THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 78:449–450, 1982

Vol. 78, No. 5
Printed in U.S.A.

RAPID COMMUNICATION

Enhancement of Specific Immunofluorescent Findings with Use of a Para-Phenylenediamine Mounting Buffer

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A recently described immunofluorescence mounting buffer containing para-phenylenediamine prevents fading of specific staining in skin sections during microscopic examination, and allows better appreciation of morphological detail. Examination of slides at high powers with intense illumination, as well as improved photomicrographs, are possible with this reagent.

Examination of skin sections stained with fluorescein-tagged antibodies under the immunofluorescence microscope is accompanied by fading of specific staining [1,2]. This fading is particularly prominent when specimens are examined with high-power objectives with microscopes equipped with epi-illumination systems and mercury light sources. As a result of this phenomenon, careful examination of subtle immunofluorescence findings at high powers and photography of these findings are difficult or impossible.

Johnson and Nogueira-Araujo have recently described a simple method of reducing fading of positive immunofluorescent findings in the examination of cells in an antinuclear antibody test [3]. This method involves the use of para-phenylenediamine (PPD) in the mounting buffer. We have used this buffer in the examination of skin specimens by direct immunofluorescence and have found fading of specific staining to be minimal. This reagent has allowed high power examination and photography of immunofluorescence findings that were not previously possible. Use of this reagent has so facilitated our immunofluorescence examinations, that we wish to bring this method to the attention of others.

MATERIALS AND METHODS

Immunofluorescence Procedure

The direct immunofluorescence procedure used by our laboratory is that described by Sams et al [4]. Skin specimens that had been snap-frozen in liquid nitrogen were imbedded in Tissue-Tek II O.C.T. Compound (Lab-Tek Products, Naperville, IL) and sectioned at 4 μ. Slides with sections were washed for 10 min in phosphate buffered saline (PBS), and stained for 30 min with fluorescein-conjugated antisera to human IgG (λ chain specific), human IgA (α chain specific), human IgM (μ chain specific), human C3, and human fibrin. All antisera were purchased from Calbiochem-Behring Corp., (La Jolla, CA) and were diluted 1:10 in PBS for use. After staining, sections were washed 2 times for 10 minutes in PBS, and mounting medium and cover slips were applied. Specimens were stored in the dark at 4° C for 1–2 days until they were examined.

Skin Specimens

Five skin biopsies which had been previously examined by direct immunofluorescence were sectioned and stained in duplicate for the immunoreactants known to be present in the specimens. One slide was prepared with the usual mounting medium; one slide was prepared with the PPD-containing medium. Specimens positive for IgG (pemphigus vulgaris), IgA (dermatitis herpetiformis, Henoch-Schönlein purpura), IgM (lupus erythematosus), C3 (erythema multiforme), and fibrin (erythema multiforme) were examined.

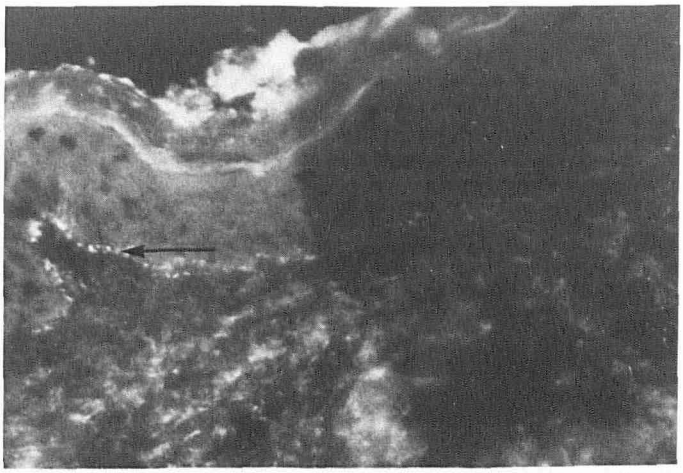

FIG 1. Normal skin of a patient with dermatitis herpetiformis stained for IgA and mounted with the usual glycerol-PBS buffer. The → marks the granular IgA along the dermal–epidermal junction. The burned-out area on the right side of the field was examined at 63× for 30 seconds. This photograph was taken through a 16× objective and exposed 150 seconds.

