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The Utility of the DNA Microarray Scanner to Simplify the Immunofluorescence Evaluation of Autoimmune Bullous Diseases

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Abstract

A DNA microarray scanner was used as a digital fluorescence microscope to simplify the diagnosis of autoimmune bullous diseases. Frozen sections of skin biopsies were taken from 3 patients with bullous pemphigoid and 1 patient each with lichen planus pemphigoides, linear immunoglobulin (Ig) A disease, and dermatitis herpetiformis. After incubation with cyanine-labeled antibodies, the tissues were scanned at 5- μ m resolution using an instrument originally designed to study gene expression. The microarray scanner's large field of view, unlike that of fluorescence microscopy, allowed a view of the entire specimen, considerably easing the orientation of tissue. All images were diagnostic and included a linear pattern along the basement membrane zone (BMZ) using anti-IgG and anti-C3 in all cases of bullous pemphigoid, a linear pattern of IgG along the BMZ in lichen planus pemphigoides, and a linear pattern of IgA along the BMZ in linear IgA dermatosis. IgA deposition along dermal papillary tips was seen in dermatitis herpetiformis, but a granular pattern was indiscernible at the 5- μ m resolution. The advantage of the microarray scanner over standard fluorescence microscopy include speed, technical ease, large field of view, potential for visualizing multiple antibodies simultaneously in a tissue section, and convenience of digital image archiving.

Keywords

microarray; bullous disease; immunofluorescence; bullous pemphigoid; linear IgA disease

INTRODUCTION

The current method of diagnosis of autoimmune bullous diseases relies on direct immunofluorescence (DIF) microscopy (1,2). This method has limitations imposed upon the user, such as the requirement of a dark room due to the low signal production from the standard fluorophore fluorescein isothiocyanate (FITC), the need for an experienced technician, and the inconvenience associated with the relatively small field of view of a standard fluorescence microscope. A disadvantage of these requirements is the difficulty of teaching medical students and residents in an efficient manner.

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We sought to decrease these limitations in ways that could be easily adopted by other investigators and pathologists/dermatopathologists. First, we tested the relatively newer cyanine fluorophores, Cy3 and Cy5, which are brighter(3), more stable(4), associated with lower background autofluorescence(5), and have sufficiently narrow emission spectra to allow the use of simultaneous fluorescence signals(6). In addition, we used a DNA microarray scanner, an instrument designed originally for the monitoring of gene expression profiling with DNA microarrays(7,8), in place of a conventional fluorescence microscope. This instrument has been shown to have the capability of imaging fluorescently labeled tissue with a very high sensitivity of signal detection, thus allowing for a larger field of view (9,10). In this initial report, we focused entirely on immunobullous diseases. Taken together, the findings reported here represent proof of principle that this experimental approach can be effective.

MATERIAL AND METHODS

Tissue Specimens

Frozen skin biopsy specimens from known cases of the more common autoimmune bullous diseases (previously diagnosed using conventional methods) were retrieved from the Division of Dermatopathology at Roger Williams Medical Center, Providence RI and the Department of Dermatology at Boston University, Boston, MA. The specimens were initially fixed in Michel's media and rinsed in neutral buffer. They were then embedded in frozen tissue media and sectioned using a cryostat into 6- μ m sections, which were then placed onto microscope slides which were stored at -20°C . Cryostat-sectioned frozen tissues of dermatitis herpetiformis were obtained from Department of Dermatology, Boston University Medical Center, Boston, MA, and these were fixed in acetone for 10 minutes before storage at -20°C . The diagnosis of all skin specimens had been previously established by standard methods of light microscopy and DIF using a standard fluorescence microscope. Control specimens included 7 specimens of tissue originating from the final layer of excised tissue during Mohs micrographic surgery and 2 skin biopsy specimens from patients with non-autoimmune bullous conditions.

Immunolabeling Technique

Slides with tissue sections were initially washed in phosphate buffered saline (PBS) at room temperature (twice for five minutes). Some tissue samples were then incubated with goat anti-human immunoglobulin (Ig) G directly conjugated with Cy3 fluorophore (Jackson Immuno Research Laboratories Inc, West Grove PA) for 30 minutes, followed by a 5-minute PBS wash and coverslipping of the slides with 90% glycerol. Because anti-IgA and anti-C3 antibodies were not readily available as direct conjugates to cyanine dyes, these antibodies were used as primary antibodies. Specifically, rabbit anti-human IgA primary antibodies (AbDserotec, Raleigh NC) or rabbit anti-human C3 (complement) primary antibodies (BioGenex, San Ramon CA) were incubated with certain tissue sections for 1 hour followed by a 5-minute PBS wash and a subsequent 20-minute incubation with goat anti-rabbit antibody conjugated to Cy5 (Jackson Immuno Research Laboratories, West Grove, PA). All incubations were performed at room temperature. After a final 5-minute wash in PBS, the slides were coverslipped with 90% glycerol. A fluorescent nuclear counterstain, Sytox (Molecular Probes, Inc., Eugene OR), was used in certain instances.

