

A STUDY OF IMMUNOPEROXIDASE TECHNIQUE IN THE IMMUNOHISTOPATHOLOGICAL DIAGNOSIS OF AUTOIMMUNE BULLOUS DISEASES

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Abstract

In a comparative study the conventional haematoxylin and eosin staining method was employed concomitantly with the peroxidase-antiperoxidase method in cases of bullous skin diseases. This study includes 40 cases of bullous diseases diagnosed on clinical grounds and confirmed by routine histology using conventional haematoxylin and eosin staining. Skin sections were stained by immunoperoxidase method for the purpose of detection of deposited auto-antibodies. Ten cases of non-bullous inflammatory dermatosis were used as controls for background staining. Our results show this technique to be very specific and sensitive in the histopathological diagnosis of autoimmune bullous diseases. It may be of particular value to the pathologist who often receives formalin fixed material and may help in diagnosing doubtful cases and also if retrospective immunopathology is required (JPMA 38:154 1988).

INTRODUCTION

The diagnosis of bullous skin diseases is usually established on clinical grounds and on the basis of the results of routine histopathological examination of the H & E stained sections. A proportion of these cases' are autoimmune in nature and may be missed by H & E technique. Currently, immunofluorescence staining is being widely employed for the detection of deposited autoantibodies for substantiating the diagnosis of autoimmune bullous disease.¹ Direct immunofluorescent methods applied to specimens of perilesional skin have been reported to be extremely reliable in demonstrating characteristic immunoreactant deposition in the different autoimmune bullous diseases². However, this technique has its limitations and drawbacks which include the requirement for fresh tissue, impermanency of stained preparations, non-specific tissue autofluorescence, and the need for special equipment.³ Nakane and Pierce in 1966 introduced immunoperoxidase method for antigen localization which largely overcame these drawbacks.⁴ Due to its compatibility with fixed and paraffin embedded tissue, this technique has gained wide acceptance. Several variations of the immunoperoxidase method were subsequently developed among which the Peroxidase-Antiperoxidase (PAP) technique elaborated by Sternberger and co-workers has been reported to be very sensitive.⁵ The PAP technique produces permanent staining which is clearly visible by light and electron microscope and provides good morphological details^{6,7}. This study was undertaken at the Armed Forces Institute of Pathology, Rawalpindi, as a part of a research project with an aim to assess the usefulness of this technique in the study of autoimmune bullous diseases by the histopathological demonstration of autoantibody deposition at various sites in these disorders.

MATERIALS AND METHODS

Skin Specimens

Biopsies were collected from bullous skin lesions in forty patients in whom a diagnosis of autoimmune bullous disease was made on the basis of the clinical features and histopathological examination of the H & E stained sections. The various diagnoses included pemphigus (n=29), pemphigoid (n=10), and dermatitis herpetiformis (n=1). The biopsy specimens were taken from fresh bullae as well as the peribullous skin using the scalpel method. Half of the total specimens were fixed in 85 and the other half in 10% formalin. These included 5 fresh specimens of which frozen sections were prepared, in addition to fixation in 85. Specimens of ten patients suffering from various non-bullous dermatoses, fixed in 10% formalin were also included as controls for background staining. Fresh adolescent tonsil sections were used as positive controls for the frozen, formalin fixed and 85 fixed sections. Fixation time was 2-4 hr. for 85 and 6-10 hr. for 10% formalin. The fixed biopsies were processed routinely. 4-5 um thick sections were cut and stained. The fresh biopsies were stored at -20°C and 5-10 um sections were cut and stained.

Fixatives and Reagents

(1) 85 (formol sublimate) (2), Aqueous mounting media. Both prepared using Brown's technique⁸. (3) Formalin (10%) was prepared by diluting concentrated formaldehyde supplied by BDH. (4) Haematoxylin and eosin (H & E) stain. (5) Mayer's haematoxylin stain. Both prepared according to the method described by Luna.⁹ (6) Phosphate Buffer saline (PBS) prepared by Dade & Lewis method.¹⁰ (7) Primary antibodies (IgG, IgM and IgA) along with PAP staining kit (Universal Kit) were supplied by Ortho Diagnostic Inc., New Jersey, USA.

