INTERMEDIATE FILAMENTS: IMMUNOCHEMICAL COMPARISON OF MAJOR POLYPEPTIDES OF ALCOHOLIC HYALIN

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1. Introduction

Intermediate filaments (IFs) are a class of cytoskeletal components found in a number of cells and tissues [1–5]. These filamentous structures (10 nm diam.) are also found in several pathological systems including alcoholic liver disease, where they accumulate in perinuclear, eosinophilic aggregates known as alcoholic hyalin (AH) [6,7]. IFs in diverse tissues appear to consist of major polypeptides of ~50 000 M_r [8–10]. A major polypeptide of 56 000 M_r has also been reported in isolated AH [11]. However, AH also contains 3 additional major components of 51 000–38 000 M_r. To determine the immunological relationship between the systems, studies were undertaken to develop an antiserum specific for the 56 000 M_r polypeptide. While assessing the specificity of antisera derived from immunization with this polypeptide, we found that significant reaction with other AH components also occurred [12]. Those results indicated that a determination of the true extent of cross-reactivity between major AH polypeptides was needed prior to the initiation of any comparative immunochemical studies of IF proteins. The present report involves such a determination.

2. Materials and methods

2.1. *Materials*

Chemicals were obtained from Sigma (St Louis MO). Electrophoresis reagents were from Biorad Labs. (Richmond CA). Peroxidase-conjugated antisera were obtained from Cappel Labs. (Cochranville PA).

2.2. *Isolation of AH*

AH was prepared from human autopsy livers using the ficoll viscosity barrier procedure [11]. This method yields AH in purities of 95–98%.

2.3. *Isolation of AH polypeptides*

Major AH polypeptides were isolated using a modification of the procedure in [13]. Following sodium dodecyl sulfate (SDS) gel electrophoresis [14], major bands, localized by a quick staining method [13], were excised, homogenized in deionized water, and extracted with 0.5% SDS for 24–28 h. Extracts were lyophilized, extracted with methanol (–20°C) to remove Coomassie blue, and dialyzed against 0.1% SDS. The dialyzed extracts were re-lyophilized and suspended in deionized water. The effectiveness of the extraction was assessed using analytical SDS gel electrophoresis (10% acrylamide).

2.4. *Preparation of antisera*

All antigens were solubilized in 0.5–1.0% SDS before use. Antigens (AH, AH polypeptides) were mixed 1:1 (v/v) with Freund's complete adjuvant and injected intramuscularly into the hind legs of male Dunkin-Hartley guinea pigs. Protein (~100 μg) in 1 ml was injected. Boosters in incomplete adjuvant were administered at day 14 and 28. Animals were bled by cardiac puncture at day 38 and sera prepared. Preimmune serum was obtained from each animal prior to immunization.

2.5. *Immunological methods*

Immunodiffusion was carried out in 1% agarose, 0.1% SDS, 0.5% Triton X-100, and 0.9% NaCl as in [4]. Samples were solubilized in 0.5–1.0% SDS before analysis. Immunodiffusion was allowed to proceed for 24–28 h at room temperature.

2.6. *Enzyme immunoassay*

A solid-phase enzyme immunoassay to assay antisera reactivity and specificity was carried out as follows. Antigen (SDS-solubilized AH or AH polypeptides) were dialyzed against deionized water to remove the bulk of the detergent. These antigens were adsorbed to polystyrene tubes as follows. Tubes were preconditioned by incubating with 0.1 M sodium carbonate (pH 9.8) for 1 h at 37°C. The buffer was removed and antigen (0.5–1.0 μg, 100 μl) in pH 9.8 buffer added. The tubes were incubated at 37°C for 3 h, followed by overnight incubation at 4°C. Unbound antigen was removed by aspiration and the tubes were washed 3 times with 0.01 M sodium phosphate–0.15 M NaCl buffer (pH 7.2) containing 0.05% (w/v) Tween 80 (PBS-T). These tubes were stored at 4°C until needed.

Serum reactivity was determined by adding 200 μl appropriately diluted antiserum (in PBS-T; all serum dilutions were made in PBS-T) to tubes containing adsorbed antigen. After incubating at room temperature for 1 h, the samples were aspirated and the tubes washed 3 times with PBS-T. After washing, peroxidase-conjugated goat anti-guinea pig IgG (HRP-GAGP, 0.4 mg/ml, 0.2 ml) was added and the tubes incubated an additional hour at room temperature. The tubes were then washed 3 times with PBS-T and the peroxidase activity remaining with the tubes determined using the 4-aminoantipyrene–phenol–H_2O_2 reagent system [15]. After 30 min color development, the absorbance at 510 nm was determined.

3. Results and discussion

3.1. *Isolation of AH polypeptides*

As reported [11], purified AH is composed of 4 major polypeptides possessing app. M_r 56 000 (56 k); 51 000 and 50 000 (doublet); 38 000 (38 k). In some experiments, the doublet was resolved further into 3 bands. Using preparative gel electrophoresis, these polypeptides have been isolated in purified form (fig.1). Isolated 38 k migrated as a broad band on re-electrophoresis suggesting that some degradation of this component occurred during isolation. Because of the difficulty in excising each member of the doublet, these polypeptides have been used as a single antigen. Each component can be obtained at ~50–100 μg/mg AH protein electrophoresed.

