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Anti-Keratin Autoantibodies and Lichenoid and Macular Amyloidosis

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Summary. **Anti-Keratin autoantibodies were detected in sera from normal persons and lichen amyloidosus and macular amyloidosis patients. In cord blood, the IgM class antibody was significantly low. But the IgG antibody in cord blood was as high as that in mother's serum. This antibody titer was also seen as low in young mice. These findings support the hypothesis that anti-keratin autoantibodies are produced during the course of aging and maturation.**

Since amyloid in lichenoid and macular amyloidosis has been suggested as deriving from degenerated epidermal keratinocytes, immunoglobulin deposits on amyloid were examined in the present study for their antibody specificity. Immunoglobulins on amyloid were eluted by using a citrate buffer, a glycine buffer, and a NaCl solution. Eluted IgG was demonstrated to react with 50 kd and/or 67 kd keratins. This finding further suggests that the amyloid protein has the antigenic determinant of keratin molecules.

INTRODUCTION

Immunoglobulins (Igs) have been immunohistologically found on amyloid deposits in cutaneous amyloidosis.[28,29,31,37] Among these amyloidoses, nodular amyloidosis (NA) is now known to have an amyloid originating from the Ig light chain. A previous study showed that Igs on amyloid deposits in NA can be removed or depleted, except for one Ig light chain, by washing frozen sections with several buffers. The study further indicated that these Ig deposits are nonspecific depositions.[22]

In lichen amyloidosus (LA) and macular amyloidosis (MA), it was demonstrated that Ig on the amyloid deposits are also diminished by washing pretreatments. It was noted, however, that this removal of Igs was sometimes incomplete or partial.[30]

In the present study, Igs on amyloid in LA and MA were eluted with such buffers as a citrate buffer, a glycine buffer and a high concentration salt solution which had been used to elute antibodies from glomerulus of the kidney.[3,11,40] These eluates were then examined for reactivity with epidermal keratin by using dot immunoblotting and SDS-PAGE immunoblotting.

It has been suggested that the anti-keratin autoantibody (AAb) is raised against keratin protein derived from degenerated keratinocytes.[20] In the present study, human sera from normal persons and patients with LA and MA and cord blood sera and corresponding mothers' sera were obtained, and their anti-keratin AAb titers were examined. As amyloid in LA and MA has been suggested as deriving from the keratin protein of degenerated epidermal keratinocytes,[15] the relationship between the anti-keratin AAb and the pathogenesis of LA and MA was discussed.

MATERIALS AND METHODS

Serum samples

Sera were obtained from ten normal persons, five patients with LA and three patients with MA. Fifteen cord blood samples and corresponding mothers' sera were also obtained. Sera from normal BALB/c mice varying in ages from 0 day to 1.5 years were collected.

Tissue sections

Skin biopsy specimens were obtained from five LA patients, three MA patients and three normal persons. Tissues were frozen with liquid nitrogen, and cryostat sections about 4 μm thick were made for further studies.

Elution study on cryostat sections

Frozen sections of LA, MA, and normal skin were placed on glass slides and surrounded by a bank of paraffin. As much of the reticular dermal portion was removed as possible under dissection microscope. Then slides were washed with PBS and covered for 2 hr at 37℃ with either 0.02M citrate buffer, pH 3.2., 0.1M glycine buffer, pH 2.2, 0.5 M NaCl in 0.01 M Tris buffer, pH 7.4, and PBS.[3,11,40] Solutions were collected as eluates and assayed for reactivity against epidermal keratin. After the elution procedure, sections were examined with immunofluorescent staining by applying fluorescein isothiocyanate (FITC)-conjugated anti-human IgG or IgM goat antibodies (Meloy Laboratories, Springfield, Virginia, U. S. A.) to determine if the Ig deposits remained on amyloid. Sections were also stained with Congo red and Dylon stain to detect amyloid deposits.[41]

Preparation of epidermal keratin

Epidermal keratin was extracted from normal human epidermis.[8,38] Briefly, the epidermis was separated from small pieces of skin tissues by using the ethylenediaminetetraacetic acid (EDTA) separation method.[4] The epidermis was minced and homogenized in 25 mM Tris HCl buffer, pH 7.4. After centrifugation, living layer keratin was extracted by homogenizing the pellet in 8 M urea in a 25 mM Tris HCl buffer, pH 7.4. Murine epidermal keratin was similarly extracted according to the method of Steinert et al.[35,36]