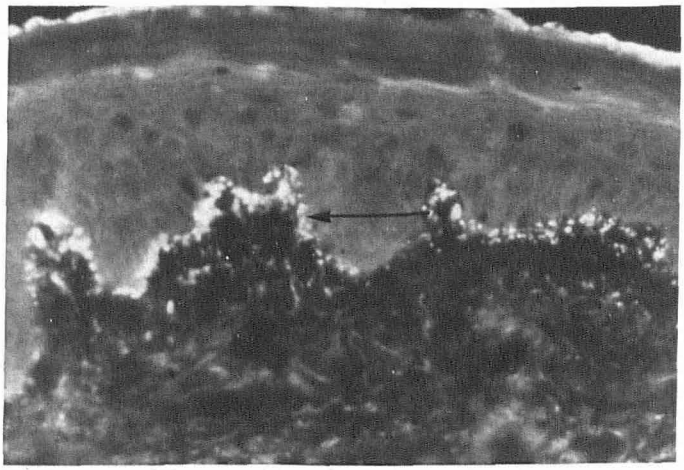

FIG 2. Normal skin of a patient with dermatitis herpetiformis stained for IgA and mounted with the PPD-containing buffer. The → marks the granular IgA along the dermal–epidermal junction. The entire field was examined for 60 seconds at 63× without loss of specific staining. Nuclei stain a brown color, and autofluorescence in the dermis is diminished. This photograph was taken through a 16× objective and exposed 150 seconds.

This work was supported by grants 5 RO1 AI 16637, 5 RO1 CA 26760, 1 RO1 AM 26427.

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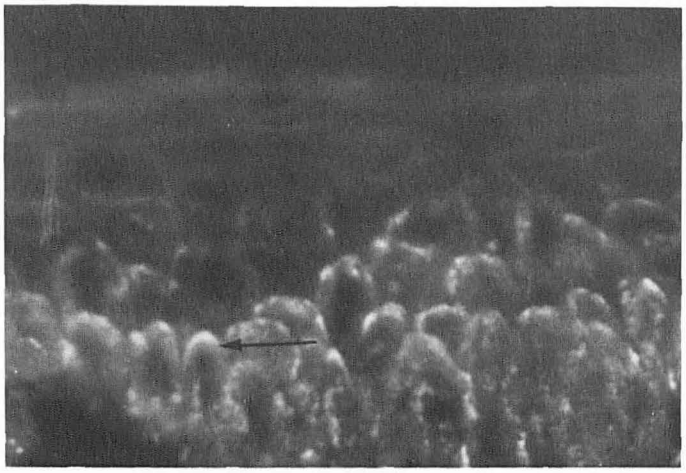

FIG 3. Perilesional skin of a patient with pemphigus vulgaris stained for IgG and mounted with the PPD-containing buffer. The → marks the intercellular IgG within the lower epidermis. No fading of specific staining in this field was seen after 60 seconds of examination at 40×. This photograph was taken through a 40× objective and exposed 60 seconds.

