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CLINICAL RESEARCH

Immunofluorescence Staining for IgG Subclass: Cause for Discrepancy in the Detection of IgG1

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Introduction: Immunofluorescence (IF) staining for IgG subclasses plays an important role in the classification of kidney disease. However, widely used IgG subclass-specific antibodies are now commercially unavailable. Thus, we compared alternative antibodies for performing IgG subclass staining.

Methods: A total of 21 cases were stained by 3 different methods: direct IF using fluorescein isothiocyanate (FITC)-conjugated polyclonal antibodies against IgG1-4 (commercially unavailable method), direct IF using FITC-conjugated monoclonal antibodies (clones HP-6091, 6014, 6050, and 6025), indirect IF using monoclonal antibodies (clones HP-6069, 6002, 6050, and 6025), and FITC-conjugated polyclonal secondary antibody. For cases with discrepancy in IgG1 staining, additional direct IF using FITC-conjugated monoclonal antibody (clone 4E3) was performed.

Results: Of 21 cases, 11 (52%) had no staining for IgG1 by direct IF using the clone HP-6091 despite ≥1+ staining by the direct IF using polyclonal antibodies. Similarly, direct IF for IgG1 using the clone 4E3 had negative result in all 10 cases with available tissue. However, indirect IF for IgG1 using the clone HP-6069 had similar staining intensity (within 1 order of magnitude) as direct IF using the polyclonal antibodies (10 of 10). Results of IF for IgG2, IgG3, and IgG4 were similar in most cases.

Conclusion: The choice of antibodies influences the result of IgG subclass staining, especially for anti-IgG1 antibodies, in which 2 monoclonal antibodies (HP6091 and 4E3) appear less sensitive. Although this may be due to unaccounted variables and requires confirmation, our results may partially explain the difference in IgG1 staining in the literature and underscore the need for careful validation.

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IF staining for IgG subclasses is a critical ancillary technique for the diagnosis of monoclonal gammopathy-related kidney lesions containing monoclonal gamma heavy chain, including proliferative glomerulonephritis with monotypic immunoglobulin deposits and heavy chain deposition disease. When routine IF reveals IgG deposits with apparent light chain restriction, a demonstration of IgG subclass restriction provides an additional layer of evidence to classify the deposits as monotypic.[1]

Kidney pathology laboratories use different antibodies to perform IgG subclass staining. Owing to discontinuation of IgG subclass-specific antibody production by The Binding Site (Birmingham, United Kingdom), a widely used source, there is urgent need to validate new methods of IgG subclass staining. However, the impact of the choice of antibodies has not been investigated to date. Thus, we compared the performance of commercially available IgG subclass-specific antibodies in kidney biopsies with the widely used method using antibodies from The Binding Site.

METHODS

A total of 21 cases from August 2022 to March 2023 were included in this study, including 15 cases where IgG subclass staining was performed for clinical indication (due to difference in staining intensity for light chains) and 6 controls (4 membranous nephropathy and 2 lupus nephritis). Diagnoses are listed in Table 1. These kidney biopsy specimens were processed

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Table 1. Results of IgG1 staining with different antibodies

		Observed intensity of immunofluorescence staining (scale of 0 to 3+)			
Cases	Diagnoses	Polyclonal (direct IF, The Binding Site)	Clone HP-6091 (direct IF, Sigma Aldrich)	Clone HP-6069 (indirect IF, Thermo Fisher)	Clone 4E3 (direct IF, Southern Biotech)[b]
1	MN, PLA2R	3+	0	3+	0
2	MN, PLA2R	3+	0	3+	0
3	MN, PLA2R	3+	0	3+	0
4	MN[a]	2+	0	2+	0
5	MN, with LC bias[a]	3+	0	3+	0
6	FGN, with LC bias	3+	0	2+	0
7	PGNMID-IgG1κ	1+	0	tr	0
8	PGNMID-IgG1λ	1+	0	1+	0
9	IgG1κ-amyloidosis	3+	0	2+	0
10	Atypical anti-GBM nephritis	2+	0	2+	0
11	LN, class IV+V	1+	0	TL	TL
12	MN, with LC bias[a]	2+	1+	2+	NA
13	FGN, with LC bias	tr	0	tr	NA
14	FGN, with LC bias	1+	1+	1+	NA
15	LN, class IV+V	3+	3+	3+	NA
16	PGNMID-IgG3κ	0	0	0	NA
17	PGNMID-IgG3κ	0	0	0	NA
18	PGNMID-IgG3κ	0	0	0	NA
19	PGNMID-IgG3λ	0	0	TL	NA
20	PGNMID-IgG1κ	1+	1+	1+	NA
21	HCDD-IgG1	3+	3+	3+	NA

