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Immuno-Electron Microscopical Investigations with a New Tracer: Peroxidase-Labeled Protein A: Application for Detection of Pemphigus and Bullous Pemphigoid Antibodies

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Peroxidase-labeled Protein A, a stable immunoenzyme tracer of high reactivity and comparatively low molecular weight, has been applied in immuno-electron microscopy for detection of bound IgG-type pemphigus and bullous pemphigoid antibodies. Comparing Protein A-peroxidase with peroxidase-labeled immunoglobulins, we obtained similar morphological results in corresponding incubation techniques, but lower nonspecific adsorption of Protein A-peroxidase complexes in tissues. The Protein A-peroxidase molecules showed good tissue penetration abilities. Our rapid one-step incubation procedure led to enhanced preservation of tissue fine structures, without the need of prior tissue fixation. It seems that Protein A-peroxidase is able to replace peroxidase-labeled anti-IgG for immuno-electron microscopical purposes.

Since the first immuno-electron microscopical observations [1,2,4], immunoglobulins in pemphigus and bullous pemphigoid skin have been well characterized [3]. A favored technique is an immunoenzyme bridging method, consisting of an incubation sequence of several specific antibodies and a marker enzyme—peroxidase [4].

We want to report our experience with a new tool for detection of immunoglobulins in immuno-electron microscopy: Peroxidase-labeled Protein A, a tracer molecule of high reactivity for "direct immunoperoxidase" investigations (in a one-step procedure). We have described the details of the labeling procedure, purification of the conjugate and its application in immunohistology (light microscopy) elsewhere [5]. There are certain characteristics and advantages of the new conjugate:

—A comparatively low molecular weight of the Protein A-peroxidase molecule (Theoretical MW 80.000–120.000); thus an enhanced tissue penetration can be expected.

—Markedly decreased nonspecific adsorption in tissues (when compared to IgG-peroxidase).

—Constant immunobiological properties of Protein A.

—Largely reduced incubation times in the one-step procedure, thus enhanced preservation of tissue fine structures. Fixatives need not to be used, thus avoiding the destruction of antigenic determinants in tissues.

Protein A (MW 42.000), a cell wall protein of certain *Staphylococcus aureus* strains, is able to bind exclusively to the Fc part of most mammalian IgG subclasses. In the recent years, it has become increasingly and successfully utilized in various techniques for detection and quantitation of IgG (for review see reference 6).

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Abbreviations:
PBS: phosphate buffered saline

MATERIALS AND METHODS

Reagents

Protein A was obtained from Pharmacia, Freiburg; Peroxidase VI from Sigma, München, 3,3′ DAB × 4HCl and BSA from Serva, Heidelberg, peroxidase-labeled anti-human IgG from Medac, Hamburg, all other reagents from Serva or Merck, Darmstadt.

Tissue specimens

Specimens were obtained from diseased skin with beginning blister formation according to the recommendations of Beutner [7]. Diagnoses were confirmed by immunofluorescence and immunoenzyme technique as described before [5].

Protein A-Peroxidase

Conjugates synthesized according to the method of Wilson and Nakane for labeling of immunoglobulins [5,8].

Immuno-electron Microscopical Procedure

1–2 mm^3 sized skin specimens were washed in phosphate buffered saline (PBS) (0.15 M, pH 7,2) and incubated for 1 hr at room temperature in PBS (0.15 M, pH 7,2), containing Protein A-peroxidase (0.1 mg Protein A/ml), 0.5% BSA, and glucose, galactose and mannose (each 0.03 M) [5]. After washing with PBS (3 × 15 min), tissues were fixed (1 hr) in Karnovsky's formaldehyde-glutaraldehyde fixative (1:1 dil.) [9]. Then followed revelation of peroxidase activity by 1 hr incubation in Graham and Karnovsky's medium, containing diaminobenzidine and H_2O_2 as substrate [9]. After washing (15 min in Tris-HCl buffer 0.05 M, pH 7,6; 2 × 15 min in PBS), postfixation (1 hr at 4°C) in OsO_4 in cacodylate buffer (0.1 M, pH 7,2) ensued. Dehydration and embedding in Araldite was executed routinely; cutting in ultrathin sections, poststaining with uranylacetate and lead citrate, and observation in a ZEISS EM 9 A followed.

