucosa is illustrated in fig 2. In UC, as previously observed in normal mucosa, the frequency of mutation in IgVH used by IgM was significantly lower than that observed in IgVH used by IgA or IgG (p=0.03 and p=0.004, respectively, using a t test). There was no significant difference in the frequency of hypermutation in UC and normal mucosa when comparisons within isotypes were made.

JH usage by IgM, IgA, and IgG in the mucosa and blood in UC

Rearrangements using JH1 were observed in mucosal IgG in this study (fig 3). This is the first time that JH1 has been observed in any sample, consisting largely of mucosal plasma cells. JH1 was not observed in IgM or IgA from the same patients. In the gut, JH6 was clearly and significantly associated with IgG compared with IgM or IgA in cells using VH5 in patient No 1 (p=7.8×10⁻⁶ and 0.0005 by χ² for IgG v IgA and IgG v IgM, respectively), and was also significantly higher in IgG than in IgM in blood (p=0.009). However, when VH1 and VH3 were also studied in patient No 1, this trend towards JH6 usage specifically by IgG was not apparent (fig 3).

Analysis of clonal distribution of IgG secreting cells and their isotype switch variants in UC

Out of a total of 11 putative clone specific primers tested, designed for specific identification of 11 different IgG secreting clones, seven detected clones against which they were raised reliably, which could be confirmed by sequencing, and were negative in five other preparations of PBLs or tonsils from other individuals.

Patient No 1

In patient No 1, four samples of mucosa (two samples of diseased mucosa and proximal and distal resection margins) and PBLs were analysed. The distribution of cells related to four IgG secreting cells from a sample of diseased mucosa was analysed using 5′ clone specific heavy chain CDR3 primers used together with 3′ primers in either Cγ or Cα to identify cells which were related to the original Ig sequence which were either IgG or IgA. The distribution of related IgG and IgA+ cells are shown in table 3.

All four IgG secreting clones were present in both available blocks of diseased mucosa in this patient. They were each also
detected in one of the two blocks of normal mucosa, and three of the four were identified in blood. IgA switch variants of each clone were detected in the diseased mucosa and blood but not in the normal mucosa. Identity was confirmed by sequencing (fig 4). The exclusive use of IgA1 in the switch variants is compared with the overall frequency of IgA subclasses observed by sequence analysis in the blood and mucosa in these patients and normal controls (table 4).

**Table 3** Distribution of IgG clones and related IgA switch variants using CDR3 clone specific primers

<table>
<thead>
<tr>
<th>Sample</th>
<th>IgG-clone 1</th>
<th>IgG-clone 2</th>
<th>IgG-clone 3</th>
<th>IgG-clone 4</th>
<th>IgG-clone 5</th>
<th>IgG-clone 6</th>
<th>IgG-clone 7</th>
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<td>-</td>
</tr>
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</table>

*Sample from which the original IgG sequences were derived.
†No IgA+ cells related to IgG were detected by this method in patient No 2.
+, strong unambiguous band; +/-, weak band.
NT, no tissue: two blocks of each of normal and diseased mucosa only for patient No 1.

**Figure 4** Confirmation of clonal relationship between genes identified by CDR3 specific primers in patient No 1 by sequencing. IgG clone 1, the original sequence against which the primers were designed, is aligned to V,32 and J,6. Aligned to this are the sequences of the polymerase chain reaction products generated using the clone specific primers. Samples tested were two areas of diseased mucosa designated sample 1 and sample 2, and peripheral blood lymphocytes (PBL). After the RNA splice site, marked with an asterisk, the products generated with the Cγ primer were aligned to the Cγ sequence and products generated with the Ca primer were aligned to Ca. The bases that determine the IgA1 subclass designation are underlined. The related IgG and IgA sequences all share considerable homology in the CDR3 before they begin to align with J,6. There was a single base difference in the related IgG genes which do not have the T to C mutation. The positions of the primers are shown in bold italics.