BC, Bowman capsule; FGN, fibrillary GN; GBM, glomerular basement membrane; HCDD, heavy chain deposition disease; IF, immunofluorescence; Ig, immunoglobulin; LC, light chain; LN, lupus nephritis; MN, membranous nephropathy; PGNMID, proliferative GN with monotypic Ig deposits; PLA2R, phospholipase A2 receptor associated; TL, tissue lost; tr, trace.
[a]Associated antigens are not known in these cases.
[b]IF for IgG1 using the clone 4E3 was positive in 2 cases of lupus nephritis.

according to standard techniques for light, IF, and electron microscopy. For conventional IF, the sample was transported in Zeus fixative, and 3 μm frozen sections were stained manually with FITC-conjugated rabbit anti-human IgG, IgM, IgA, C3, C1q, and κ and λ light chain (Dako Agilent, Santa Clara, CA).

Using the residual frozen tissue from the samples submitted for IF, IgG subclasses were determined using FITC-conjugated polyclonal sheep antibodies to IgG1-4 (catalog# AF006, AF007, AF008, AF009, The Binding Site, Birmingham, United Kingdom), with the following dilutions: 1:10 for IgG1 and IgG4 and 1:20 for IgG2 and IgG3. These sections were cut at 2 μm in thickness, air dried, and incubated with antibodies for 30 minutes.

In addition, IgG subclasses were determined using FITC-conjugated monoclonal mouse antibodies against IgG1 (catalog #F0767, clone HP-6091, also known as 8c/6-39), IgG2 (catalog #F4516, clone HP-6014), IgG3 (catalog #F4641, clone HP-6050), and IgG4 (catalog #F9890, clone HP-6025) (Sigma-Aldrich, St. Louis, MO). These sections were cut at 4 μm in thickness, air dried, and incubated for 60 minutes using 1:20 dilution of each antibody, because 1:40 dilution used by the others[2] did not produce sufficient staining intensity for sections cut at both 2 and 4 μm and with 30-minute incubation (data not revealed).

IgG subclasses were also determined by a 2-step (indirect IF) protocol, using unconjugated monoclonal mouse antibodies against IgG1 (catalog #A-10630, clone HP-6069), IgG2 (catalog #05-3500, clone HP-6002), IgG3 (catalog #MH1031, clone HP-6050), and IgG4 (catalog #A-10651, clone HP-6025) (Thermo Fisher Scientific, Waltham, MA). Frozen sections were cut at 3 μm in thickness, air dried, and incubated for 45 minutes with 1:200 dilution of each primary antibody. Sections were then incubated for 30 minutes with FITC-conjugated AffiniPure polyclonal goat anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, catalog #115-095-062) at 1:50 dilution. Two cases stained with secondary antibody alone (as negative control) revealed no significant staining.

Last, in cases where IgG1 staining results differed between the direct IF method using polyclonal antibodies and the direct IF method using clone HP-6091, additional staining for IgG1 was performed using FITC-conjugated mouse monoclonal anti-IgG1 hinge antibody (catalog #9052-02, clone 4E3, Southern Biotech, Birmingham, AL). These sections were cut at 4 μm in thickness and incubated for 30 minutes, using 1:20 dilution of the antibody.

Antibodies were stored as recommended by the manufacturers, and all staining was performed manually at room temperature. Phosphate-buffered saline was used as diluent, except for antibodies from Sigma-Aldrich for which a diluent (catalog #S080983-2) from Agilent Dako was used. Phosphate-buffered saline was

used to wash between the steps. Direct IF using the polyclonal antibodies and monoclonal antibodies (clones HP-6091, HP-6014, HP-6050, and HP-6025) were examined by 2 of 7 pathologists (SK and the diagnosing pathologist), with general agreement in all non-control cases (without blinding). Staining intensities for all immunoreactants were graded semi-quantitatively using a scale (0, trace, 1, 2, or 3+) using Olympus BX41TF microscope.

This study was approved by the Institutional Review Board of Columbia University Irving Medical Center.

RESULTS

Results are found in Table 1 and Supplementary Table S1. Representative images are shown in Figure 1. Diagnoses included a wide spectrum of kidney diseases.

