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Characteristics of Human IgA and IgM Genes Used by Plasma Cells in the Salivary Gland Resemble Those Used in Duodenum But Not Those Used in the Spleen

Deborah K. Dunn-Walters,* Margaret Hackett,§ Laurent Boursier,* Paul J. Ciclitira,† Peter Morgan,‡ Stephen J. Challacombe,§ and Jo Spencer2* 

Immunologically, the parotid salivary gland is an effector site that secretes large quantities of polyspecific Abs into the saliva, mainly of the IgA isotype (1). It is considered to be part of the common mucosal immune system (2), but the inductive site for the Ab-producing cells of the salivary gland has not yet been clearly identified. It has been suggested that the palateine and nasopharyngeal tonsils may be the inductive sites, because they may be the equivalent of the rodent nasal-associated lymphoid tissue (3), which is the inductive site for salivary glands in mice (4). It has also been shown that direct immunization of palatine tonsils in the rabbit results in specific IgA Ab-producing cells in the salivary gland (5). Nasopharyngeal IgA Ab levels have been shown to be decreased after tonsillectomy and adenoidectomy of young children (6). However, a more recent study of older pre- and postoperative tonsillectomy patients showed a decrease in specific IgG saliva Abs, but no significant change in IgA Abs, after removal of the tonsils (7). Duct-associated lymphoid tissue, which occurs in the ducts of both major and minor salivary glands, may also be an inductive site for the salivary glands (8, 9).

The diversity of cells of B lineage can be investigated by analyzing their Ig heavy chain genes (IgH). We have obtained sequences of IgM and IgA V\textsubscript{H}4–34 genes from plasma cells in human salivary gland, duodenal lamina propria, and splenic red pulp. Related sequences were found in different areas sampled within each tissue studied, indicating that the plasma cells carrying these genes are widespread with limited diversity. Examples of related Ig\textsubscript{H} genes that are isotype switched were also seen in the salivary gland. The genes from plasma cells of the salivary gland were highly mutated, as were duodenal plasma cell sequences. The level of mutation was significantly higher than that seen in splenic plasma cell sequences. Analysis of CDR3 regions showed that the sequences from salivary gland had significantly smaller CDR3 regions than sequences from spleen, due to differences in number and type of D\textsubscript{H} regions used. Sequences from duodenum also had smaller CDR3 regions. Therefore, plasma cells from human duodenum and salivary gland showed characteristics that differed from those of human splenic plasma cells. The Journal of Immunology, 2000, 164: 1595–1601.

The diversity of cells of B lineage can be investigated by analyzing their Ig heavy chain genes (Ig\textsubscript{H})\textsuperscript{10, 11}. The diversity of human Ig genes is achieved through the processes of gene rearrangement and somatic hypermutation. As Ig variable (IgV\textsubscript{H}), diversity (D\textsubscript{H}), and junctional (J\textsubscript{H}) gene segments rearrange in the bone marrow, additional nucleotides (N nucleotides) are added randomly in the junctions by the enzyme TdT. In this way, a unique junctional complementarity-determining region (CDR) sequence, CDR3, is created. Therefore, cells sharing the same CDR3 sequence are considered to be progeny of a single clone, which may have been amplified during a germinal center response (12). Ig\textsubscript{H}4 genes of B cells can also undergo somatic hypermutation after activation by Ag, thus naive B cells have germline Ig\textsubscript{H}4 gene sequences, whereas mutated Ig gene sequences are seen in cells that have been through a germinal center reaction (13, 14). The mean number of mutations seen in postgerminal center cells of the tonsil, spleen, and lymph node vary between 5.9 and 7.5 mutations per Ig\textsubscript{V}\textsubscript{H} gene (15). Ig\textsubscript{H} genes generally have higher numbers of mutations than IgG genes, which in turn have a higher number of mutations than IgM genes (16–18). We have previously found, in humans, that B cells and plasma cells from the duodenum, ileum, and colon carry Ig\textsubscript{H} genes that have a much higher level of mutation (10, 11, 17, 19). The reasons for the higher level of mutation are not yet known.

