Monoclonal gammopathies (MGs) are hematological diseases characterized by high levels of a monoclonal immunoglobulin (Ig) or M-protein. Within this group are patients with more than one M-protein, referred to as double MGs (DMGs). The M-proteins in DMG patients may have different heavy chain (HC) isotypes that are associated with different light chains (LCs), or different HCs that are LC matched. In this study, we examined the clonal relatedness of the M-proteins in the latter type in a cohort of 14 DMG patients. By using PCR, we identified 7/14 DMG patients that expressed two Ig HC isotypes with identical Ig HC variable (IGHV), diversity (IGHD), and complementarity determining region (HCDR3) sequences. Two additional DMG patients had two Ig transcripts using the same IGHV, IGHD and IGHJ genes but with slight differences in variable region or HCDR3 mutations. LC analysis confirmed that a single LC was expressed in 3/7 DMG patients with identical HC transcripts and in the two DMGs with highly similar transcripts. The PCR findings were confirmed by immunofluorescence for HC and LC expression. Clonally related HC-dissimilar/LC-matched DMGs may occur often and defines a new subtype of MG that may serve as a tool for studies of disease pathogenesis.

INTRODUCTION

Monoclonal gammopathies (MGs) encompass a number of disorders characterized by the presence of a clonal expansion of malignant plasma cells (PCs) typically located within the bone marrow (BM). In addition, these patients generally have high levels of monoclonal serum immunoglobulin (Ig) of a single isotype, which is referred to as the M-protein. As with normal PCs, MG PCs are derived from B cells that have undergone rearrangement of the Ig heavy chain (HC) and Ig light chain (LC) variable (V) region genes.

Further diversification arising from somatic hypermutation (SHM) of the Ig V regions and class switch recombination (CSR) during germinal center (GC) reactions results in a unique Ig clonotypic signature. Indeed, in post-follicular malignancies including the MGs multiple myeloma (MM), MG of undetermined significance (MGUS), LC amyloidosis (AL) and Waldenstrom’s macroglobulinemia (WM), the malignant PCs have acquired Ig HC and LC V region somatic mutations. In addition, MM, MGUS and AL have typically undergone IGH isotype switch to IgG, IgA or IgE, and studies in MM suggest that the malignant PCs no longer undergo SHM as evidenced by lack of intraclonal diversity.

Despite being considered a clonal malignancy expressing single rearranged Ig HC and LC V regions, 3–4% of all patients with MGs express more than one M-protein in serum and urine, a condition termed bclonal gammopathy or double MG (DMG). These patients can express M-proteins with different isotypes of HCs that are LC isotype-matched, the same HC isotypes with different LC isotypes, or different HC isotypes with different LC isotypes. The latter situation would signify true bclonal or polyclonal gammopathy, most likely arising from two or more distinct cell populations. Within DMGs, the most frequently observed combination of HCs is IgA and IgG followed by IgG and IgM. However, when LC expression was considered, 73% of those patients that expressed IgG/IgA also expressed the same LC compared with 60% of IgG/IgM combinations. These results suggest that DMGs may include patients with independent malignant PC clones, as well as those that have clonally related malignant PCs resulting from CSR that has occurred at some point during disease pathogenesis. However, thorough molecular studies of the Ig HC and LC V region genes in patients with DMGs are extremely limited and published reports in this area are largely restricted to analysis of single patients. Thus, detailed analysis of the clonal relationship between these cell types is lacking. Indeed, Fair and Krueger reviewed several early studies of individual DMG cases analyzed with the techniques available at the time, but concluded that more detailed and sensitive methods were needed to determine the clonal relationships (if any) and whether the clones are from a common precursor cell or distinct clones arising from different malignant PCs. In a more recent study, Bakkus et al. used PCR to analyze an IgA/IgE DMG MM patient and found that the nucleotide sequence of the Ig HC V (IGHV) genes in the IgA and IgE clones were identical, including somatic mutations and the clone-identifying third HC complementarity determining region (HCDR3). They could find no evidence of pre-switched clonally related B cells, and therefore suggested that a malignant transformation most likely occurred in an IgA precursor cell at the post-switch stage allowing for further CSR to IgE. Because this analysis was restricted to a single patient, it currently remains unknown how frequently LC-matched DMGs indeed reflect clonal disorders that include class switch variants.