STAINING METHOD

Paraffin sections

Paraffin sections 4-5 um thick were rehydrated and those fixed in 85 were treated to remove mercury pigment before staining. These were incubated in 3% H₂O₂ in cold methanol for 30 min. to reduce endogenous peroxidase staining. Sections were washed in PBS for 5 min. and then incubated with sheep nonnal serum to reduce non-specific staining. This was poured off and the sections were then incubated with primary antibody (rabbit antihuman IgG, IgA and IgM). In negative controls the primary antibody was replaced by control serum, i.e., non-immune rabbit serum. These were subsequently incubated with linking antibody (Sheep antirabbit Ig), labelling antibody, a peroxidase antiperoxidase enzyme complex (raised in rabbit), then finally with 2% AEC (3-amino-9-ethyl carbazole) a chromogen substrate. The sections were thoroughly rinsed in PBS for 5 min. after each step. The slides were then incubated for 1 min. in distilled water, followed by 5 min. in Mayer's haematoxylin and, after blueing, mounted with glycerin jelly mounting media while the sections were still wet.

Frozen sections

5-10 um thick sections were cut, rehydrated and stained in the same way as the paraffin sections with the only difference being the substitution of 3% H₂O₂ by 0.4% H₂O₂.

RESULTS

The criteria for diagnosis in immunoperoxidase staining was the deposition of reddish chromogen substrate at the sites of antibody deposition. The negative and positive controls helped to distinguish the diagnostic staining from background staining.

Table. Results of Immunoperoxidase Staining.

Diagnosis	Antisera					
	IgG		Ig A		IgM	
	ICS	BMZ	ICS	BMZ	ICS	BMZ
Pemphigus (n=29)	23	00	00	00	12	00
Pemphigoid (n=10)	00	05	00	04	00	01
Dermatitis (n=01) herpeti- formis	00	00	00	00	00	00
Non-bullous (n=10) dermatoses	00	00	00	00	00	00

ICS: Intercellular cement substance.

BMZ: Basement membrane zone.

Table gives a summary of the results of the immunoperoxidase staining using specific antisera.

Pemphigus

Out of 29 cases of pemphigus group diagnosed clinically and confirmed on H & E staining 79.3% (n=23) showed unequivocal positive staining in that deposits of reddish granular material were clearly visualized in the intercellular spaces of the epidermis (Figure 1 and 2).