There were 11 cases (52%) that had no staining for IgG1 by direct IF using clone HP-6091 despite at least 1+ staining by direct IF using polyclonal antibodies from The Binding Site (Table 1; mean difference and SD 2.3 ± 0.9). In addition, cases 12 and 13 had reduced staining by direct IF using clone HP-6091 compared with direct IF using the polyclonal antibodies. Of these 11 cases, direct IF staining result against IgG1 using clone 4E3 was negative in all 10 cases with available tissue. In contrast, of these 11 cases, direct IF using clone HP-6069 revealed staining consistent with that observed with the polyclonal antibodies from The Binding Site in all 10 cases with available tissue (i.e., difference in intensity ≤1 order of magnitude; mean difference and SD 0.3 ± 0.4).

Direct IF for IgG2, IgG3, and IgG4, using clones HP-6014, HP-6050, and HP-6025 stained similarly to direct IF using the polyclonal antibodies from The Binding Site (i.e., difference ≤1 orders of magnitude), except for case 2 (IgG2, Supplementary Table S1). Of note, staining intensities by the former method were less intense in 4, 3, and 3 cases for IgG2, IgG3, and IgG4, respectively.

Last, for IgG2, IgG3, and IgG4, staining intensities of direct IF using polyclonal antibodies from the Binding Site and indirect IF were also similar in all cases except for case 10 (IgG4, Supplementary Table S1). Of note, the indirect IF for IgG2 and IgG3 revealed more intense staining in 7 and 3 cases, respectively, by one order of magnitude, as compared with the direct IF using the polyclonal antibodies from The Binding Site.

DISCUSSION

In our experience, the choice of antibodies influences the results of IgG subclass staining. This is particularly true for anti-IgG1 antibodies where direct IF using clones HP-6091 and 4E3 seems less sensitive than direct IF using polyclonal antibodies from The Binding Site, a widely used method that is no longer commercially available. Indirect IF for IgG1 using clone HP-6069 was most similar to the staining for IgG1 using the polyclonal antibodies. IF results for IgG2-4 using antibodies from clones HP-6014, HP-6050, and HP-6025 were

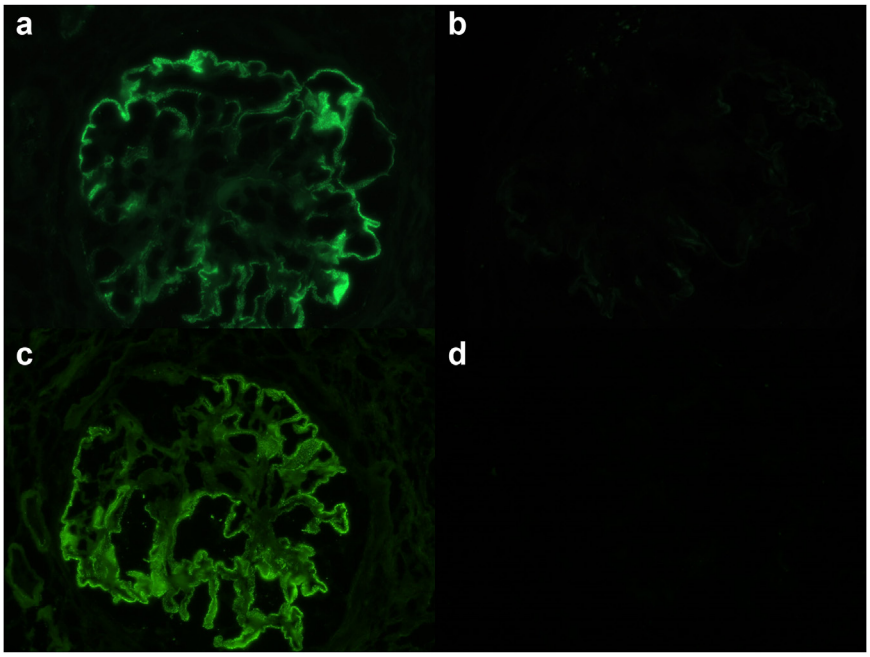

Figure 1. An example of membranous nephropathy (case 4) stained for IgG1 by (a) direct IF using polyclonal antibodies from The Binding Site, (b) direct IF using the clone HP-6091, (c) indirect IF using the clone HP-6069, and (d) direct IF using the clone 4E3. Staining intensities were graded as 2+, 0, 2+, and 0, respectively.

somewhat less sensitive in general than direct IF using antibodies from The Binding Site and indirect IF, although these differences were minor compared with the differences for IgG1.