In this study, therefore, our goal was to exploit our extensive tissue bank of MGs and examine a larger cohort of HC-dissimilar/LC-matched DMG patients.
LC-matched DMGs for clonal relationships. Using molecular and immunofluorescence (IF) methods, we present evidence suggesting that class switch variants in the DMGs may be more frequent than anticipated. Our observations provide further insight into the clonal origins of DMG and possible mechanisms of disease progression.

MATERIALS AND METHODS

Ethics statement

Mayo Clinic Institutional Review Board approval was granted to the Mayo Clinic Dysproteinemia Cell Bank for collection of BM from Mayo Clinic patients with malignant PC disorders. A BM specimen was collected from the iliac crest only after the patients provided written informed consent in accordance with the Declaration of Helsinki. Review of the clinical records identified a cohort of 14 MG patients for whom cryopreserved cells were available for analysis with a hematological diagnosis, and for which immunofixation electrophoresis showed more than one HC of different isotypes and a monoclonal LC. Patients with these criteria that had not undergone transplant or other current treatment were considered for further study.

Cell isolation

BM mononuclear cells (BM MNCs) obtained from donors were isolated by Ficoll Paque (GE Healthcare, Piscataway, NJ, USA) density centrifugation or Ficoll Paque (GE Healthcare, Piscataway, NJ, USA) density centrifugation or BM mononuclear cells (BM MNCs) obtained from donors were isolated by ACK lysis (Life Technologies, Grand Island, NY, USA). For most patients, PCs were enriched by magnetic bead sorting using Human CD138-Positive Selection kits (StemCell Technologies, Vancouver, BC, Canada) and a Robosep instrument (StemCell Technologies). The purity of the PC samples was >98% as assessed by morphology.

RNA isolation, cDNA synthesis, Ig HC/LC V region PCR and sequence analysis

RNA was isolated by the TRIzol method (Life Technologies) and 2 μg of RNA was converted to cDNA using the GE Healthcare First Strand Synthesis kit. To determine the IGHV, Ig HC diversity (IGHD), and Ig HC joining (IGHJ) gene usage, 2 μl of cDNA was amplified using the Qiagen HotStarTaq MasterMix kit (Qiagen, Valencia, CA, USA) in a multiplex PCR reaction using 0.5 μM of each of seven sense primers, representing the seven IGHV families in conjunction with 0.5 μM of antisense primer to either the IgG, IgA or IgM constant region. An additional reaction for β-actin was included for each cDNA. For most patient samples, individual PCR reactions were also performed using IGHV family-specific primers to detect any secondary clones and to ensure that the resulting products were not a result of PCR recombination events (hybrids).

To determine Ig LC V region gene usage, individual PCR reactions for each Ig LC family using 0.5 μM of the sense primer in conjunction with 0.5 μM antisense primer to the κ or λ constant region were performed. Amplification was carried out in a Perkin Elmer 9700 thermocycler (Perkin Elmer, Waltham, MA, USA) using the following conditions: 95°C for 15 min; 35 cycles of 95°C for 30 s, 60°C for 60 s, 72°C for 60 s and a final cycle of 72°C for 10 min. Amplified products were visualized on a 1.5% agarose TAE gel with ethidium bromide, excised and purified with the Purelink Gel Extraction kit (Life Technologies). Purified products were directly sequenced on an ABI PRISM 3100 × L DNA Analyzer (Applied Biosystems/Life Technologies, Grand Island, NY, USA). Resulting sequences were aligned to germline (GL) Ig HC and LC V region gene sequences using IMmuGeneITics Information (IMGT) System reference sets and IMGT/V-Quest software (http://imgt.cines.fr).