Dot immunoblotting

The dot immuoblotting method was used to detect anti-keratin AAbs in serum samples and eluates recovered from elution procedure by using a Bio-Dot Microfiltration apparatus (Bio-Rad Laboratories, Richmond, California, U.S.A.).[5,17] Every 100 μl of epidermal keratin sample solution was blotted onto nitrocellulose paper. After the paper dried, the paper was incubated with 1% bovine serum albumin (BSA) in Tris-buffered saline (TBS) to block nonspecific binding sites of the paper. After drying, 150-200 μl of serum samples diluted 100 times with PBS were applied on the paper and incubated. After this incubation, the paper was washed with a 0.05% Tween-20 TBS washing solution. The paper was then incubated with biotinylated secondary antibodies directed to human IgG or IgM (Vector Laboratories, Inc., Burlingame, California, U. S. A.). After subsequent washing, the paper was incubated with an avidin-biotin-peroxidase complex (ABC) solution. Immunoreaction was visualized with 4-chloro-1-naphthol (Sigma Chemical Company, St. Louis, Missouri, U.S.A.). The eluates were analyzed by a similar method, substituting eluates for sera.

Analysis of dot immunoblotting by using an image analyzer

In order to estimate the reactivity of antibodies in sera, results of the dot blotting were analyzed with a computerized image analyzer (model TIF-256R, Immunomedica Co., Shizuoka, Japan) to visualize the intensity of the reaction. Nitrocellulose paper after immunoreaction was monitored through a video camera. The image of the paper was processed through an image analyzer. Dots of reaction were scanned, and the density was measured and indicated on densitometric graphs.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

Keratin proteins were electrophoresed in 12% SDS-PAGE according to the method of Laemmli.[27] Proteins were then transferred onto nitrocellulose paper.[39] Each lane was cut into strips. The paper strips were incubated with 1% BSA in TBS and incubated with eluates from the tissues. After washing in a 0.05% Tween-2 0 TBS solution, the strips were further incubated with secondary anti-human IgG or IgM biotinylated antibody. After this incubation, an ABC solution was applied, and the reaction was colored with 4-chloro-1-naphthol.

RESULTS

Anti-keratin AAb activity in serum samples

Dot immunoblotting demonstrated that all the serum samples had an anti-keratin AAb activity. Sera from normal individuals and LA and MA patients showed moderate to strong IgG and IgM antibody activity as indicated by the densitometric analysis on the dot immunoblotting (Fig. 1). Between patients with amyloidosis and normal persons, there was no significant difference in titers. However, in cord blood samples, IgM antibody titers were significantly low, as shown in Fig. 1B. They were lower than their mothers' titers. The IgG antibody activity in cord blood was almost the same as that of corresponding mother's serum.

In mouse sera, both IgG and IgM class anti-keratin AAbs were significantly low in mice younger than 1 week (Fig. 2).

Elution of Igs from sections of LA and MA

When the eluates were tested for their anti-keratin activity, although the reactivity was weak, it was definitely shown that the eluates of a citrate buffer, a glycine buffer, and a NaCl solution from the tissues of LA had anti-keratin IgG antibody activity (Fig. 3A). On the other hand, the IgM antibody activity was not detected in the eluates.

SDS-PAGE and immunoblotting of eluates against epidermal keratin

SDS-PAGE immunoblotting with eluates demonstrated that eluates from LA tissues contained an IgG class antibody activity against keratins of 50 kd and 67 kd. No IgM activity was detected (Fig. 3B).

Immunofluorescent staining of sections

Amyloid deposits were positive for IgG and IgM by immunofluorescent staining (Fig. 4A). This immunofluorescence for IgG and IgM on amyloid deposits in LA and MA were depleted after the elution procedure (Fig. 4B). Even after the elution, the deposits were consistently demonstrated with Congo red and Dylon staining (Fig. 4C).