These differences in staining may be due to the higher sensitivity of polyclonal compared with monoclonal antibodies, and the higher sensitivity of indirect compared with direct IF,[3] especially for IgG2-4. However, the differences in the staining intensity for IgG1 may also reflect differing avidity of these antibodies and/or variable accessibility of the epitopes present in the tissue, because staining intensity for IgG1 using the clone HP-6091 and 4E3 could not easily be amplified by increasing the incubation time, concentration, or section thickness. In addition, although higher sensitivity of polyclonal antibodies could be due to their lack of specificity, prior documentation of insensitivity of HP-6091, compared with HP-6069 by the World Health Organization/International Union of Immunological Societies collaborative studies for both enzyme-linked immunosorbent assay and IF using HP-6069 and HP-6091, and apparent agreement between indirect IF using HP-6069 and direct IF using polyclonal sera argue against this possibility.[4,5] Reduced sensitivity may also be due to variables we did not account for, such as variations in the method of staining (i.e., manual vs. automated), diluent, incubation time, and mounting media.

Of note, our results may explain, at least in part, some of the differences in the results of IgG subclass staining in the literature. For example, compared with the study using the polyclonal antibodies from The Binding Site[6] and monoclonal antibodies from clone HP-6069,[7] a greater proportion of primary membranous nephropathy cases do not appear to stain for IgG1 when the clone HP-6069 or 4E3 is used (positivity for IgG1 in 97% and ∼90% using the polyclonal antibodies from The Binding Site and clone HP-6069 vs. 1%, 15%, 50%, 66% and 85% using clone HP-6091 or 4E3),[8-12] although the differences in staining intensity also could be due to other factors (such as immunohistochemistry vs. IF). Similarly, in fibrillary glomerulonephritis, less frequent staining for IgG1 is found with HP-6091[12,13] than with the polyclonal antibodies from The Binding Site (positivity for IgG1 in 15% and 40% vs. 95% and 100%, respectively).[6,14] However, assessment of the full extent of these differences is limited by insufficient description of the methodology and staining data for individual cases. Regardless, careful attention to the methodological detail is needed to compare results between studies.

Detection of IgG subclasses by IF is not without challenges. Because of their highly (≥95%) homologous primary structure, there are only 3 to 9 unique epitopes specific for each subclass that can serve as potential targets for IF-based detection.[15] The reactivity of these epitopes can be further affected by light chain utilization, allotype, idiotype, and, accessibility of the selected epitope(s) in the tissue.[15,16] This issue may be further compounded by choice of monoclonal antibodies,[4,5,15] and, in the case of polyclonal antibodies, lot-to-lot variations and method used for adsorption by the manufactures.[17] As many renal pathology laboratories will now be forced to adopt new reagents for IgG subclass staining, there is a clear need for critical evaluation of these antibodies in a wider range of cases, optimally with complementary techniques and assessment of interobserver variability.

Our study has several limitations. Given the limited resources and tissue, we did not test more antibodies from more vendors, especially those against IgG1. Similarly, we could not test all permutations of possible variables that theoretically could influence the performance of these antibodies. Given the limited amount of remaining antibodies from The Binding Site and of archived frozen tissue, only a relatively small number of cases could be stained concurrently with other antibodies. In addition, although subjective grading is routinely performed in the clinical setting, grading of the staining intensity and evaluation of interobserver variability were not performed in an objective manner. Last, our results were not validated by an orthogonal technique. Thus, although our results implicate the choice of antibodies as a reason for the discrepancy in reported rates of IgG1 staining in the literature and provide a method to replace the widely used polyclonal antibodies, our study does not necessarily prove whether the widely used method is truly the correct one, without further validation. Whether comparatively low sensitivity of HP-6091 and 4E3 is observed in other applications, such as enzyme-linked immunosorbent assay,[18] also deserves further investigation, as similar findings were observed in flow cytometry.[19]

In summary, we found that the choice of antibodies influences the results of IgG subclass staining, especially for IgG1. Our findings may partially explain the interlaboratory variability for both clinical and research applications and underscore the need of greater cooperation and, possibly, standardization among kidney pathology laboratories.

AUTHOR CONTRIBUTIONS

SK and DS designed the study and drafted the paper. SK made the figures. All authors contributed to the acquisition and interpretation of data, revised the paper, and approved the final version of the manuscript.

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DISCLOSURE

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SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

ARRIVE Checklist.

Table S1. Results of IgG subclass staining with different antibodies.

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