Immunofluorescence

Total BM MNCs were collected onto glass slides using a Cytospin 2 (Thermo Shandon, Waltham, MA, USA) and stored at –20°C until needed. For cytoplasmic Ig staining, the slides were fixed in 95% ethanol for 5 min, incubated in PBS + 0.1% Tween 80 (Sigma-Aldrich; St Louis, MO, USA) for 1 min and then rinsed in PBS alone. For staining, antibodies were diluted 1:100 or 1:200 in antibody diluent with background reducing components (Dako, Carpinteria, CA, USA). The following antibodies that were used in combination are as follows: anti-IgM FITC, anti-IgA TRITC, anti-κ, FITC (all from Southern Biotech, Birmingham, AL, USA), anti-κ AMCA (Vector Labs, Burlingame, CA, USA) and an anti-IgG antibody (Southern Biotech) that was labeled with CF350 using a Sigma Max-n-Stain kit. Slides were incubated with appropriate antibodies at 37°C for 45 min under humidified conditions. After incubation, the slides were rinsed in PBS + 0.1% Tween 80 for 5 min, rinsed in PBS for 3 min, and then air dried. A coverslip was mounted using Vectashield (Vector Labs) mounting medium with or without propidium iodide depending upon the combination of antibodies used. Slides were visualized using an Olympic Provis AX79 microscope (Olympus, Center Valley, PA, USA), equipped with necessary fluorescent filters, and images were acquired with an Olympus DP71 digital camera with Olympus DP Manager software.

RESULTS

DMG patient cohort

Review of clinical data for patient samples in our MG tissue bank identified 14 patients with DMGs characterized by two HCs and a single monoclonal LC upon immunofixation electrophoresis (example shown in Supplementary Figure 1). Importantly, none of these patients had undergone prior transplant or had received treatment within 6 months of BM collection. Of the 14 patients, nine were diagnosed with MM or its precursor disease MGUS, three with AL, one with WM and one with WM/MGUS (Table 1). The percent BM PCs ranged from 2 to 74%, with 9/14 patients having ≥10% PCs and the PC labeling index ranged from 0 to 7.7% (when data was available). The M-protein (M-spike) for IgA,
IgG or IgM ranged from 0.2 to 3.2 g/dl when able to quantify. In three cases, the M-spikes were evident, but one or both were too small to quantify, and in four others, the two M-spikes was recorded as a single value with no distinction between the two IgGs.

Ig HC V region gene PCR and isotype variant comparisons in DMG patients

The Ig HC V region gene expression was determined by multiplex PCR with sense primers to the seven IGHV gene families in conjunction with antisense primers specific for IgA, IgG or IgM isotypes (Supplementary Figure 2). Some DMG samples were also amplified using individual PCR reactions for each of the seven IGHV families. In 13 of the 14 DMG samples, a rearranged Ig HC V region gene was amplified using antisense primers to two different HC isotypes, four patients (patients 9, 12, 13, 14) had two or more distinct IGHV genes and different HCDR3s for each HC amplified. This analysis suggests that the DMGs in these patients most likely reflect the presence of two or more divergent malignant (or normal) PCs that are not clonally related at least at the level of detection by Ig HC V region PCR (Table 2).

Of great interest, molecular analysis revealed that the IgG and IgA transcripts from 9/14 (64%) DMG samples used the same IGHV gene as well as the same IGHD and IGHJ genes when aligned to GL reference sequences using IMGT/V-Quest (Table 2). In 7/9 patients, the entire IgH region and HCDR3 (including deviations from the GL sequence) were identical at the nucleotide and amino acid (AA) levels, suggesting a clonal relationship despite different HC usage. Patient 2 is shown as an example of this pattern of identity in Figure 1a. In the remaining two patients (patients 6 and 11), although the Ig HC V region sequences were not identical, the IgG and IgA transcripts were highly homologous. Patient 11 had an identical HCDR3 at both the nucleotide and AA levels in the IgG