DISCUSSION

It has been shown that human as well as murine sera contain anti-intermediate filament AAbs.[6,7,14,18,19,23,32,34] Initially these AAbs were found in diseased patients.[1] However, when sensitive methods, such as enzyme-linked immunosorbent assay (ELISA), were used, it was shown that even normal individuals have anti-intermediate filament AAbs.[24,33] It has been speculated that these AAbs are raised against antigens from degenerated cells from various tissues. Therefore, degenerated epidermal keratinocytes are suggested to be the antigens to anti-keratin AAbs.[20]

The present study revealed that adult sera contained substantial antibody activity of both IgG and IgM. However, the IgM antibody showed a very low titer in cord blood compared with mothers' sera. This finding supports the hypothesis that anti-keratin AAb is generated against keratin from degenerated keratinocytes during maturation and aging. High IgG AAbs in cord blood may reflect the IgG AAbs in mothers' sera because of the permeability of IgG through the placental barrier. Low titers of anti-keratin AAbs in young mice, demonstrated by dot

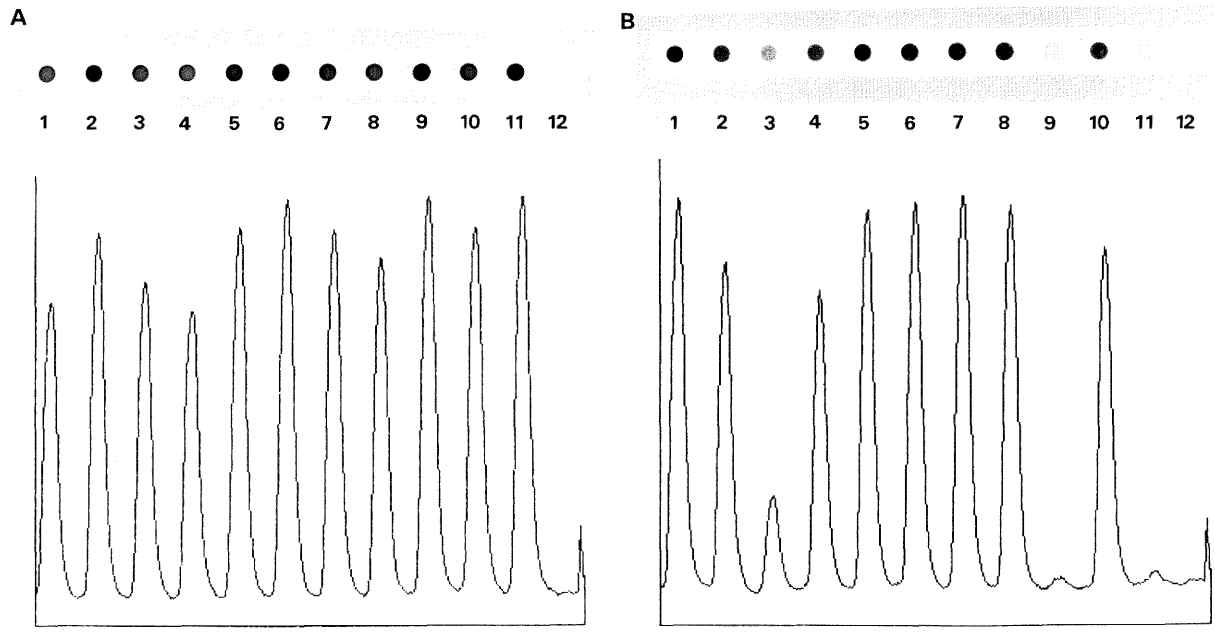

Fig. 1. Dot immunoblotting and its densitometry of human sera. IgG class (A) and IgM class (B) anti-keratin autoantibodies in the sera were detected. Density of each dot represents antibody titer shown in densitometry below. 1-4: normal persons, 5, 6: lichen amyloidosus, 7: macular amyloidosis, 8, 10: mothers' sera, 9, 11: cord blood sera, corresponding to mothers' sera of 8 and 10 respectively, 12: PBS as control. All the sera showed moderate or strong IgG class antibody activity, while IgM class antibody in cord blood sera was significantly lower than in corresponding mothers' sera.

