Granulomatosis with polyangiitis involves sustained mucosal inflammation that is rich in B-cell survival factors and autoantigen

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Abstract

Objective. Granulomatosis with polyangiitis (GPA) is a rare chronic autoimmune disease that may be triggered by upper airway infection. ANCAs specific for PR3 that is expressed by activated neutrophils and macrophages are associated with GPA. Our aim was to investigate regional immune mechanisms that might induce or support the autoimmune response in GPA.

Methods. Biopsy samples from 77 patients including 8 with GPA were studied by immunohistochemistry. B-cell homing subsets in blood samples from 16 patients with GPA and 11 healthy controls were studied by FACS. The distribution of B-cell clones was searched in paired biopsies and blood samples from one patient by analysing immunoglobulin heavy chain gene (IGH) junctional sequences.

Results. Activated B cells were located alongside PR3-expressing cells and B-cell survival factors BAFF and APRIL in mucosa from patients with GPA. We detected APRIL production by the granulomas and giant cells. B cells were proliferating in all cases and persistent for 5 years in biopsies obtained from one patient. However, there was no evidence of B-cell clones from the mucosal biopsies circulating in peripheral blood in GPA or any numerical or proportional change in B-cell subsets expressing markers of regional homing in blood in GPA.

Conclusions. Our study illustrates chronically activated B cells alongside autoantigens and B-cell survival factors in the mucosa in GPA.

Key words: granulomatosis with polyangiitis, B cells, granuloma, APRIL, immunoglobulin genes.

Introduction

Granulomatosis with polyangiitis (GPA) is a rare, multi-organ inflammatory disease that may affect lungs, bronchi, oral or nasal mucosa, eyes, kidneys, nerves, brain, joints and skin [1, 2]. GPA is associated with the production of autoantibodies to neutrophil cytoplasmic antigens (ANCAs) in particular antibodies to the protease 3 (PR3) autoantigen expressed by neutrophils and activated macrophages [3–6]. Friederich Wegener first observed that patients with this disorder almost universally had upper airway disease especially affecting the facial sinuses, and he proposed that the disease was initiated in the upper airways [7, 8]. It has been suggested that Staphylococcus aureus infection may be associated with the pathogenesis of GPA and persistent S. aureus nasal carriage is associated with a risk of disease relapse [9, 10]. The chronological sequence of upper airway symptoms followed by systemic manifestations, including a high incidence of thromboembolic events [11], is consistent with the notion that GPA may evolve as a consequence of upper airway inflammation and that other systemic, potentially life-threatening symptoms are a...
direct consequence of the inflammation and activity of pathogenic autoantibodies.

The immunological features of the mucosal microenvironment in GPA have not been studied extensively. Classical features of the disease include a mixed infiltration of acute and chronic inflammatory cells, vasculitis, granulomas and small numbers of giant cells [12–14]. It has been shown that B cells are present in nasal mucosa in GPA and that the immunoglobulins they express are encoded by genes that are mutated in their immunoglobulin variable regions, a feature of germinal centre (GC) origin [15, 16]. Despite this, conventional germinal centres have not been identified as a consistent feature of the inflamed airway mucosa in GPA, though GC-like structures have been described [17]. B cells in normal oro-nasal mucosa and bronchial mucosa have been identified in organized lymphoid structures termed nasal-associated lymphoid tissue (NALT) or bronchus-associated lymphoid tissue (BALT), respectively [18]. In addition, isolated activated B cells can be identified beneath the stratified/pseudostratified epithelium in oral mucosa that are increased in frequency and are associated with plasma cell differentiation in local inflammatory responses [19].

In this study, we have characterized the B-cell infiltrate in mucosa in GPA. We have related the distribution of B cells to the distribution of cells expressing or binding the PR3 autoantigen and to the local production of B-cell survival factors, B-cell activating factor (BAFF) and a proliferation-inducing ligand (APRIL) by immunohistochemistry. We observe that large activated B cells in GPA exist alongside an abundance of cells expressing PR3. The B cells in GPA mucosa express BAFF receptor and are therefore potentially able to receive survival signals from locally produced BAFF. APRIL is expressed abundantly by many cell types in GPA mucosa, but most notably by the granulomas and giant cells that are a feature of the disease.

Immunoglobulin heavy chain gene (IGH) analysis allows the identification of B-cell clonality by comparison of junctional sequences that are unique to each B cell and its clonal progeny. By immunoglobulin variable region gene analysis, we observed evidence for local and sustained B-cell clones in the upper bronchial mucosa in GPA in a single case studied. We also studied subsets of blood lymphocytes and saw no evidence for preferential expansion of cells associated with any anatomically defined circulation pathways. By IGH analysis, there was no evidence of the B-cell clones identified in the mucosa circulating in the blood.