Table 2. IGHV, IGHD, and IGHJ gene usage for DMG isotypes

| Patient | Ig  | IGHV | IGHD | IGHJ  | % Mut\(^a\) | HCDR3                  |
|---------|-----|------|------|-------|-------------|------------------------|
| 1       | IgG | 1–46 | 1–26 | J4*02 | 13.9        | TFLGGGGDF              |
|         | IgA | 1–46 | 1–26 | J4*02 | 13.9        | TFLGGGGDF              |
| 2       | IgG | 2–5  | 6–19 | J5*02 | 8.3         | AGTRLGAVSGKEKSAWDFP    |
|         | IgA | 2–5  | 6–19 | J5*02 | 8.3         | AGTRLGAVSGKEKSAWDFP    |
| 3       | IgG | 4–39 | 5–5  | J6*02 | 10          | ASQAYSSLPLSV           |
|         | IgA | 4–39 | 5–5  | J6*02 | 10          | ASQAYSSLPLSV           |
| 4       | IgG | 3–48 | 6–19 | J6*03 | 7.1         | ARDNWSK55YFHMDDV       |
|         | IgA | 3–48 | 6–19 | J6*03 | 7.1         | ARDNWSK55YFHMDDV       |
| 5       | IgG | 3–73 | 2–21 | J6*03 | 11          | TVPMYRADFGLGYYMDV      |
|         | IgA |      |      |       |             | No clonal sequence determined |
| 6       | IgG | 3–48 | 2–15 | J4*02 | 6.7         | AREAPIAVASTPFDS        |
|         | IgA | 3–48 | 2–15 | J4*02 | 7.9         | AREAPIAAATPFDY         |
| 7       | IgG | 4–39 | 6–6  | J5*02 | 13.6        | ARDGLTARAVEKNLFDP      |
|         | IgA | 4–39 | 6–6  | J5*02 | 13.6        | ARDGLTARAVEKNLFDP      |
| 8       | IgG | 5–51 | 3–22 | J5*02 | 0.8         | ARRGRYDSSGQYT          |
|         | IgM | 5–51 | 3–22 | J5*02 | 0.8         | ARRGRYDSSGQYT          |
| 9\(^b\) | IgG | 3–30 | 2–8  | J5*02 | 11.7        | ATGGFDSD               |
|         | IgM | 3–7  | 5–12 | J4*02 | 10.8        | VRLGSYPGD              |
| 10      | IgG | 1–2  | 2–15 | J2*01 | 4.7         | ARDAGWGGLYWHFDL        |
|         | IgA | 1–2  | 2–15 | J2*01 | 4.7         | ARDAGWGGLYWHFDL        |
| 11      | IgG | 3–23 | 2–8  | J4*02 | 8.6         | ASSLGYYTSGFPGDI        |
|         | IgA | 3–23 | 2–8  | J4*02 | 10.0        | ASSLGYYTSGFPGDI        |
| 12\(^b\) | IgG | 4–31 | 3–22 | J6*01 | 15.6        | ARVVPREAALVFPLRRGMDV   |
|         | IgM | 4–61 | 4–23 | J4*02 | 0.4         | AREATVPPYFN            |
| 13\(^b\) | IgG | 4–39 | 3–22 | J4*02 | 5.8         | ARHRSGYDTSQYFDSD       |
|         | IgM | 3–7  | 6–19 | J4*02 | 2.5         | ASNWAVPGD              |
|         | IgM | 4–61 | 6–13 | J4*02 | 6.6         | ARVGRQVSD              |
| 14\(^b\) | IgG | 3–30-3 | 4–17 | J4*02 | 6.3         | ARDAGYDTGYYLADYW       |
|         | IgM | 3–7  | 2–8  | J5*02 | 12.1        | ARNGGCVL               |
|         | IgM | 4–30-4| 5–18 | J5*02 | 3.3         | AREPWGYSGN1            |
|         | IgM | 5–51 | 6–19 | J4*02 | 5.2         | ARSAFREGWRDGWY5VDY     |

Abbreviations: Ig, immunoglobulin; IGHV, Ig HC variable; IGHD, Ig HC diversity; IGHJ, Ig HC joining. Changes in HCDR3 amino acid sequence in Patient 6 are in bold. Patient numbers are identical to numbers shown in Table 1. \(^a\)\% Mut = percent mutation from GL sequence in the IGHV region. \(^b\)True bi- or polyclonal.
Figure 1. DMG sequence alignments. (a) Sequence alignment of patient 2 representing those patient samples that had identical sequences in the variable and HCDR3s in two different HC transcripts. (b) Sequence alignment of patient 11 showing shared and distinct somatic mutations in the Ig HC V region, but identical HCDR3s in IgG and IgA transcripts. (c) Sequence alignment of patient 6 showing shared and distinct somatic mutations, and highly homologous but not identical HCDR3s in IgG and IgA transcripts. Blue denotes replacement mutations resulting in AA change; green denotes silent mutations resulting in AA preservation; HFR, heavy chain framework regions; HCDR, heavy chain complementarity determining regions.

