

Evaluation of Cutaneous Amyloid Employing Anti-Keratin Antibodies and the Immunoperoxidase Technique (PAP Method)

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Thirty-one cases of primary cutaneous amyloidosis were studied by the immunoperoxidase technique (PAP method) employing anti-keratin antibodies. All specimens were examined using consecutive paraffin sections to confirm the correspondence between amyloid existing area and reactive sites. All of the sections examined were non-reactive in amyloid deposited sites whereas the epidermis always showed strong reaction with anti-keratin antibodies. (Received August 14, 1983.)

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Increasing number of investigators have suggested that amyloid in primary cutaneous amyloidosis originates from necrotizing keratinocytes (1, 2, 3, 4, 5). This was first suggested by Black & Wilson Jones in 1971 (1) and has been strengthened by reports of the existence of 'colloid-amyloid bodies' in PUVA-treated psoriatic dermo-epidermal junctions (2) and positive reaction of amyloid with anti-keratin antibodies using immunofluorescence microscopy (3, 4, 5).

In recent years, however, Kobayashi (6) and Hashimoto (7), using immunofluorescence microscopy, reported the negative reaction of amyloid with monoclonal anti-keratin antibodies derived against each layer of the epidermis. Based on these reports, the following interpretations are possible: 1) The monoclonal anti-keratin antibodies used were made from only a portion of whole keratin antigen which was usually acquired from human callus. If monoclonal anti-keratin antibodies covering the whole spectrum of keratin material were prepared, the test would become positive. 2) Cutaneous amyloid in primary cutaneous amyloidosis does not contain keratin-like materials or, at most, only small amounts. In addition, Norén et al. (8) recently demonstrated negative reactions of amyloid with anti-keratin using immunofluorescence microscopy in papular and macular amyloidosis.

Further investigations using a variety of methods are needed to evaluate these hypotheses and to re-examine the results of Norén et al. We have utilized the immunoperoxidase technique (peroxidase, anti-peroxidase method, PAP method) to study cutaneous amyloid, and report the results herein.

PATIENTS AND METHODS

Amyloid tissue

Twelve cases of lichen amyloidosis, 12 cases of macular amyloidosis and 7 cases of biphasic amyloidosis were confirmed with stains of thioflavin T and dylon (9). By routine processing, paraffin sections were obtained from all confirmed specimens in 31 patients.



Immunoperoxidase technique

Immunoperoxidase staining was performed after the modified method of Sternberger et al. (10). Sections were deparaffinized in xylene, placed in absolute and 95% alcohol, then sequentially incubated for 20, 5, 20, 120, 20, 20, 20 min with each of the following reagents: 1) 0.1% protease type VII (SIGMA) at 37°C; 2) 3% hydrogen peroxide, at room temperature; 3) normal swine serum prediluted in 0.05 M Tris buffer pH 7.6, at room temperature; 4) rabbit antihuman keratin antiserum prediluted in 0.05 M Tris, at room temperature; 5) swine antirabbit serum immunoglobulins prediluted in 0.05 M Tris, at room temperature; 6) PAP (soluble horseradish peroxidase-rabbit antihorseradish peroxidase) complex prediluted in 0.05 M Tris at room temperature; 7) substrate solution, mixture of 3-amino-9-ethylcarbazole and 0.3% hydrogen peroxide in 0.1 M acetate buffer pH 5.2 at 37°C. After each incubation, except for normal swine serum, slides were rinsed and bathed with Tris buffer or water. After incubation with the substrate solution, they were rinsed with water, counterstained with Mayer's hematoxylin, rinsed with water, dipped ten times in ammonia water, rinsed with water and mounted in glycerol gelatin. All reagents from 2) to 7) were purchased from DAKO Corporation (a subsidiary of Dakopatts A/S of Copenhagen, Denmark), Santa Barbara, USA, as 'DAKO PAP KIT 518'. For a control study, 31 slides from all the patients examined were stained in the same manner with 120 min incubation with Tris buffer instead of with primary antibodies. For a control study, 31 slides from all the patients examined were stained in the same manner with 120 min incubation with Tris buffer instead of with primary antibodies.