Imaging with the DNA Microarray Scanner

A diagrammatic overview of the immunolabeling and scanning method is shown in Figure 1. Immunolabeled slides were scanned with a DNA microarray scanner (Genepix 4000B; Axon Instruments/Molecular Devices/MDS Analytical Technologies, Sunnyvale, CA) connected to a Dell Dimension 8250 computer running Genepix_Pro 4.1 software (Axon Instruments/Molecular Devices). Scanner settings were adjusted for each specimen, with typical laser power

settings between 10% and 33% and typical photomultiplier tube settings between 400 and 700 units. After scanning at a 5- μ m/pixel resolution, the image was visualized on a Hewlett Packard 2335 monitor. Brightness, contrast, and magnification were adjusted to optimize the image quality using the Genepix software. The images were saved and exported as TIFF files. In some instances, a Zeiss fluorescence microscope workstation (Everest Workstation, Intelligent Imaging Innovations, Denver CO) equipped with fluorescence cubes for Cy3 and Cy5 was adjunctively used to confirm the deposition patterns of the immune deposits.

RESULTS

Bullous Pemphigoid

Incubation with anti-IgG antibody directly conjugated to Cy3 followed by scanning with the microarray scanner showed a clear linear signal along the basement membrane zone (BMZ) of all 3 specimens of bullous pemphigoid and 1 of lichen planus pemphigoides. Typical scanned images are shown in Figure 2. A similar pattern of linear signal along the BMZ was demonstrated using anti-C3 antibody, which was visualized by a secondary antibody conjugated to Cy5 (Fig. 3). No signal was noted when no primary antibody was used, nor when an anti-IgA primary antibody was used. The nonspecific background fluorescence seemed no higher than that encountered in standard direct fluorescence microscopy. The image produced by the microarray scanner possessed a significantly larger field of view than one that could be obtained through a standard fluorescence microscope. The scanner could portray a histological image of the entire tissue specimen on one screen, yet the image still retained a sufficient signal-to-noise ratio such that the immune deposits could easily be discerned at the BMZ.

Linear IgA Dermatitis

Incubation of anti-IgA antibody followed by a secondary antibody conjugated to Cy5 revealed a clear linear signal along the BMZ (Fig. 4).

Dermatitis Herpetiformis

Incubation of anti-IgA antibody followed by a secondary antibody conjugated to Cy5 showed increased signal along the tips of dermal papillae. The pattern was distinctly different from that of the continuous linear signal along the BMZs of bullous pemphigoid and linear IgA dermatitis. Notably, there was a reproducible signal at the tips of dermal papillae (Fig. 5) that corresponded precisely to the pattern noted when the same specimens were examined by standard immunofluorescence microscopy. The 5- μ m resolution of our microarray scanner, however, was insufficient to display the characteristic granular pattern of deposition that is generally seen with a fluorescence microscope.

Controls

No signal was seen in normal skin specimens or with non-autoimmune specimens incubated with antibodies to IgG, IgA or C3 (Fig. 6 is a representative image for this control).

DISCUSSION

In this communication, we have shown proof of principle that the DNA microarray scanner is effective in producing diagnostic images of immune deposits characteristic of the more common autoimmune bullous disorders. We found complete concordance of the results visualized by the microarray scanner with those demonstrated by conventional immunofluorescence microscopy. The main advantage of the microarray system is the practicality associated with its use. This should be easily appreciated by those who routinely use fluorescence microscopy. The microarray scanning technique, due to its high degree of

sensitivity, large field of view, and the ability to generate images directly onto a computer screen, circumvents the need for a high power objective and darkened room, as needed for standard fluorescence microscopy. These advantages could be used to make DIF more accessible and easier to demonstrate to those in training, including medical students and residents.

There are several reasons for considering our described technique as easy to use. First, cyanine dyes are more intense than the standard FITC fluorophore used in routine DIF. Second, the microarray scanner is more sensitive than a fluorescence microscope in capturing the fluorescence signal. Both the higher signal intensity and increased sensitivity help the scanner to expand its field of view to capture an image of the entire tissue specimen in one view, with immune deposits clearly highlighted at the BMZ. In our hands, this feature allows an easier orientation of the tissue compared with standard fluorescence microscopy. Third, microarray scanners are able to scan in more than 1 wavelength, thereby allowing the evaluation of multiple different antibodies on the same tissue. Another advantage is the ease of image archiving. In conventional clinical practice, images of DIF microscopy of autoimmune bullous diseases are not always routinely archived. The digital microarray images can easily be stored by standard methods of electronic archiving.