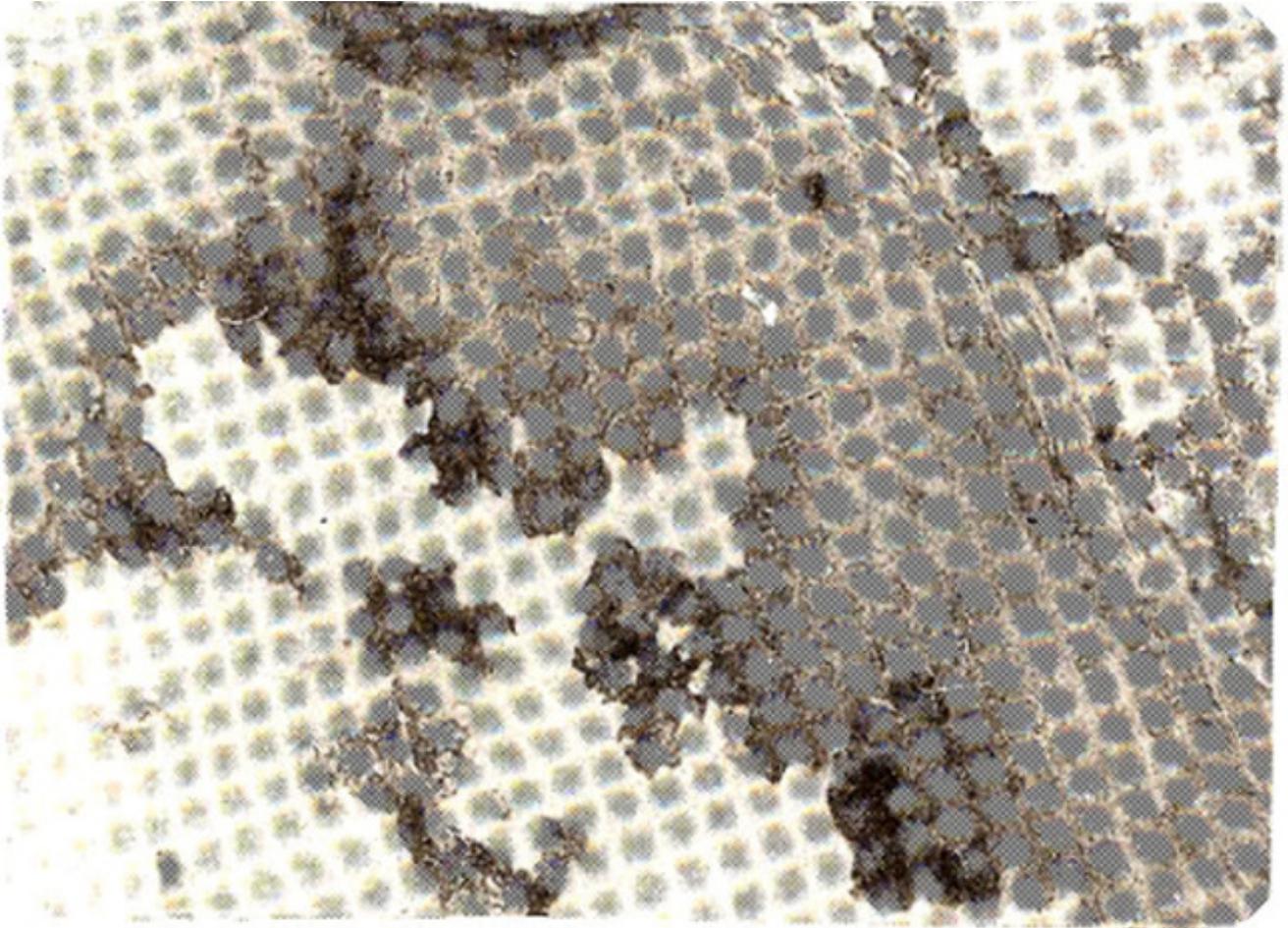


Figure 1. Paraffin section showing deposition of reddish chromogen substrate around acantholytic and epidermal cells surrounding the bullous cavity in a case of Pemphigus vulgaris. Nonspecific background staining is also seen around the RBCs. X 400.