**Patient No 2**

In patient No 2, eight samples of mucosa and a sample of blood were available for study (table 3). Of the eight samples of mucosa, four were of diseased colon, taken at approximately 10 cm intervals. The additional samples were of normal colon, caecum, and two samples of the normal ileum. Three clones of IgG originally identified in the most proximal sample of disease mucosa could be traced using clone specific primers. All three clones were present in all of the blocks of diseased mucosa and two of the three were identified in blood. The
IgG and IgA expressing cells did exist in this patient because detected in this patient using CDR3 primers. However, related specific and therefore IgA switched variants could not be vascular adhesion protein 1 by endothelium in areas of assays, and expression of peripheral node addressin and with the observed ability of mucosal lymphoid cells in UC to migrate via blood and home specifically to the site of disease in suggest a model of UC in which the IgG secreting clones of plasma cells that home to mucosal sites. These observations observed are not part of the normal population of precursors mucosa from the patient suggests that the IgG secreting cells However, the fact that they were relatively rare in normal blood and the presence of these cells in blood per se does not same individual were studied. Mucosal cells traffic via the and the presence of these cells in blood per se does not allow us to conclude that they are of peripheral origin. However, the fact that they were relatively rare in normal mucosa from the patient suggests that the IgG secreting cells observed are not part of the normal population of precursors of plasma cells that home to mucosal sites. These observations suggest a model of UC in which the IgG secreting clones migrate via blood and home specifically to the site of disease in UC but not to the normal mucosa. This scenario is consistent with the observed ability of mucosal lymphoid cells in UC to bind to both mucosal and peripheral venules in adherence assays, and expression of peripheral node addressin and vascular adhesion protein 1 by endothelium in areas of diseased mucosa in UC. These observations together suggest that the barriers that normally exclude plasma cell precursors of peripheral origin from homing to the gut are broken down in UC, allowing invasion of the mucosa with cells producing proinflammatory immunoglobulins with potential to cross react with components of the flora and mucosal autoantigens.

Assuming that migration of the aberrant IgG expressing population observed in UC is regulated by receptor ligand systems that facilitate the movement of cells into peripheral sites, these plasma cells would also be expected to localise to peripheral sites. There is evidence for this in that patients with UC may also suffer from peripheral antibody mediated problems including arthritis, autoimmune thyroid disease, and autoimmune haemolytic anaemia. The observation of related IgG secreting cells in diseased mucosa and blood in UC does not necessarily lead to the conclusion that this response is of peripheral origin as mucosal responses are known to disseminate via blood. However, exclusion of these cells from normal intestinal mucosa suggests that these cells are not components of a normal mucosal response. To pursue this question further, we examined other features that discriminate between the mucosal and peripheral B cell responses. There is a difference in the relative use of the IgA subclasses in the peripheral and mucosal repertoires; IgA, being the dominant subclass in the periphery. We were interested to identify and characterise any IgA switch variants of the IgG+ clones. An example of related IgG1 and IgA1 sequences was identified by alignment of CDR3 sequences. These shared seven mutations but were individually diversified. The IgA sequence may have arisen by isotype switching from the IgG sequence as the α constant region is 3′ of γ. Alternatively, they may have both arisen from the same IgM+ precursor as the Vγ of IgM may undergo hypermutation before the class switch event. The fact that we were able to identify these clonally related sequences suggests that this clone is relatively common. Four further IgA sequences, all IgA1, which were clonally related to IgG, were identified using clone specific primers. Like the IgG+ clones, these were identified in the diseased mucosa and blood only, suggesting that their migration is restricted by the same criteria. The fact that all IgA sequences related to IgG were IgA1 is evidence that this response is peripheral in origin. The Ig genes isolated from inflamed mucosa were not from blood, either through contamination or from blood vessels, because the Ig secreting tissue plasma cells and their immediate precursors have substantially more RNA than cells in blood. Thus plasma cells contribute most substantially to total RNA. Consistent with this, as discussed below, the IgM sequences from these preparations were mutated. If they were derived from blood, many would be unmutated sequences from the virgin peripheral blood B cell population.

It is now apparent that Jμ segment usage varies between the mucosal and peripheral repertoires. We have accumulated published data on Jμ1 usage for comparison with Jμ1 usage in

<table>
<thead>
<tr>
<th>Table 4</th>
<th>Analysis of IgA subclasses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotype</td>
<td>Control mucosa</td>
</tr>
<tr>
<td>---------</td>
<td>----------------</td>
</tr>
<tr>
<td>IgA1</td>
<td>8</td>
</tr>
<tr>
<td>IgA2</td>
<td>10</td>
</tr>
<tr>
<td>%IgA1</td>
<td>44</td>
</tr>
</tbody>
</table>

UC, ulcerative colitis.