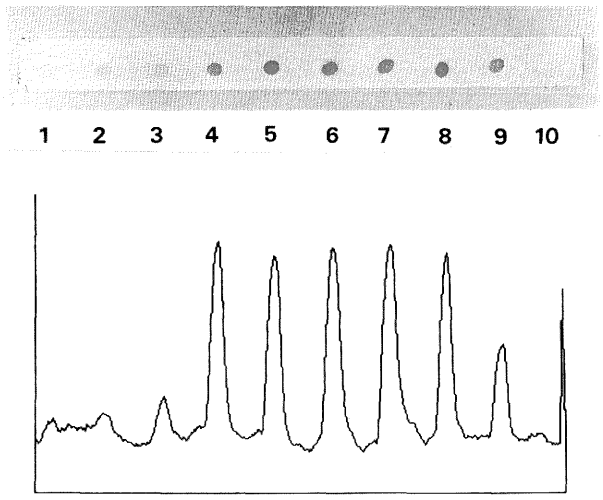

Fig. 2. Dot immunoblotting of mouse sera and its densitometry. Sera from mice younger than 1 week old showed weak antibody activity of IgG class against epidermal keratin. Ages of mice are 0 day (1), 3 days (2), 1 week (3), 2 weeks (4), 1 month (5), 3 months (6), 6 months (7), 1 year (8) and 1.5 years (9). TBS was applied for control instead of serum (10). Peaks in the densitometry represent density of the dots shown above.

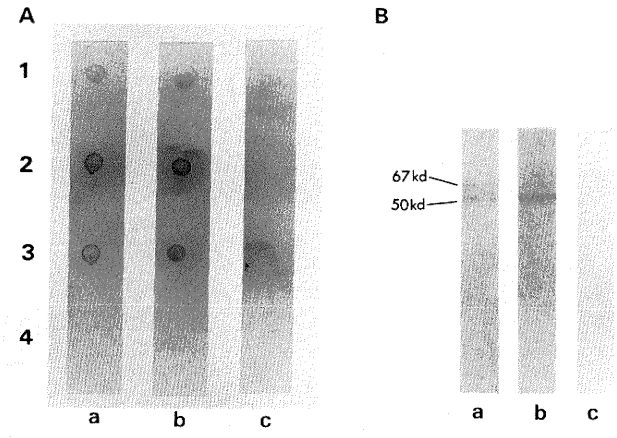

Fig. 3. Dot immunoblotting and SDS-PAGE immunoblotting of eluates from the tissues. A: Dot immunoblotting of eluates from the tissues of 2 cases of lichen amyloidosus (lanes a and b) and normal skin (lane c) against epidermal keratin. Eluates of 0.02 M citrate buffer, pH 3.2 (row 1), 0.1 M glycine buffer, pH 2.2 (row 2), 0.5 M NaCl in 0.01 M Tris buffer, pH 7.4 (row 3), and PBS (row 4). Eluates of citrate buffer, glycine buffer, and NaCl solution show positive reaction for epidermal keratin. B: SDS-PAGE immunoblotting of eluates against epidermal keratin. Glycine buffer eluates from lichen amyloidosus (lanes a and b) show positive reaction for 50 kd and/or 67 kd keratins. Eluates from normal skin do not show positive reaction (lane c).

immunoblotting, further supports the hypothesis.

Amyloid deposits in cutaneous amyloidosis contain Ig deposition.[28,29,31,37] In NA, the amyloid material is now thought to be derived from the Ig light chain. Other Ig fragments are adsorbed on amyloid material nonspecifically.[22] In LA and MA, washing studies on Ig deposits on amyloid have been performed and have