Overall, our study supports the concept that the mucosa in GPA is a niche containing chronically activated B cells and autoantigen. A local autoantibody response as described previously [20] could be sustained by the abundance of local B-cell survival factors.

Materials and methods

Tissues

Paraffin blocks of inflamed mucosal biopsies from 77 patients, including 8 patients with clinically confirmed GPA, were selected to include a range of characteristics from mild lymphocytic infiltrates considered within normal histological limits through to marked inflammation (detailed location of the tissue sampled and the diagnoses are included in supplementary Table S1, available as supplementary data at Rheumatology Online). The group of patients with orofacial granulomatosis (OFG) were considered to be an important control group because this is a chronic granulomatous mucosal lesion, but it is not associated with acute inflammation or autoimmunity. Specimens were graded according to the inflammatory infiltrate present to produce a semi-quantitative score (Nil, Mild and Marked). This was undertaken blind by two authors (P.P. and E.O.).

In addition, peripheral blood mononuclear cells (PBMCs) were isolated from fresh blood samples donated by 16 patients with GPA (M:F ratio 1:1, age between 25 and 73 years, average age 53 years) and 11 healthy controls (M:F ratio 1:1, age between 22 and 68 years, average 45 years). The details of patients’ clinical information are listed in supplementary Table S2, available as supplementary data at Rheumatology Online.

Flow cytometry (FACS)

PBMCs were isolated and incubated with fluorochrome-conjugated mAbs on ice for 40 min. To isolate Beta7low CLA+ CCR10– B cells, PBMCs were stained with CD19-PE (BD Biosciences 10 μl/100 μl), Beta7-PeCy5 (BD Biosciences 10 μl/100 μl), CLA-FITC (Biologend, 15 μl/100 μl), CCR10-APC (R&D Systems, 7 μl/100 μl). After staining, cells were washed and resuspended in sterile PBS and immediately separated into different subpopulations with the BD FACS Aria I cell sorter or BD FACSCanto.

Immunohistochemistry

Paraffin embedded tissue sections (5 μm) were submitted to antigen retrieval by heating retrieval solution (DAKO, Glostrup, Denmark) at 95 °C for 40 min. Endogenous peroxidase and alkaline phosphatase were blocked. Sections were single stained for CD20, APRIL, BAFF, BAFF-R, PD1, CD57, IgD, IgG, IgA, IgM and IgE and double stained for CD138 with CD20, Ki67 with CD20, neutrophil elastase with CD20, PR3 with CD20, PR3 with neutrophil elastase and E-selectin with CLA using the EnVision single and double stain systems (DAKO). After staining, sections were counterstained with haematoxylin.

Analysis of immunoglobulin gene sequences

IGH from blood cells was amplified from the variable (IGHV) to constant (IGHC) regions. IGH from paraffin-embedded biopsy sections was amplified from the framework region 3 (FR3) to the joining (J) region. Semi-nested PCR was applied (details in supplementary Table S3, available as supplementary data at Rheumatology Online). To search the clone relationships between biopsies and different circulating blood subsets, clone-specific primers were applied to all blood subsets and biopsies from the same patient using the FR3-IGHJ PCR program.
All primers used are listed in supplementary Table S4, available as supplementary data at Rheumatology Online.

Ethics approval
This study was approved by the National Research Ethics Service (Approval no. 10/H0715/3). All patients donated blood and agreed to allow access to biopsy and tissue samples that were surplus to clinical investigation after fully informed written consent.

Statistical methods
Data sets are expressed with average values and comparisons were made using the Mann-Whitney test. Statistical analysis was carried out using GraphPad Prism4.

Results
B cells and plasma cells as components of chronic/acute inflammation in GPA
B and T cells were consistently present in mucosal biopsies from eight patients with GPA. B cells in most cases tended not to be components of any organized lymphoid structure but were scattered submucosally, and the infiltrate showed no evidence of GC formation. One nasal biopsy from a patient with GPA contained abundant organized mucosa-associated lymphoid tissue (MALT), but the affected mucosa in the remaining cases had no evidence of GC formation. The B cells in GPA had properties of activated cells; they did not express IgD but were large cells with extensive dendritic processes that extended and made contact with many adjacent cell types. Approximately 5% expressed the Ki67 nuclear proliferation antigen. This description of B-cell morphology, phenotype and proliferative behaviour does not fit easily within the current schemes of B-cell biology. These cells were therefore compared with those seen in a range of inflammatory conditions of different levels of severity involving mucosal surfaces, including those around the oral cavity and upper airways. This B-cell subset was indistinguishable from that consistently seen in other biopsies of nasal and oral mucosa stained as controls, demonstrating that the presence of this B-cell subset was not a disease-specific observation. Unlike the controls, used in this study, however, the B-cell infiltrate in GPA was generally intimately mixed with acute inflammatory cells that included cells expressing the GPA-associated autoantigen PR3 and neutrophil elastase.