<table>
<thead>
<tr>
<th>Table 5</th>
<th>Usage of Jμ1 in mucosal and peripheral repertoire</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral</td>
<td>Mucosal</td>
</tr>
<tr>
<td>No of Jμ1s/No of rearrangements in sample (%)</td>
<td>Isotype sampled</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>1/17 (5.9)</td>
<td>IgM</td>
</tr>
<tr>
<td>1/15 (6.7)</td>
<td>IgA</td>
</tr>
<tr>
<td>1/18 (5.6)</td>
<td>IgG</td>
</tr>
<tr>
<td>1/29 (3.4)</td>
<td>IgA</td>
</tr>
<tr>
<td>1/99 (1)</td>
<td>ND</td>
</tr>
<tr>
<td>1/71 (1.4)</td>
<td>ND</td>
</tr>
<tr>
<td>3/235 (1.3)</td>
<td>IgM</td>
</tr>
<tr>
<td>0/84 (0)</td>
<td>ND</td>
</tr>
<tr>
<td>Grand total 9/568*</td>
<td></td>
</tr>
</tbody>
</table>

SG, salivary gland.

*χ² comparisons: 9/568 vs 0/324, p=0.023.
IgG in UC (table 5). All of the heavy chain sequences from the mucosal repertoire analysed were isotype switched or mutated, or both, and had therefore probably all undergone selection for specificity or functional viability at some time in their history. None used JH1. When the population of B cells from the blood was considered as a whole, which includes naïve cells and memory B cells, JH1 usage was observed. In UC, three examples of JH1 usage were observed, all in association with IgG. This is consistent with selection of the IgG in UC according to the criteria that shape the repertoire in the periphery.

We have previously observed that J6 is preferentially associated with the plasma cell repertoire in UC. It has since been implied by analysis of IgA producing cells that J6 is associated with the peripheral immune response. We have therefore examined J6 usage in three different VH families, in the mucosa and blood in UC. There was a clear and significant association between J6 and IgG in V\(_{\mu}5\) expressing B cells in both patients studied, but not in genes using V\(_{\mu}1\) or V\(_{\mu}3\). Consistent with this, there was no evidence of JH6 bias in V\(_{\mu}3\) genes used by anticolon antibodies in UC in another study. Although the bias towards J6 usage in IgG in UC is not common to all families, the fact that this bias was clear in V\(_{\mu}5\), and has been associated with the peripheral repertoire in another study, is consistent with the concept that the IgG originated from a peripheral response.

Analysis of hypermutation showed that, as in normal bowel, all isotypes were predominantly highly mutated, with IgA and IgG significantly more highly mutated than IgM. None of these populations in UC differed significantly from isotype matched controls from normal mucosa. It has been shown previously that IgG genes used by peripheral B cells have a lower frequency of mutation than those used by mucosal B cells. In a sample of 13 IgG sequences from the spleen that used V\(_{\mu}6\), Varade et al. showed that IgG sequences had an average of 14 mutations from germline (range 2–25), which is lower than that observed in the sample of IgG producing cells in normal bowel or those from the colon in UC described in this study. However, as the site of expansion of the IgG producing cells in UC is not known, it is possible that once localised in the gut, a response of peripheral origin might be subjected to the same mechanisms which induce the high load of mutations in V regions observed in the Ig genes used by mucosal plasma cells. It is possible that the IgG expressing cells that were identified in the blood and mucosa in this study arose via a clonal expansion in the mucosa, and were also destined for extravasation in the mucosa.

We therefore propose that the proinflammatory antibody response in the mucosa in UC includes clones of IgG/IgA1 cells which expand within and disseminate through the diseased mucosa via the blood. Evidence suggests that this response originates in the periphery. It is almost certain that the bacterial flora is involved in the pathogenesis of UC, and in animal models of disease, local T cell responses are also implicated. It is not clear therefore whether the recruitment of proinflammatory autoreactive immunoglobulins to the mucosa in UC is a primary cause of disease or a consequence of the other immunological imbalances. However, it is highly likely that the antibody response is a significant factor in the evolution of mucosal inflammation and peripheral symptoms in UC. This study has increased our understanding of how this occurs.

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**ACKNOWLEDGEMENTS**

This work was funded by grants from the National Association for Colitis and Crohn’s and Crohn’s in Childhood Research Association.

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Related IgA1 and IgG producing cells in blood and diseased mucosa in ulcerative colitis

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Gut 2002 51: 44-50
doi: 10.1136/gut.51.1.44

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