We have used RT-PCR, cloning, and sequencing to obtain sequences of IgM and IgA genes from plasma cells in human salivary gland. IgM and IgA genes were also obtained from plasma cells in human duodenal lamina propria and splenic red pulp, as examples of mucosal and systemic populations, for comparison. To examine the local diversity of Ab-forming cells, separate samples were taken from each tissue, and CDR3 regions were

1 Abbreviations used in this paper: Ig\textsubscript{H}, Ig heavy chain gene; CDR, complementarity-determining region.

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Table 1. Number and origin of IgV H gene sequences

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Patient No.</th>
<th>Sample</th>
<th>No. of IgA Sequences</th>
<th>No. of IgM Sequences</th>
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<tr>
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<tr>
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<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>d</td>
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<tr>
<td>IgM</td>
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Materials and Methods

Histologically normal parotid salivary gland was obtained from tissue surgically removed due to pleomorphic adenoma. Duodenal biopsies taken during investigations for coeliac disease, but found to be normal, were used. Histologically normal spleen was obtained alongside resections of bowel for carcinoma and perforation (patient 5) or from surgery due to trauma (patient 6). The spleen from patient 5 showed mild acute inflammation characteristic of a response to the flora from the perforated bowel. All samples were obtained fresh and were snap frozen and stored in liquid nitrogen until required. Samples from two patients for each tissue were used, and two or four samples were taken from each patient so that dissemination of cells could also be investigated (Table I).

RNA was made from ~1-1 mm³ quantities of each tissue, prepared by chopping the block while still frozen. Efforts were made to avoid sampling any organized lymphoid tissue; prior histological examination of sections from the same block for duodenum and salivary gland and selection of red pulp only for spleen. cDNA was prepared using Moloney murine leukemia virus reverse transcriptase and oligo(dT) primers. IgVH4-34–34 genes were PCR amplified using a seminested strategy as previously described (17). Briefly, 5’ primers specific for V H 4 family leader region (5’-ATGAAA-

Results

A total of 93 different sequences were obtained, of which 66 were unique V-D-J rearrangements. The remaining 27 sequences could be grouped into 11 families with the same CDR3 region, resulting in a total of 77 different V-D-J rearrangements. Of the 11 groups of related sequences identified (2 from duodenum, 3 from spleen, and 6 from salivary gland), 6 contained Ig genes isolated from different areas of tissue (Fig. 1), indicating that there is dissemination of related plasma cells or their precursors. Examples of this were seen in all three tissue types. In the salivary gland, related sequences associated with both Ca and Cb were identified in the different sites studied within each sample. All three of these genes were highly mutated, indicating that class switching can occur after diversification of the gene by somatic hypermutation.

Frequency of somatic hypermutation in IgV H genes from salivary gland

Fig. 2 shows the number of mutations in each gene of IgA and IgM isotype in all three tissues. There is a significant increase in the number of mutations in Ig genes that have switched to IgA, compared with the number of mutations in IgM genes. When comparing the frequency of mutation between genes from different tissues, but the same isotype, it can be seen that the mean number of mutations in IgM genes from spleen (6.7) is lower than that from salivary gland (18.3) and the duodenum (16.2). These differences are significant (p = 0.006 and p = 0.005, respectively). Similarly, IgA genes from spleen had a lower mean number of mutations (14.9) than IgA genes in salivary gland (28.9) and duodenum (21.7), although only the former difference reached significance (p = 0.0002). There were no significant differences in the number of mutations between isotype-matched IgV H genes from salivary gland and duodenum.

Size of CDR3 and NDN analysis

There was no difference in the mean length of CDR3 regions in IgV H genes of the two different isotypes in any tissue. Neither was there any difference in the mean CDR3 length of IgV H genes from salivary gland and duodenum. However, the mean CDR3 lengths of IgV H genes from both salivary gland and duodenum, for both isotypes, were significantly shorter than that of the spleen (Fig. 3).

Fig. 4 shows the results of analysis of all CDR3 regions in terms of length of J H and D H regions, number of N additions, and number of D H regions used. The increase in CDR3 size in spleen cannot be accounted for by an increase in N additions (Fig. 4a) because the mean number of N additions per Ig gene in the spleen is 6.0 bp, compared with 6.6 bp and 6.4 bp for Ig genes of duodenum and salivary gland, respectively. The mean sizes of J H region for duodenum, salivary gland, and spleen are 13.9, 11.4, and 15.6 bp, respectively (Fig. 4b). Only the difference between spleen and salivary gland is significant (p = 0.02). There is an increased usage of the largest J H segment, J H 6, in the spleen, which may account for this, although the difference is not significant (Table II). In contrast, the mean total length of D H gene segments per CDR3
FIGURE 1. Related IgH genes found in salivary gland, duodenum, and spleen. Sequences are shown aligned with the germline V_{H}4–34 gene and the appropriate germline J_{H} gene. Dashes indicate identity with the germline sequence, d signifies a deletion. The name of each sequence indicates its origin and type. The first number and letter refer to the patient and sample from which the sequence was isolated (Table I), the capital A or M refers to the isotype of the gene, and the final number and letter refer to the number of the particular gene rearrangement and sequence. Three groups of related sequences are shown from salivary gland in patient 1, one from patient 3 and one from patient 4 in duodenum, and one from patient 5 in spleen.
The size of CDR3 regions in Ig genes from duodenum, salivary gland, and spleen was examined. The mean values for IgA genes from salivary gland (n = 16), duodenum (n = 9), and spleen (n = 15) are shown, along with the values for IgM genes from salivary gland (n = 12), duodenum (n = 9), and spleen (n = 17). The p values given are the results of statistical comparison with the values for splenic genes of the same isotype. Some, but not all, of the differences can be accounted for by the use of a slightly larger number of D\(_{\text{H}}\) segments in CDR3 regions of Ig genes from spleen (mean number of 2.4 D\(_{\text{H}}\) segments per gene) compared with duodenum or salivary gland (mean numbers of 2.1 and 1.8 D\(_{\text{H}}\) segments per gene, respectively), as shown in Fig. 4d. Some D\(_{\text{H}}\) segments were incorporated into the CDR3 in the reverse orientation; the proportion of D\(_{\text{H}}\) segments that were reversed was the same in all three groups.