The plasma cell and B-cell infiltrates tended to occupy adjacent areas of mucosa. Plasma cells were often observed in the infiltrate that was predominantly B cells, but B cells were rare in the plasma cell-dominated zones. The majority of plasma cells expressed IgG rather than IgM or IgA, consistent with the profile observed in the control samples (Fig. 1).

Analysis of local B-cell clonality by Ig gene analysis
DNA was prepared from serial sections of diagnostic biopsies from one patient (patient P5 in supplementary Table S2, available as supplementary data at Rheumatology Online) who donated surplus tissue following surgical procedures in 2004, 2009 and 2010 and a blood sample. Ten PCRs to amplify IGH from FR3 to J were carried out from each of the biopsies. Clones with the same junctional sequence from the same PCR were considered to be the same sequence and were counted as one. A total of 74 different rearrangements were identified; 40 from bronchial mucosa in 2004, 13 from bronchial tissue in 2009 and 21 from nasal mucosa in 2010. The different numbers were a reflection of different B-cell numbers in the different-sized tissue samples studied. Clone-specific primers were then prepared to the V proximal N-D sequence to specifically identify the clone in the target DNA sample by PCR. Design ensured that some non-templated sequence was present between the clone-specific primer and the J segment primer to allow confirmation of clonal identity by sequencing. Of 14 primers designed, 8 were found not to amplify non-specifically in a DNA preparation of tonsil cells from an unrelated individual and were considered to be potentially clone specific. When PCR products of the correct size were generated by clone-specific PCR, clonal identity was confirmed by sequencing.

Although the B-cell population was polyclonal, these methods identified three examples of B-cell clonal
expansion in the inflamed mucosa. Two examples were restricted to the 2009 biopsy. Remarkably, one clone was observed to be present in bronchial tissue in both 2004 and 2009 (Fig. 2). The same method was also applied to detect circulating B-cell clones in peripheral blood. However, we did not observe any evidence that B-cell clones in the lesion were circulating in peripheral blood.

Local production of B-cell survival factors in GPA
Since we saw evidence of local B-cell division and sustained clonal expansion as described above, we investigated the local distribution of B-cell survival factors APRIL and BAFF and the BAFF receptor [21–24], all of which can be detected by immunohistochemistry in paraffin-embedded tissue. APRIL was produced abundantly in the B-cell microenvironment in GPA and in other control mucosal biopsies. It was observed that granulomas and giant cells, both of which are features of most cases of GPA, contained abundant APRIL. Granulomas in OFG and sarcoid also contained APRIL. Granulomas are, therefore, a previously unrecognized source of APRIL that may contribute to the support of the local B-cell response. The B cells in GPA expressed the BAFF receptor, and cells producing BAFF were also identified in the mucosa, indicating that this pathway of promoting B-cell survival is also relevant to the local response in GPA (Fig. 3).

Analysis of lymphocytes expressing regional homing receptors in GPA
Evidence presented above is consistent with a local and sustained B-cell response in the mucosa in GPA. We investigated whether the chronic expansion of B cells in the mucosa was associated with any changes in the proportion of total lymphocytes or B cells in blood, which express molecules that facilitate regional lymphocyte homing. Cells that home to the nasal and bronchial mucosa have been reported to express the cutaneous lymphocyte antigen (CLA), which binds the endothelial antigen E-selectin, but not the chemokine receptor CCR10 [25–28].

We confirmed the expression of E-selectin by flat endothelium in mucosal biopsies in GPA. We then identified cells expressing CLA, the receptor for E-selectin, either among B cells or in the total lymphocyte gate by flow cytometry. There was no difference between patients with GPA and healthy controls in the proportion of B cells or lymphocytes expressing CLA or any other lymphocyte subset associated with regional homing (Fig. 4).

The clone-specific PCR method as described above was applied to the B-cell subsets isolated from blood, using primers designed to the CDR3 sequences observed in the biopsy taken from the patient at the same time. No evidence of the B-cell clones identified in the affected mucosa was observed in the blood in any sorted subsets.

Discussion
In this study we observed that inflamed mucosa in GPA contains chronically activated B cells intimately mixed with cells expressing PR3, a characteristic target of autoantibodies in most cases of GPA. The mucosal microenvironment is rich in B-cell survival factors APRIL and BAFF and the expression of APRIL by granulomas and giant cells is described here for the first time.