Preparation and characterization of antigen and antibody

The keratin fraction was extracted from stratum corneum obtained from calluses of several patients (11). Each extract obtained during step by step extraction processes was examined by SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis (12). The electrophoretic patterns of the keratin fraction and its characteristics in formation of filaments were similar to those reported by Sun & Green (11). Antiserum for keratin fraction was obtained by ordinary immunization techniques in New Zealand White rabbits. Double diffusion tests showed two precipitin bands between the keratin fraction and the antiserum. The keratin fraction did not react with the preimmune rabbit serum.

RESULTS

No reaction products of PAP were found in all the control sections but all the sections treated with primary antibodies revealed strong brown to red-brown reactivity in the epidermis, eccrine sweat ducts and hair follicles. Although we have used consecutive sections to confirm the correspondence between reacted PAP and amyloid deposits (one section for thioflavin T or dylon staining and a consecutive section for the immunoperoxidase reaction), none of the amyloid deposits reacted with anti-keratin antibodies and no reaction products were stained (Fig. 1*a, b*, Fig. 2*a, b*).

DISCUSSION

The PAP method has the following merits: 1) consecutive sections can easily be used for comparative staining; 2) in normal paraffin sections details of the tissue specimens are

Fig. 1. (a) Lichen amyloidosis. Dylon stain. Abundant amyloid deposits (A) are stained red in the papillary dermis. $\times 220$. *(b)* Same case of lichen amyloidosis. PAP method was used in the serial section from the section of Fig. *a* (asterisked rete ridges of Fig. *b* correspond to those of Fig. *a*). Although a strong red brown positive reaction is shown in the whole epidermis, amyloid deposits (A) are completely non-reactive. $\times 220$.

Fig. 2. (a) Macular amyloidosis. Dylon stain. Scattered or grouped amyloid islands (A \rightarrow) are stained red in the papillary dermis. $\times 275$. *(b)* Same case of macular amyloidosis. PAP method was used in the serial section from the section of Fig. *a* (asterisked rete ridges of Fig. *b* correspond to those of Fig. *a*). Although a strong red brown positive reaction is shown in the whole epidermis, amyloid deposits (A \rightarrow) are completely non-reactive. They can be found as small round islands weakly stained compared with the background light brownish colour of collagen fibers. $\times 275$.

better preserved than in frozen sections which are used for immunofluorescence microscopy, and the antigen localization can be detected with more accuracy; and 3) the procedure has high sensitivity for antigen detection (100 to 1000 fold higher than immunofluorescence) (13).

In our study, amyloid stained negatively for keratin determinants while the epidermis always stained strongly positive. Some denaturation of antigen determinants may occur during the process of fixation and embedding for paraffin sections. However, as all the slides showed strongly positive reaction products in the epidermis, we do not believe that only amyloid deposits were denaturated and therefore stained negatively.

From these findings, it may be suggested that the amyloid deposits of primary cutaneous amyloidosis either contain no keratin-like antigens, or only a small amount of keratin-like antigens which are derived from degenerated keratinocytes. These results differ from those of immunofluorescence microscopy which revealed a positive reaction of anti-whole keratin antibodies with amyloid deposits (3, 4, 5), but the reason is unknown. We suggest that: 1) since whole keratin antigens were collected from a variety of calluses, pure antigens are difficult to obtain; 2) materials other than anti-whole keratin antibodies may contaminate the antibody making processes; 3) techniques, reagents and the processes between immunofluorescence and immunoperoxidase methods are quite different.

In addition, since amyloid deposits can easily absorb immunoglobulins, C₃ etc. (14, 15), amyloid deposits in frozen sections may non-immunologically react with anti-keratin antibodies, if the frozen sections better preserve this non-specific absorbability than the paraffin sections. These possibilities are speculative, and further investigations and considerations are needed.

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