Although the novel technique reported here seems to be very promising and could lead to new standards in visualizing DIF, some technical hurdles need to be overcome. The disadvantage of this method is the limited resolution of commercially available scanners that were originally designed for a different purpose, namely, for gene microarray analysis. Therefore, their 5- μ m resolution falls just short of allowing visualization of the typical granular pattern found in dermatitis herpetiformis. Yet despite this lowered resolution, the microarray image for our case of dermatitis herpetiformis is a distinctive one, consisting of discontinuous signals highlighted at the tips of the dermal papillae. It seems that this disadvantage may soon be overcome because just recently released scanners have a resolution of 3- μ m. Another obvious disadvantage is the cost of the scanner. At this time, the method proposed here can be justified at research institutions where the scanner is also being used for gene microarray. However, as in other areas of medicine, it is reasonable to predict that specific modifications of the scanner and technological advances will allow it to be more cost effective in the not-so-distant future. With these considerations in mind, our purpose was to introduce a new modality and show proof of principle for its utility in human pathological diagnosis. At this time, we are not yet suggesting that this novel technique be used to replace standard DIF. Despite its lower resolution, this microarray scanner technique still has several advantages (as listed above) that, in our opinion, outweigh this problem that is now being solved with the new generation of scanners to be released. As the price of new scanners drops, it will be important to analyze the cost effectiveness of this novel approach. We suspect that the price differential will be in favor of traditional DIF methods for the foreseeable future but that important issues related to research and teaching may in time drive larger institutions and laboratories to the scanner approach.

In summary, our results demonstrate that this novel approach can lend potential efficiency to the diagnostic process and possibly lead to a more timely treatment intervention for patients suffering from autoimmune bullous disorders. The simplicity of the technique is such that we have found it practical to use in a Mohs micrographic surgery clinic where a cryostat is readily available. The turnaround time of an hour or two can be considerably less than that of a standard pathology lab where logistics can prolong the turnaround time to days. We have created an algorithm (Fig. 7) based on only 3 antibodies that may be useful for the evaluation of the more common autoimmune conditions encountered in a general dermatology clinic. We are testing the simultaneous use of these antibodies with separate fluorophores using a 3-laser version of the scanner.

Additionally, this approach can be used to evaluate salt-split skin to distinguish bullous pemphigoid from epidermolysis bullosa acquisita. The technique may also be useful for the evaluation of other conditions where immunofluorescence microscopy is used, such as connective tissue diseases, vasculitides, and renal diseases. Furthermore, we have early indication that this technique could be useful in identifying metastases to lymph nodes by melanoma or breast carcinoma, using appropriate melanocytic or carcinoma markers (S. Iwamoto, personal observation, 2008)

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Figure 1. Diagram of the microarray scanner method to evaluate direct immunofluorescence
Frozen sections of skin are cut onto slides and then incubated with either a primary antibody directly conjugated to a fluorophore or a primary antibody followed by a fluorescently labeled secondary antibody. The slides are then scanned with a DNA microarray scanner to create an image on the screen.

Figure 2 **Figure 2. Microarray scan of Bullous Pemphigoid, IgG deposition**

A, Large field of view showing the entire biopsy section, allowing easy orientation of the tissue and visualization of the linear IgG deposition at the BMZ. Note that the large field of view is not obtainable with a fluorescence microscope. B, Close-up view of the same section at the BMZ with a clearer view of the linear IgG deposition.

Figure 3

Figure 3. Microarray scan of Bullous Pemphigoid, C3 deposition

Linear pattern of signal at the BMZ after incubation with anti-complement C3 followed by Cy5-conjugated secondary antibody.

Figure 4

Figure 4. Microarray scan of Linear IgA Dermatitis

The scan of the entire skin punch biopsy specimen shows the expected linear deposition of IgA when incubated with anti-IgA antibody followed by Cy5 conjugated secondary antibody.

Figure 5 **Figure 5. Microarray scan of Dermatitis Herpetiformis**

The IgA deposition shows the expected discontinuous pattern of signal predominating at the tips of the dermal papillae (see arrows). In this scan, the resolution of the scanner may have been too low to optimally highlight a granular deposition pattern.

Figure 6

Figure 6. Microarray scan of control skin

Representative sample demonstrating the complete absence of antibody deposition when incubated with anti-IgG antibody conjugated to Cy3. The arrow indicates the position of BMZ.

Figure 7. Algorithm of direct immunofluorescence patterns of the common bullous disorders
Simplified algorithm using 3 antibodies (anti- IgG, anti-IgA and anti-C3) for the more common bullous disorders encountered. Note that C3 deposition is similar to that for IgG within the BMZ, except that C3 can also be found as a linear pattern in bullous lupus erythematosus, erythema multiforme, and paraneoplastic pemphigus. The asterisk (*) denotes the fact that in herpes gestationis, C3 is more likely than IgG to be found at the BMZ. Copyrighted (S. Iwamoto, 2008).