Fig. 2 IGH VDJ junction sequences from a patient with GPA.

(A) and (B) are alignments of sequences of VDJ obtained from different DNA samples of a biopsy obtained in 2004. The presence of the same sequence in different PCRs confirms B-cell division and local presence of members of a B-cell clone. (C) One VDJ rearrangement was identified in both the 2004 and the 2009 biopsies from this patient, implying that this B-cell clone was persistent in the lesion for at least 5 years.
combination of mixed acute and activated chronic inflammatory cells, alongside survival factors including APRIL produced by granulomas and giant cells, were only seen in GPA.

The B cells observed in GPA were indistinguishable as a population from recently identified subepithelial dendritic B cells in oral mucosa [19]. In GPA and a varied panel of controls, B cells were activated cells that lack IgD, contain a small but consistent proliferating fraction and have irregular cytoplasmic processes that extend and contact diverse adjacent cells. They are not associated with any follicular structures but are present in increased numbers and exist alongside a plasma cell component in inflammation. Such cells are not a general feature of the physiological process of plasma cell differentiation from B cells. For example, the lamina propria of the normal gut, a site of plasma cell differentiation, does not have a significant population of cells that resembles those observed in the mucosa described here. In GPA, the most common Ig isotype observed was IgG, consistent with the profile observed in the diverse set of control tissues.

Local B-cell division observed by immunohistochemistry was supported by the detection of clonally related cells in a single bronchial biopsy studied by Ig gene analysis. Clonally related cells were detected in a polyclonal B-cell background by comparison of CDR3 sequences. The presence of a clonal population of B cells persisting for 5 years in one patient suggests that B-cell stimulation is chronic.

In GPA, the B cells were often intimately associated with cells expressing PR3. Some PR3-positive cells were clearly neutrophils, but not all. It is possible that the abundance of PR3 antigen locally provides an immunological stimulus to potentially initiate or sustain the chronic B-cell anti-PR3 response. However, some cases studied were negative for autoantibodies to PR3. This demonstrates that while this juxtaposition could possibly support the generation of autoantigen reactive B cells, this does not necessarily occur.

B-cell responses that result in GC formation are generally T-cell dependent [29]. T-cell involvement places stringent regulation on the discrimination between self and non-self as a consequence of thymic education [30, 31]. The chronic B-cell activity but lack of GC formation in most disease-relevant mucosal microenvironments in GPA might indicate that the anti-PR3 response in the mucosa could be driven in a T-cell independent way.

B-cell survival factors APRIL and BAFF were present in the nasal mucosa in GPA. We observed for the first time in this study that granulomas and giant cells are both significant potential sources of APRIL. It is possible that the granulomas, that are also linked to macrophage fusion and giant cell formation, may subsequently provide a source of factors to support an aberrant B-cell response. This observation is relevant to granulomatous diseases other than GPA and is therefore not restricted to this autoimmune condition. Studies have not observed correlation between concentrations of APRIL in serum and ANCA titres [32], and the relevance of serum BAFF to ANCA status and vasculitis remains unclear in a study of GPA serum BAFF varied inversely with ANCA levels [32–34]. It is not known if BAFF and APRIL produced in mucosa acts locally or if it may contribute to the serum pool of B-cell survival factors.

The B cells in mucosal lesions in GPA have been observed to have mutated IGHV genes, yet in the majority of cases no GC formation was observed in the mucosa [15, 16]. We therefore considered that the B-cell fraction that localizes to the mucosa might be expanded as a population in blood in GPA as part of a migratory continuum. It is also possible that recruitment to an inflammatory site might result in depletion of a subset of inflammatory cells from the blood. When we analysed the subsets of total lymphocytes or B cells in blood that expressed markers associated with tissue-specific
homing, no differences between GPA and healthy controls were apparent.
Overall our data are consistent with the hypothesis that a chronic, local B-cell response in inflamed mucosa, in the presence of abundant PR3 antigen and supported by locally produced B-cell survival factors BAFF and APRIL, could support the production of the autoantibodies that are associated with life-threatening systemic symptoms in GPA.

**Supplementary data**

Supplementary data are available at Rheumatology Online.

**References**

6. Rarok AA, Stegeman CA, Limburg PC et al. Neutrophil membrane expression of proteinase 3 (PR3) is related to

**Rheumatology key messages**

- B cells in the mucosa in GPA are mostly isolated, activated cells.
- In a single case studied, local mucosal B cells included long-lived clones.
- B-cell survival factor APRIL produced by granulomas could promote B-cell survival in granulomatous lesions.

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