**FIGURE 2.** Numbers of point mutations per IgV\(_{\text{H}}\) gene in duodenum, salivary gland, and spleen. The number of point mutations in each IgV\(_{\text{H}}\) gene is indicated by an individual point on the graph. Values are grouped according to the tissue from which the genes were isolated (duodenum, salivary gland, spleen) and to the isotype of each gene (IgA or IgM). The bars indicate the mean values for each group.

**FIGURE 3.** Size of CDR3 regions in Ig genes from duodenum, salivary gland, and spleen. Mean values for the CDR3 size of IgA genes from salivary gland (n = 16), duodenum (n = 9), and spleen (n = 15) are shown, along with the values for IgM genes from salivary gland (n = 12), duodenum (n = 9), and spleen (n = 17). The p values given are the results of statistical comparison with the values for splenic genes of the same isotype.

**FIGURE 4.** Analysis of CDR3 regions in Ig genes from duodenum, salivary gland, and spleen. The mean values for (a) number of N additions, (b) length (in bp) of J\(_{\text{H}}\) region, (c) total combined length (in bp) of D\(_{\text{H}}\) segments, and (d) number of D\(_{\text{H}}\) segments used per Ig gene are shown for Ig genes from duodenum (n = 16), salivary gland (n = 24), and spleen (n = 31). * Significant difference in the spleen value compared with that of the salivary gland. ** The value for spleen is significantly different from those of both duodenum and salivary gland.
The DH regions used and the number of nucleotides used from each DH are shown in Table III. The DH segment D4 was significantly more common in spleen than gut \((p = 0.03)\). The D4 DH segments tended to be larger, which contributed to an overall greater average DH length in the spleen. The difference in D4 usage, the higher average number of DH regions used per gene, and the bias toward the use of JH6 are major factors resulting in the significantly longer CDR3 in the sequences isolated from splenic cells.

Discussion
This is the first study of IgH genes from plasma cells of the human salivary gland; IgH genes from lamina propria of the duodenum and red pulp of the spleen were studied for comparison. Related, mutated sequences were found in different sites within the salivary gland, duodenum, and spleen. This included isotype-switched variants in the salivary gland. Examples of related plasma cells have been seen before in the gastrointestinal tract (10, 11, 17), where the related cells can either be clustered locally (more commonly seen) or disseminated along the bowel. The salivary gland preparations were taken from opposite sides of the tissue block and were \(5–10\) mm apart. Because there was no evidence of organized lymphoid tissue within the salivary glands used, it is presumed that the clonal expansion occurred elsewhere and related plasma cell precursors migrated to the same site in the salivary gland. If this is the case, then the plasma cell population must be widespread but be of limited diversity. An alternative explanation is that expansion, hypermutation, and isotype switching may occur within the salivary gland, outside the germinal center environment.

Related clones, which are highly mutated but switched to different isotypes (Fig. 1a), indicate that isotype switching can occur after the onset of hypermutation. This has previously been

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**Table II. JH usage of Ig genes**

<table>
<thead>
<tr>
<th>JH Segment</th>
<th>Duodenum IgM</th>
<th>Duodenum IgA</th>
<th>Salivary Gland IgM</th>
<th>Salivary Gland IgA</th>
<th>Spleen IgM</th>
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* Other indicates unknown JH region.

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**Table III. Details of D region usage**

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<th>D Regions in Size Order</th>
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<td>21/10</td>
<td>37</td>
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Average size of D used (bp): 10.5
Average size of D used (excluding D4 (bp)): 10.0
Average size of Ds used (bp) which are 31 bp in germline: 11.9
Average size of Ds used (bp) which are 31 bp in germline (excluding D4): 11.9

* D4 was used significantly more frequently in the spleen than the gut \((p = 0.03\) by \(\chi^2\) analysis).
observed in duodenum, spleen, and tonsil (16–18) and suggests that Abs of the same specificity can be associated with different isotypes. It is interesting to observe a highly mutated sequence associated with both IgA and IgM, where there are more mutations in the IgM gene, because data in this paper and others (16–18) show that IgA genes generally have a higher number of mutations. Therefore, it is possible for a sequence to become highly mutated before it switches from IgM to IgA and for the IgM gene to continue to accumulate more mutations than the IgA gene, though this is not likely to be a common event.