and IgA transcripts, and the majority of the V region mutations were shared. However, the IgA transcript included five additional somatic mutations, four of which resulted in an AA replacement, whereas the IgG transcript had one additional mutation resulting in an AA replacement (Figure 1b). In patient 6, the IgG and IgA lg HC V region transcripts were also very similar. As shown in Figure 1c, the same IGHV gene was used in both transcripts (IGHV4-48). Similar to patient 11, the pattern and number of mutations in the variable region gene differed between the IgG and IgA transcripts (Figure 1c). However, unlike the identical HCDR3s observed in patient 11, the HCDR3s in the IgG and IgA transcripts of patient 6 were not identical but remarkably homologous. Specifically, both transcripts: (1) used the same D (IGHD2-15*01) and the J region gene (IGHJ4*02); (2) have the same number and similar pattern of nontemplated (N) and palindromic (P) nucleotides and (3) share the same CDR3 AA length. Each of these features has a crucial role in defining the HCDR3 and clonality. 

Further PCR confirmation of identical Ig isotype transcripts in DMG patients

The multiplex PCR method for Ig HC V region analysis lends itself to possible PCR recombination events (hybrids), which may result from amplification of multiple but similar IGHV genes. To strengthen the PCR results suggesting clonal relatedness, alternative PCR strategies were used. Specifically, patients 1 and 4 were further evaluated using a single-tube PCR reaction for each IGHV gene family in conjunction with IgG or IgA antisense primers. In patient 1, the IgA reactions resulted in 1 amplified product (IGHV 1) and the IgG reactions amplified two products (IGHV 1 and 7; Supplementary Figure 3). When aligned to GL reference sequences using IMGT/V-Quest, the three amplicons had identical homology to each other and to the product amplified in the multiplex reaction. In patient 4, both the IgG and the IgA reactions resulted in multiple amplification products. However, only one IgA and one IgG sequence was readable, and both were homologous to each other and the sequence generated by the multiplex PCR. Therefore, while normal PCs or a second malignant clone may be
Ig LC region gene expression in DMG patients with different HCs

The Ig LC V region results were more complex, but nonetheless revealing. Of the nine patient samples using the same IGHV gene with two different HCs, we could clearly detect a single LC transcript corresponding to the LC detected by immunofixation in five of the patients, thereby providing further evidence that these transcripts are from clonally related cells (Table 3). Within this group were patients 6 and 11 that were highly homologous by HC analysis but not identical (Figures 1b and c), again further demonstrating a clonal link between the cells expressing those two transcripts. Patients 3, 4, 7 and 8 had identical transcripts (patients 1–6, who expressed IgG or IgA on immunofixation, were stained as in Figure 2). Both LCs were found by PCR in patient 2. (× 60 magnification).

Immunofluorescence of Ig HC and LC in DMGs

To further support the PCR results, we also included a PCR to amplify IGHV genes using an Ig isotype not detected by immunofixation electrophoresis. Supplementary Figure 4 shows that in patients 1–6, who expressed IgG or IgA on immunofixation electrophoresis, a multiplex Ig HC V region PCR with IgM either yielded no amplification products (patients 1–3 and 5), or the amplified products did not result in a readable sequence (patients 4 and 6), indicating that a PCR hybrid with the prominent Ig HC V region and IgM constant region did not occur. Rather any amplicons represent nonspecific amplification or polyclonal background of normal PCs.

Table 3. LC gene usage for κ (IGKV) and λ (IGLV) isotypes in DMG patients

| Patient | Clinical LC | IGKV  | Mutation | IGLV  | Mutation |
|---------|-------------|-------|----------|-------|----------|
| 1<sup>a</sup> | κ | IGKV3-11 | M | ND | NA |
| 2<sup>a</sup> | λ | ND | NA | IGLV1-47 | M |
| 3<sup>a</sup> | κ | IGKV1-27 | UM | IGLV4-69 | M |
| 4<sup>b</sup> | λ | Multiple | M/UM | Multiple | M/UM |
| 5<sup>b</sup> | λ | IGKV1-39 | M | Multiple | M/UM |
| 6<sup>b</sup> | κ | IGKV1-33 | UM | ND | NA |
| 7<sup>a</sup> | λ | IGKV2-28 | UM | Multiple | M |
| 8<sup>a</sup> | λ | IGKV4-1 | UM | IGLV6-57 | UM |
| 9<sup>a</sup> | κ | Multiple | M | ND | NA |
| 10<sup>b</sup> | λ | ND | NA | IGLV2-23 | M |
| 11<sup>b</sup> | λ | ND | NA | IGLV3-25 | M |
| 12<sup>a</sup> | κ | IGKV1-5 | M | IGLV6-57 | M |
| 13<sup>a</sup> | λ | Multiple | M/UM | Multiple | M |
| 14<sup>a</sup> | κ | IGKV1-16 | M | ND | NA |