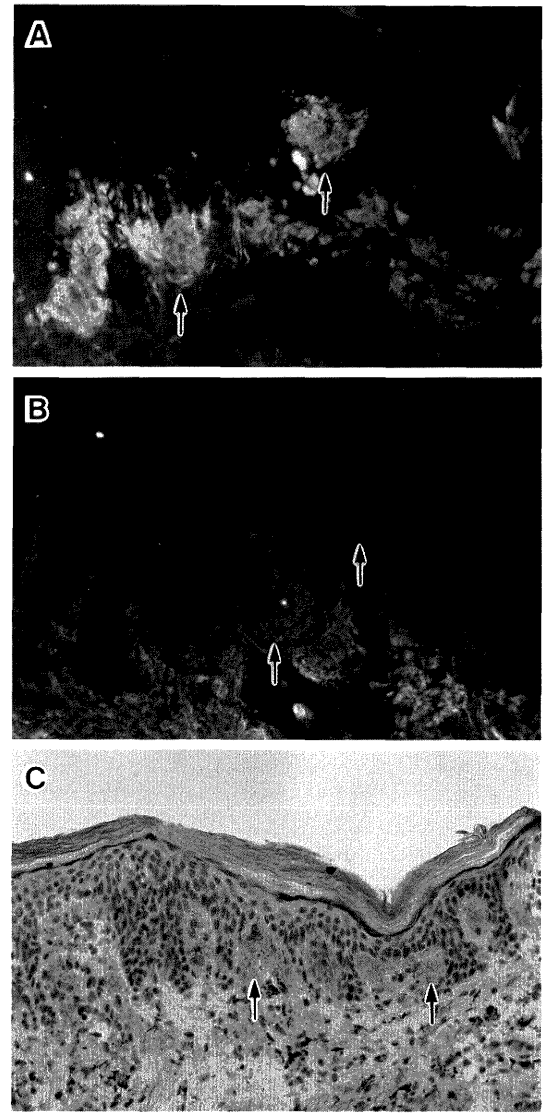

Fig. 4. Immunofluorescent staining and Dylon stain on frozen tissues of lichen amyloidosus. A: IgG is strongly positive on amyloid deposits (arrows) before elution procedure. (×185) B: Positive reaction on amyloid (arrows) is removed after the elution process. (×185) C: Dylon staining demonstrates consistent amyloid deposits (arrows) after elution procedure. (×185)

revealed that the Igs on amyloid were diminished to some extent by the treatment of sections with Tween-20, PBS, and a glycine buffer, pH 7.2.[30] In the present study, this depletion was efficient using buffers used for Ig elution from glomerulus to dissociate antigen-antibody complex.[3,11,40] This finding suggests that the Igs on amyloid are not only nonspecific depositions but also depositions of anti-keratin AAbs specific for antigen.

In the present elution procedure, the IgM AAb activity was not detected in the eluates in spite of the sensitivity of dot immunoblotting. Immunofluorescent staining after elution demonstrated a depleted reaction for IgM on amyloid deposits. Therefore, the failure of detecting the IgM antibody activity may be due to very low levels of IgM on amyloid. The role of IgM antikeratin AAb still needs to be examined.

It has been speculated that the amyloid protein in LA and MA is derived from epidermal keratin from degenerated epidermal cells from ultrastructural and immunohistochemical studies. Ultrastructural observations have shown that tonofilaments in the degenerated apoptotic keratinocytes are closely associated with amyloid fibrils.[26] Immunohistological examinations indicated antigenic similarity of amyloid protein with epidermal keratin. Amyloid deposits are reactive with anti-keratin polyclonal[25] and monoclonal antibodies. EKH-4, which reacts with 50 kd keratin, and MA 904, which reacts with 67 kd keratin, monoclonal antibodies were shown to react with amyloid in LA and MA.[9,10,12,16] Therefore, it has been suggested to call amyloid in LA and MA amyloid K.[15]

In the present study, the eluates were shown to react with 50 kd and/or 67 kd keratin. This finding suggests that AAbs for these keratin molecules are trapped in amyloid protein by the antigen-antibody binding capacity and supports the hypothesis that amyloid protein has antigenic determinants of keratin proteins.

In a normal epidermis, it was found that the apoptotic epidermal cells are covered with anti-keratin AAbs.[13] These immunoglobulins are shown to promote the opsonization of degenerated apoptotic keratinocytes.[21] Therefore, these apoptotic epidermal cells are depleted by phagocytes in normal tissues. In lichen planus, cytoid bodies are also covered with an Ig deposition.[2] However, cytoid bodies do not change into amyloid. On the other hand, in LA and MA, although the amyloid deposits are covered with immunoglobulins, degenerated keratinocytes change into amyloid material probably because there may be some defects in removing such degenerated products from the epidermis. This process still remains to be studied.

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