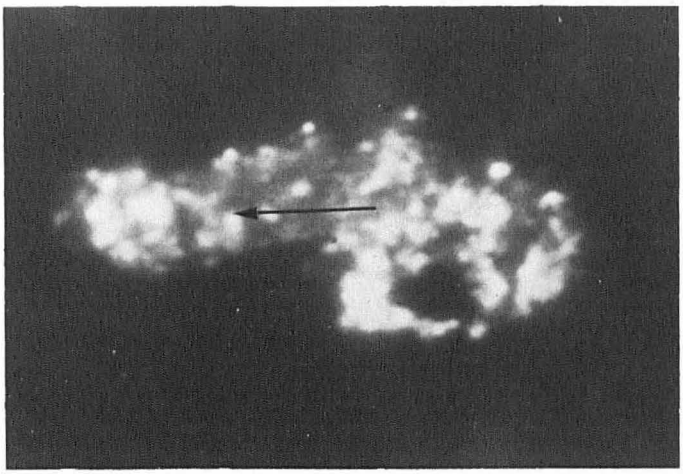

FIG 4. Lesional skin from a patient with erythema multiforme stained for C_3 and mounted with the PPD-containing buffer. The → marks granular C_3 staining in a papillary blood vessel. No fading was seen after 60 seconds of examination at 63×. This photograph was taken through a 63× objective and exposed 45 seconds.

Mounting Buffers

The regular mounting medium consisted of a 1:9 mixture of PBS and glycerol. The special mounting medium described by Johnson and Nogueira-Araujo consisted of 10 ml of PBS containing 100 mg of PPD (Fisher Scientific Co., Fairlawn, NJ) and 90 ml of glycerol (J.T. Baker Chemical Co., Phillipsburg, NJ). The pH of the solution was adjusted to 8.0 with a carbonate-bicarbonate buffer, pH 9.0. This mounting buffer was placed in brown dropper bottles and was stored in the dark, at −20°C. One drop of each buffer was placed over the stained sections before application of cover slips.

Examination and Photography of Slides

Duplicate slides were examined under a Zeiss immunofluorescence microscope equipped with an epi-illumination system, an HBO-100 mercury light source, and a filter-reflector combination for fluorescein (BP 450–490, FT 510, LP 520). Slides were viewed with 16×, 40×, and 63× objectives. Photography was performed with a Praktina camera loaded with Ektochrome 400 film (Eastman Kodak, Rochester, NY).

RESULTS

With all slides prepared with the usual mounting medium, positive immunofluorescence staining faded with prolonged examination. With 40× and 63× objectives, specific staining faded 50–90% after 30 seconds of examination in one field. Although positive findings could be photographed at 16× and 40×, the fading phenomenon during the prolonged exposure time led to photomicrographs much less impressive than the findings seen under the microscope. Specific staining could not be satisfactorily photographed at 63× because of the remarkable fading observed.

With all slides prepared with the PPD-containing buffer, positive immunofluorescence staining did not fade with prolonged high-power examination in one field for 5 min or longer. Furthermore, the PPD stained cell nuclei a brick-red color and allowed recognition of cells and better appreciation of the histological features of the specimen being examined. In addition, the PPD-containing buffer imparted some staining to the connective tissue of the dermis, and with diminished autofluorescence in the background, specific staining within the dermis was accentuated. In photomicrographs taken of specimens prepared with the PPD-containing buffer, specific staining was impressively documented, even through high power objectives (Figs 2,3,4). However, the specific staining in slides with the PPD-containing mounting buffer did gradually fade after a week or longer of storage at 4°C.

DISCUSSION

The specific actions of this buffer in enhancing immunofluorescence findings are not entirely understood. The somewhat diminished background and better morphological detail are not insignificant, but the primary advantage achieved with use of this reagent is the persistence of specific staining through prolonged, intensive irradiation of the slides. Because PPD is easily oxidized at an alkaline pH to a colored substance [5], its role in the mounting medium may be that of an antioxidant. Although an alkaline pH for the mounting medium may accentuate specific immunofluorescence and lessen fading [2], the pH alone does not account for the favorable characteristics of the PPD-containing buffer.

The advantages of this reagent in immunofluorescence procedures includes the following: First, morphological detail can better be appreciated on slides stained by an immunofluorescence procedure. Second, specific immunofluorescent findings, even faint staining, can be examined, even at high powers, without fading. Quality immunofluorescence photomicrographs are therefore possible when this reagent is used. Finally, because specific staining is so well recognized and preserved, higher dilutions of expensive immunofluorescence reagents may be possible with use of this buffer. We feel that others who use immunofluorescence techniques will find this reagent to be of great value.

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