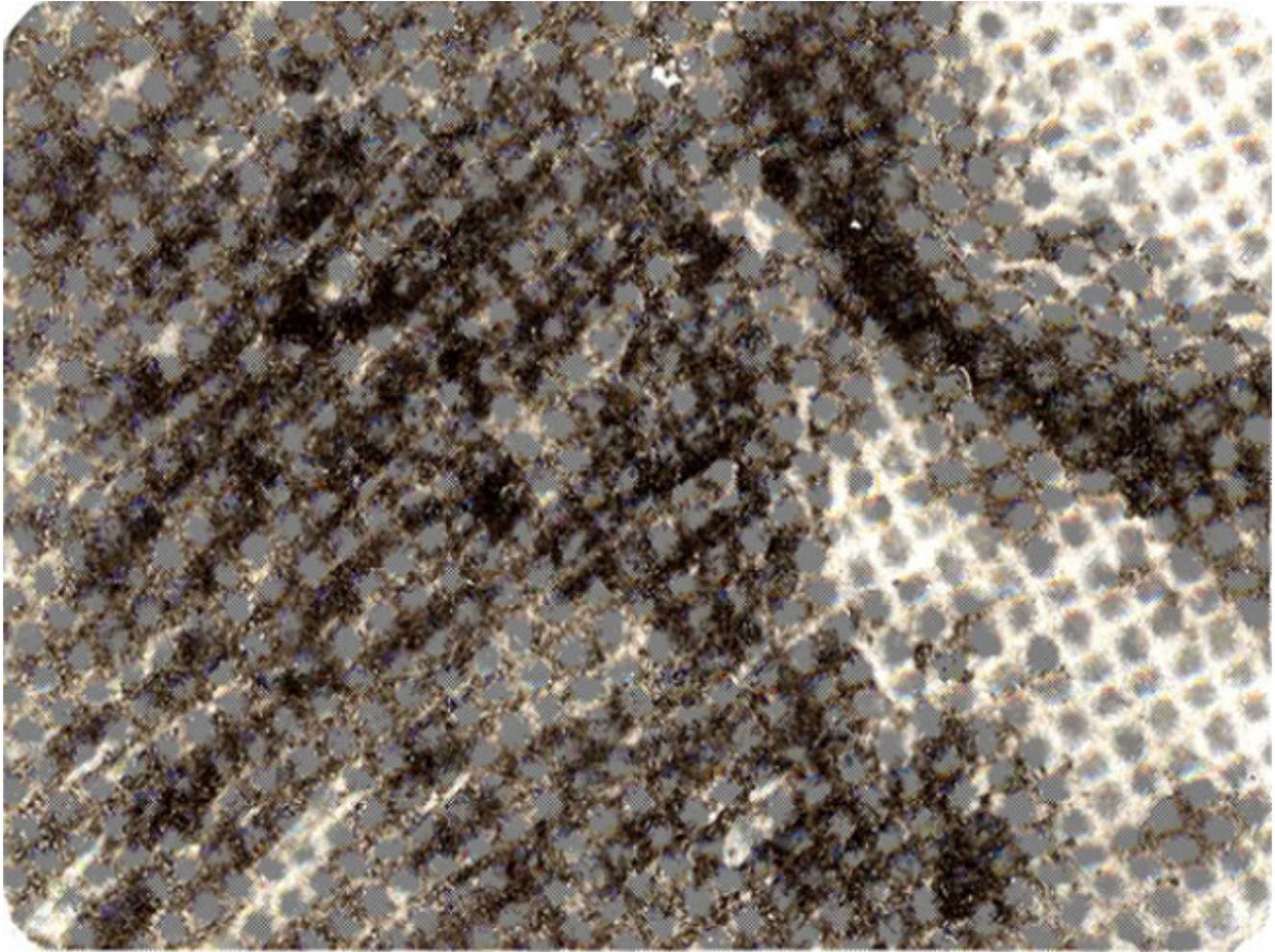


Figure 2. Frozen section showing granular deposits of chromogen substrate at the intercellular spaces staining the deposited autoantibodies in a case of Pemphigus foliaceus. X 1000.

In 4 cases (13.8%) the staining results were equivocal and 2 cases (6.9%) showed no staining and were considered negative. All positive cases showed auto-antibodies of IgG class and in addition about half (52%) showed IgM autoantibodies as well.

Pemphidoid

Ten provisionally diagnosed cases of bullous pemphigoid were stained. Five (50%) were positive for pemphigoid and showed continuous linear deposits of chromogen substrate at the basement membrane zone (Figure 3).