Controls

1. Before incubation with Protein A-peroxidase, a "blocking step" with unlabeled Protein A was included to prove the specificity of the autoantibody-Protein A-peroxidase reaction.
2. Endogenous peroxidase reaction was controlled by incubating the tissue specimens only in Graham and Karnovsky's medium.
3. For comparison, incubations were done with anti-IgG-peroxidase and with uninvolved skin.

RESULTS

In Pemphigus lesions, immunoglobulins (Fig 1) were localized in between the epidermal cells. Electron-dense material, indicating the presence of bound IgG, was found as lumpy deposits partially filling the widened intercellular space. There was no obvious local correlation to desmosomes, but the material seemed to stick to the epidermal cell surfaces. Controls (uninvolved skin, dermis, and in blocking experiments) were negative.

Immunoglobulins in bullous pemphigoid lesions were preferentially found in the region of the lamina lucida, extending to the inferior cellular surfaces of the basal layer of epidermis cells. In regions of beginning blister formation, most of the electron-dense material stuck in a band-like pattern underneath the basal layer of keratinocytes, whilst the basal lamina remained mainly with the dermis, with less of the material attached to it (Fig 2). Controls (blocking, dermis, uninvolved skin) were negative.

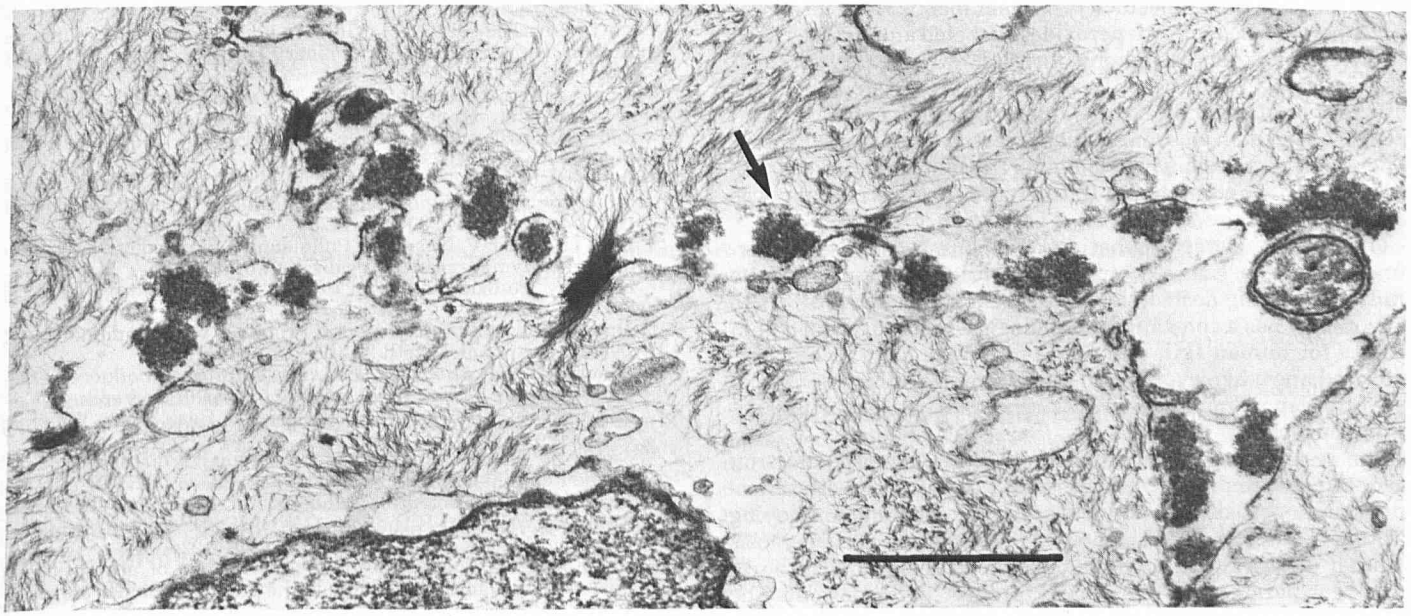

FIG 1. Pemphigus. Immunoglobulins as lumpy deposits of electron dense material (*arrow*) in widened intercellular space. (Protein A-peroxidase, direct method, ×28,000. *Bar* indicates 1μ).