Previous studies have indicated that the number of mutations in IgH genes of gastrointestinal plasma cells is high (10, 11, 17, 19) compared with published reports of the level of mutation in spleen, tonsil, and peripheral blood (16, 20–25). However, we have shown that the number of mutations in IgA genes in lamina propria plasma cells of the duodenum is higher than the number in IgM genes (17). Because the ratio of IgA:IgM plasma cells in the gastrointestinal tract is much higher than in the periphery (26), a random sampling of cells from either tissue would result in a bias in the overall frequency of mutation observed. In this study, we have isolated RNA and amplified IgH genes from cDNA using primers specific for IgA and IgM genes to compare isotype-matched genes from different tissues. The IgH genes from both IgM and IgA plasma cells of the salivary gland carry a surprisingly high number of mutations per gene, considering that polyclonality is often encoded by unmutated Ig genes (27), and Abs found in saliva may be polyclonal. The frequency of mutation of IgM VH genes in the spleen is comparable with the previously published data on frequency of mutation in IgM and IgG of tonsil (16, 21), splenic memory B cells (25), splenic marginal zone B cells (13, 24), and lymph node marginal zone B cells (24). The frequency of mutation of IgVH genes from salivary gland is highest of all groups, and is comparable with that of the duodenum, rather than the spleen. This observation is consistent with the salivary gland being part of the common mucosal immune system, which is separate from the peripheral immune system. It has been shown that in rabbits the tonsil can be an inductive site producing the effector cells of the peripheral immune system. Mucosal IgH genes generally have a higher number of mutations than IgM VH genes previously reported in other peripheral sites (13, 16, 21, 24, 25).

It is also interesting to note that in this group of 32 splenic Ig genes there are two that use JH1 segments, whereas no sequences from duodenum, salivary gland, or over 200 previously reported Ig genes from lamina propria plasma cells (10, 11, 17, 19) were found that used JH1. Because JH1 is so rarely used anyway, a much larger sampling from spleen than this one would be required to determine whether this is significant. Therefore, we propose that IgH genes in a mucosal plasma cell population have characteristics distinguishable from those seen in the peripheral immune system. The fact that any differences can be seen at all supports the theory that the common mucosal immune system is separate from the peripheral system. Mucosal IgH genes generally have a higher number of somatic mutations in their VH regions and a shorter CDR3 region. Due to the high variance for these parameters in the populations studied, it would be difficult to be able to use them to distinguish between cells originating in the peripheral immune system and not the mucosal immune system. However, there does seem to be a “cut off” point for the number of mutations found in IgM genes in the periphery. We have not seen any IgM genes with more than 24 mutations isolated from the periphery, while we have quite often seen IgM genes with a larger number of mutations in mucosal tissues (Fig. 2 and Ref. 17).

By studying the IgM and IgA isotypes separately, we have shown that the differences in characteristics between different tissues are not due to variations in isotype. The reasons for these differences are not clear. It has previously been suggested that the highly antigenic nature of mucosal surfaces may result in the constant stimulation of B cells to enter germinal centers and accumulate a higher number of mutations (11). If overstimulation were the
cause of the high numbers of mutations seen in mucosal tissues, then a similar level of mutation would be expected in the peripheral tissues as a result of chronic systemic Ag challenge. Patient 5 had a perforated bowel and the spleen was histologically normal but showed signs of mild acute inflammation consistent with challenge by luminal Ag. Even so, the IgH genes from this spleen were still less mutated than those seen in duodenum and salivary gland. These data suggest that there are fundamental differences in the mechanisms of mutation and/or selection in the peripheral and mucosal compartments.

The significance of the difference in CDR3 length and D<sub>H</sub> region usage between the mucosal and peripheral Ig<sub>H</sub> genes is unclear. Differences in CDR3 region length could arise as a result of codon deletions arising during somatic hypermutation or some other process, Ag selection, or origin of precursor B cells from a population that has a slightly different mechanism of Ig gene rearrangement. Although it has been documented that deletions and insertions can occur during somatic hypermutation, it is unlikely that the change in CDR3 length is due to this phenomenon, because we saw no evidence of deletions within D<sub>H</sub> segments in any Ig gene studied. Because this study sequenced cDNA, the Ig genes are expressed and therefore may have been selected by Ag. It is possible that the Abs encoded by Ig<sub>H</sub> genes with shorter CDR3 lengths are due to this phenomenon, because we saw no evidence of deletions within D<sub>H</sub> segments in any Ig gene studied.

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