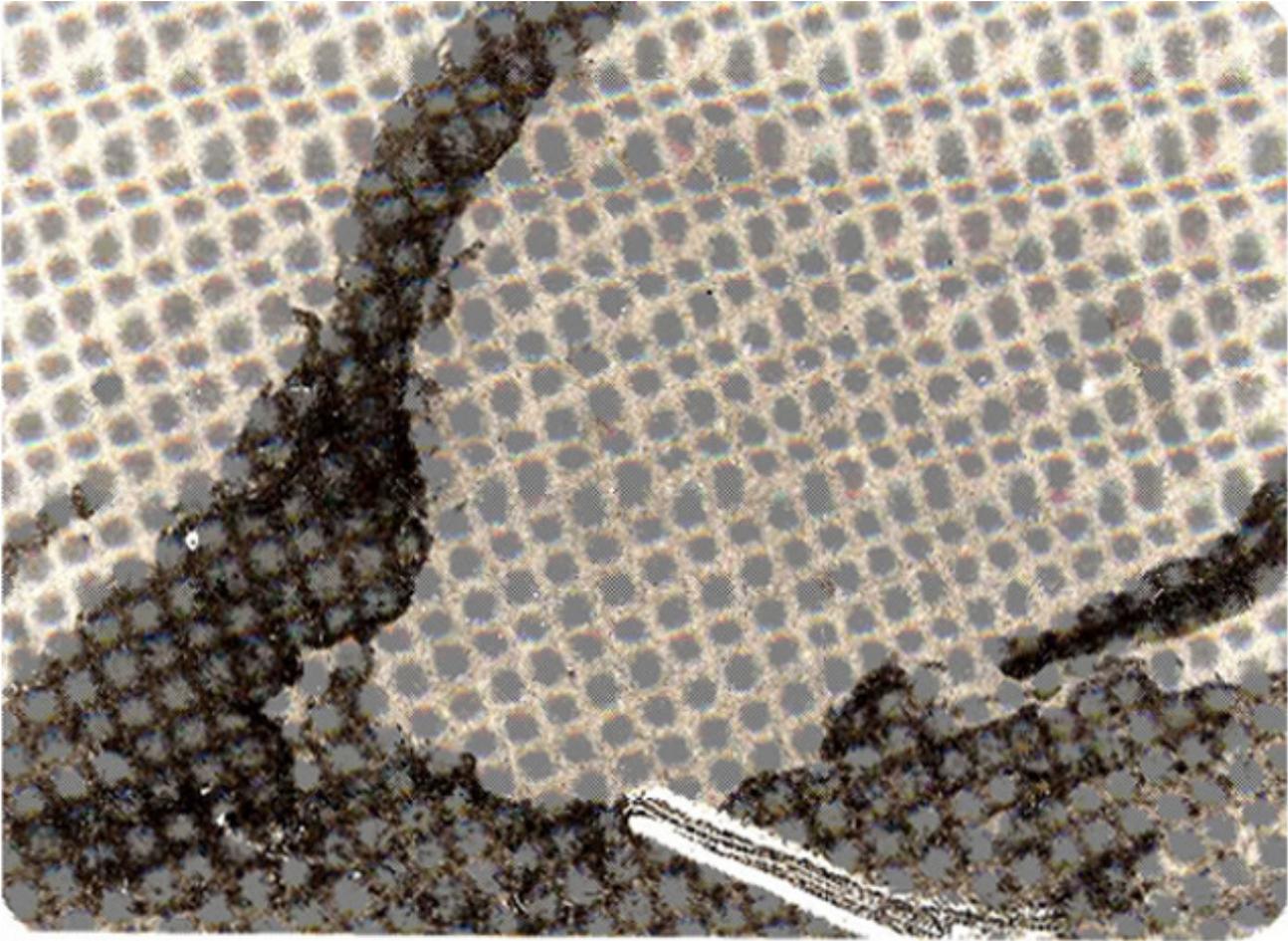


Figure 3. Paraffin section showing staining of autoantibodies deposited at the basement membrane zone in a case of Bullous pemphigoid. X 40.

All positive cases showed IgG autoantibody deposition. In addition, four cases showed IgA and one IgM deposition as well.

Dermatitis herpetiformis

One case of dermatitis herpetiformis was stained. No staining of IgA class at the papillary tips was seen and the staining results were considered negative.