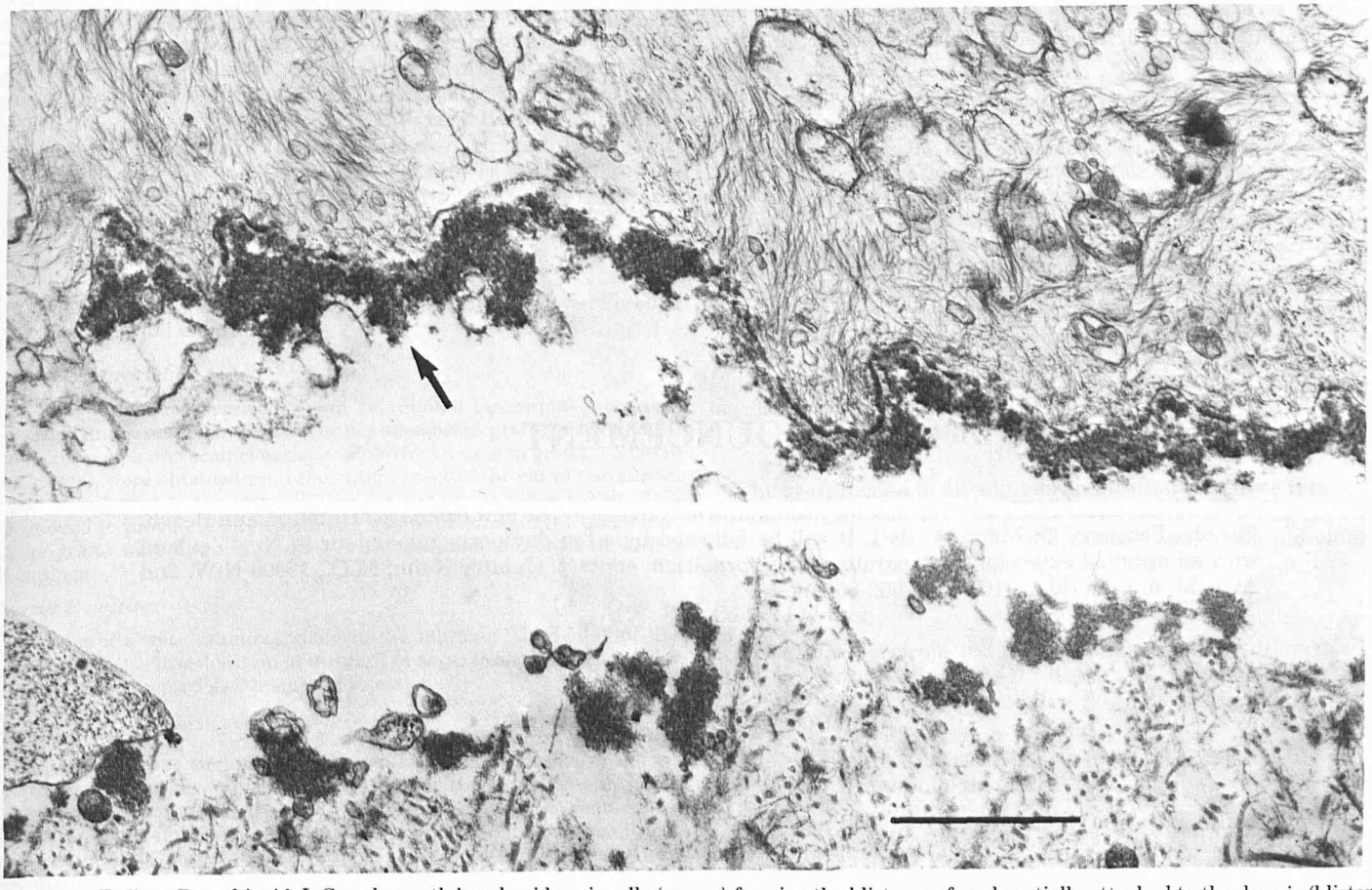

FIG 2. Bullous Pemphigoid. IgG underneath basal epidermis cells (*arrow*) forming the blister roof, and partially attached to the dermis (blister space narrowed). (Protein A-peroxidase, direct method, ×25,000. *Bar* indicates 1μ).

DISCUSSION

Peroxidase-labeled anti-immunoglobulins have proven reliable tracers for immunoglobulin detection in light and electron microscopy, yet the quest still remains for the "ideal" conjugate of low molecular weight (enhanced tissue penetration), high reactivity (minimal functional impairment by the labeling technique), purity (preferably purification by affinity chromatography), and easy immunochemical characterization.