Abbreviations: IGKV, Ig Kappa variable; IGLV, Ig lambda variable; LC, light chain; M, mutated (>2% deviation from germline (GL) sequence); ND, no readable sequence detected; NA, not applicable; UM, unmutated (<2% deviation from GL sequence). <sup>a</sup>HC transcripts were identical for these patients. <sup>b</sup>HC transcripts were not identical, but clonally related for these patients.

DISCUSSION

The presence of two or more M-proteins in a MG is an infrequent occurrence. However, the existence of such a phenomenon affords an opportunity to examine DMG disease pathogenesis and possible origins of the two clones. Although DMGs may be present in different combinations with respect to HCs and LCs, in this study we chose to focus on those DMGs that exhibited HC-dissimilar/LC-matched M-proteins. Given that there is no known mechanism for LC switch, HC-dissimilar/LC-matched DMGs have a greater probability of being clonally related than DMGs with dissimilar LCs, hence the rationale for our focus on this subtype of DMGs. Of importance, we selected only those patients that had
IGHJ genes, suggesting a clonal relationship (Table 1). Of those nine patients, seven had identical Ig HC V region nucleotide sequences (and thus AA sequences), thereby confirming the clonal relatedness of the two M-proteins (Figure 1a). Early studies of DMGs that looked at clonality by AA identity with techniques of sequences (and thus AA sequences), thereby confirming the clonal relatedness of the two MM patients is very rare, and instead, it would be of two or more B cells resulting in two unrelated M-proteins in the same MM patient would be very rare, and that instead, it would be more likely that two M-proteins within the same MG patient would be clonally related.35

Of interest, we also identified two patients whose M-proteins were encoded by the same IGHV, IGHD and IGHJ genes but with differences in the number and pattern of somatic mutations. Thus, patient 11 expressed two transcripts with the same IGHV, IGHD and IGHJ genes and therefore identical HCDR3s, but had differences in the Ig HC V region somatic mutations. Patient 6 is newly diagnosed and untreated with MM, where the IgA and IgG clones used the same IGHV gene but differed by a few mutations in the variable region. In addition, in this patient the HCDR3s in the two clones were highly homologous and were of the same length but could be distinguished by four nucleotide changes resulting in three different AAs (one resulted in a silent mutation). Despite these differences, these two cases are also highly suggestive of a clonal relationship between the two transcripts, and are particularly interesting because they suggest the possibilities that either some of the malignant cells have had additional transits through the GC resulting in CSR and further diversification; or, the malignant B-lineage cell acquired limited Ig HC V region somatic mutations and underwent CSR after the transforming event. Although we were not able to perform analysis on memory B cells to evaluate either of these scenarios, it is of interest that Bakkus et al.11 were not able to detect clonally related B cells in their study of an IgAκ/κ IgEx: DMG MM. A third possibility is that the mutations in patients 6 and 11 may reflect random somatic mutations independent of GC reactions. This is of importance as MM has recently been shown to undergo considerable genetic evolution at the genome level36 and intraclonal heterogeneity due to SHM37 or mutations independent of GC reactions. This is of importance as MM occurs and includes only a single patient case study.11,33 Our study is the first to look at a larger cohort of DMG patients with detailed molecular analysis, and we have found that more than half of these DMGs are clonally related. However, two of our 14 DMGS were WM, a MG in which the PCs always secrete IgM.34 In both cases of WM, the IgG and IgM HC isotypes were not clonally related and served somewhat as an internal control for the detection of nonrelated clones. Therefore, we demonstrate that 9/12 non-WM DMGs are clonally related. Indeed, the low incidence of the MG MM, in general, suggests that malignant transformation of two or more B cells resulting in two unrelated M-proteins in the same MM patient would be very rare, and that instead, it would be more likely that two M-proteins within the same MG patient would be clonally related.35

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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