Nonbullous dermatoses

The ten specimens of non-bullous dermatoses stained with immunoperoxidase PAP method showed similar background staining patterns. No autoantibody deposition was seen at the basement membrane, at the papillary tips, or in the intercellular areas of epidermis. No significant difference in the appearances of the PAP stained sections was observed between the frozen or paraffin embedded specimens. Also no dissimilarity was noted in the formalin or B5 fixed specimens.

DISCUSSION

With the advent of enzyme labelled antibodies the immunoperoxidase methods which include the various procedures utilizing the horseradish peroxidase, have found wide application in investigative and diagnostic pathology and are being increasingly used for the detection of antigens in different

localizations. The overall positive results in this study were 70%. This is in close agreement with the results reported by Turbitt et al. who obtained 72% overall positive results.¹¹ The discrepancy between the H & F and immunoperoxidase staining results may partly be due to poor selection of biopsy site and/or improper sampling in the referred cases. The older the lesion, the lesser are the chances of detecting autoantibodies due to repair mechanisms. Another reason for the discrepancy might be due to the different aspects of the disease process that each technique helps to define. The specific diagnostic criteria defined by H & F staining are the morphological changes whereas peroxidase staining helps detect deposition at specific sites. The morphological appearances may not always be specific and might be seen in other dermatological disorders. For example acantholysis is seen in transient acantholytic dermatosis,¹² as well as in benign familial pemphigus¹³. Any other condition with subepidermal bulla formation can mimic pemphigoid or dermatitis herpetiformis.¹⁴⁻¹⁶ The only case of dermatitis herpetiformis in this study failed to show the characteristic granular deposition of IgA. This might be due to the fact that the normal biopsy site for histological details is not the area of choice for demonstrating the immunoglobulins. The specimen from perilesional skin is more likely to yield positive results.¹¹ A marked increase in specificity over the conventional methods of diagnosis, i.e., the clinical picture and the H & E stained section appearances was achieved by this technique. The appearances of PAP stained sections were highly specific and almost always provided a clear-cut picture which was of great help in doubtful cases. The exact site of antibody deposition aided in differentiating between the various bullous diseases, and the detection of different classes of antibodies may prove helpful in understanding their pathogenesis. The characteristic pattern and site of antibody deposition seen in the positive cases of pemphigus and bullous pemphigoid was similar to the specific appearance as described by Turbitt et al. Certain fixatives have been reported to abolish or alter the staining patterns probably due to alteration or destruction of the immunoglobulins.¹⁷ However, our results show good correlation between the formalin and B5 fixed tissues as both fixatives produced comparable results. Further, no difference in immunostaining between the frozen and the paraffin embedded sections was seen. However, it was observed that the morphology was better defined in the paraffin sections of the same biopsy specimen. This indicates that the antigens present survive fixation and that this technique is very suitable for immunostaining of fixed biopsy specimens. Similar results have been reported by Kuhlman and Miller.¹⁸ The biggest advantage of immunoperoxidase technique is the clarity of cellular and structural details, in addition to demonstration of antigens in the same tissue section. The excellent morphological details together with the specific antigen localization helps in understanding the histogenesis of the disease process which is not possible with H & E or immunofluorescence techniques. No special procedure is required and the same paraffin block can be used for H & E and immunoperoxidase staining. The use of paraffin embedded material allows retrospective studies on stored material. It is also useful for pathologists who often receive fixed biopsy specimens. The stain is permanent and can be stored at room temperature.¹⁹ The background staining at stratum corneum, epithelial cells, and at collagen and reticulin fibres poses a problem. It was present in almost all the cases in spite of all the efforts made to reduce it. The negative controls, however, were of great help in this regard. The immunoperoxidase technique has proved to be useful in the diagnosis of autoimmune bullous disorders. However, it is not the replacement of H & E staining which still remains the basic and most useful stain. In conclusion, the routine PAP technique described here should allow easier and more precise correlation between the clinical and the immunological events in the autoimmune bullous diseases. This study emphasizes the importance of the immunoperoxidase techniques for routine use in suspected cases of auto-immune bullous diseases.

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