In combination with the labeling technique suggested by Wilson and Nakane [8], and purification procedures by Boorsma and Streefkerk [10], the application of Protein A-peroxidase in immunoenzyme techniques seems to offer most of these requirements [5]. Compared to peroxidase-labeled immunoglobulins, the theoretical molecular weight of the Protein A-peroxidase molecules is low (MW 80.000–120.000, versus 200.000–240.000 MW of IgG-peroxidase).

For comparison of specificity, parallel incubations with Protein A-peroxidase and with peroxidase-labeled anti-IgG (as used in conventional immunoenzyme methods) were executed. In all cases investigated hitherto, reaction patterns were consistent with those obtained by anti-IgG-peroxidase. In contrast, nonspecific adsorption was markedly decreased when using Protein A-peroxidase, especially when adding the monosaccharides glucose, galactose and mannose to the buffer [5]. No reaction was seen in cases of tissue-bound IgA or IgM.

The rapid one-step incubation procedure facilitated preservation of tissue fine structures, and prior fixation could be omitted, avoiding destruction of antigenic determinants.

Protein A has a constant immunological reactivity and a high affinity for human IgG, and does not always have to be characterized anew like every fresh antiserum. Its ability to bind to IgG of other mammalian species renders it useful for investigative purposes.

Our results are in accordance with the immuno-electron microscopical findings of other groups [3], obtained with different immunoperoxidase techniques. We are currently employing Protein A-peroxidase conjugates in investigation and diagnosis of other diseases with immunological features. In our hitherto existing experience it seems that Protein A-peroxidase may be able to replace peroxidase-bound immunoglobulins for immuno-electron microscopical purposes.

REFERENCES

1. Wolff K, Schreiner E: Ultrastructural localization of Pemphigus autoantibodies within the epidermis. Nature 229:59–61, 1971
2. Schaumburg-Lever G, Rule A, Schmitt-Ullrich B, Lever WF: Ultrastructural localization of in vivo-bound immunoglobulins in Bullous Pemphigoid—A preliminary report J Invest Dermatol 64:47–49, 1975
3. Albini B, Holubar K, Shu S, Wolff K: Enzyme antibody methods, in Immunodermatopathology. Immunopathology of the skin. Edited by EH Beutner, TP Chorzelski, SF Bean. John Wiley and Sons, New York, 1979, pp 93–134
4. Holubar K, Wolff K, Konrad K, Beutner EH: Ultrastructural localization of immunoglobulins in Bullous Pemphigoid skin. J Invest Dermatol 64:220–227, 1975
5. Trost TH, Weil HP, Noack M, Pullmann H, Steigleder GK: A new immunoenzyme tracer for localization of antibodies in immunohistology: Peroxidase-labeled Protein A. J Cutaneous Pathol, 7: 227–235, 1980
6. Goding JW: Use of Staphylococcal Protein A as an immunological reagent. J Immunol Meth 20:241–253, 1978
7. Beutner EH: Indications for skin and serum immunofluorescence in Dermatology, Immunopathology of the skin, 2nd edition. Edited by EH Beutner, TP Chorzelski, SF Bean. John Wiley & Sons, New York, 1979, pp 3–28
8. Wilson MB, Nakane PK: Recent developments of the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies, Immunofluorescence and Related Staining Techniques. Edited by W Knapp, K Holubar, G Wick. Elsevier/North Holland Biomedical Press, Amsterdam 1978, pp 215–224
9. Graham RC, Karnovsky MJ: The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291–302, 1966
10. Boorsma SM, Streefkerk JG: Improved method for separation of peroxidase conjugates, Immunofluorescence and Related Staining Techniques. Edited by W Knapp, K Holubar, G Wick. Elsevier/North Holland Biomedical Press, Amsterdam 1978, pp 225–235

ANNOUNCEMENT

The 8th Annual Pediatric Dermatology seminar will convene at the new Eden Roc Hotel, Miami Beach, Florida, February 26–March 1, 1981. It will be followed by a ten day postseminar tour to New Zealand with an optional extension to Australia. For information, contact: Guinter Kahn, M.D., 16800 N.W. 2nd Ave., Miami, Florida 33169. (305-652-8600)