3.2. *Immunological studies*

The presence of active antisera was ascertained by immunodiffusion. Diffusion of anti-AH antiserum against the various antigens resulted in the formation of precipitin lines against AH, 56 k, doublet, and 38 k material (fig.1B). Moreover, fusion of the lines indicated that, with respect to this antiserum, the various AH polypeptides possess common antigenic determinants. Cross-reactivity was also indicated by the fact that anti-56 k and anti-doublet antisera reacted not only with AH and their respective antigens, but also with the other major AH polypeptides (fig.1C–F).

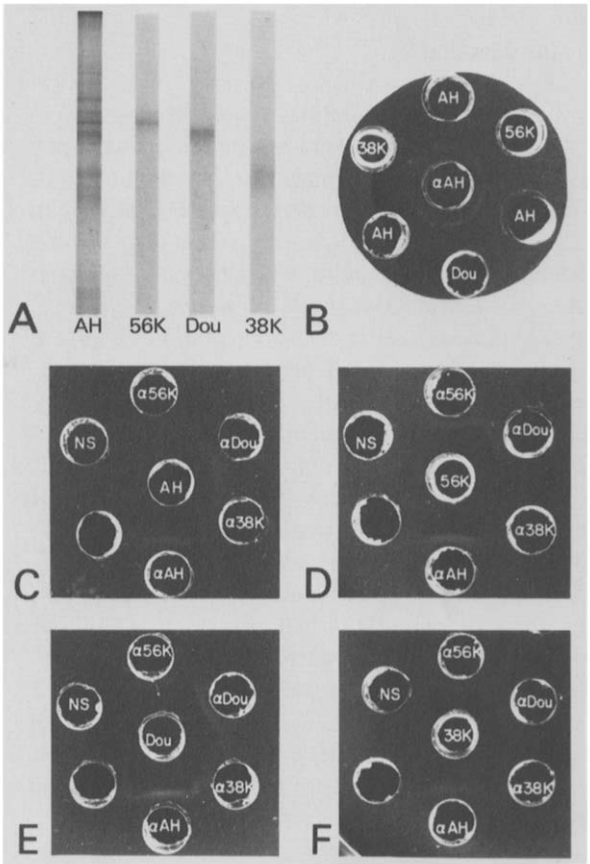

Fig.1. Immunological comparison of AH polypeptides. (A) SDS–polyacrylamide gel electrophoresis of isolated AH polypeptides. AH polypeptides were prepared by preparative electrophoresis and rerun on analytical gel systems. (B–F) Immunodiffusion. Diffusion was carried out in a system containing 1% agarose, 0.1% SDS, 0.5% Triton X-100 and 0.9% NaCl [4]. Samples were solubilized in 0.5–1.0% SDS prior to analysis. Antisera (e.g., αAH, anti-AH antiserum) were used undiluted. Each sample well contained 20 μl (5–20 μg protein). Immunodiffusion proceeded at room temperature for 24–28 h. NS refers to preimmune guinea pig serum.

Anti-38 k antiserum and preimmune guinea pig serum did not form visible lines against any of the antigens tested.

Quantitative information concerning antisera reactivities was obtained using an enzyme-linked solid-phase immunoassay. All 4 antisera reacted with tubes containing adsorbed AH with binding detectable at serum dilutions as low as 1:5000 (fig.2). Furthermore, anti-AH antisera bound to tubes containing each of the isolated AH polypeptides (fig.3). In addition, anti-56 k, anti-doublet, and anti-38 k antisera reacted positively not only with their immunogens but also with the other AH components (fig.3). Although similar reactivities were noted with 56 k and doublet polypeptides, binding to 38 k material was lower. Preimmune guinea pig sera did not bind to any of the antigens under the conditions tested.

These results indicate that the major polypeptide comprising purified AH possess considerable immunological cross-reactivity. A relationship between AH polypeptides is also indicated by another line of evidence. A number of studies have indicated that AH contains a lectin-like activity of broad specificity [16,17]. In an attempt to identify this lectin activity using an affinity labeling technique, we found that all major polypeptides appeared to display activity [17], suggesting that these components possess common properties.

Since human AH preparations are obtained from autopsy material, we have considered the possibility that the multiple polypeptides seen are derived from postmortem proteolytic breakdown of a single protein. Although direct evidence on this point is not attainable, indirect evidence suggests that the complex pattern seen is a function of the material and not an artifact. A similar polypeptide pattern has been described in AH-like filament aggregates isolated from mouse liver [12], a system where postmortem modi-

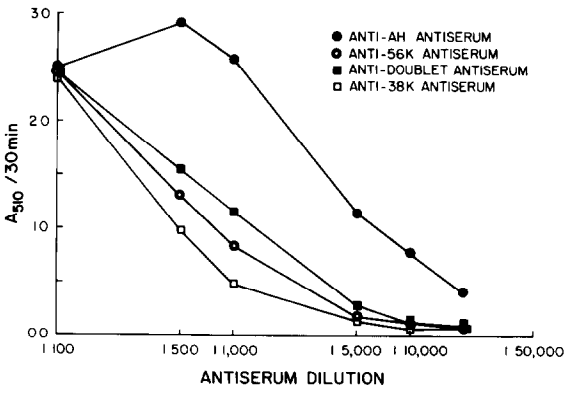

Fig.2. Enzyme immunoassay for antibody. AH was prepared in soluble form, adsorbed to polystyrene test tubes (1 μg/tube), and employed in an enzyme immunoassay as in section 2.6.

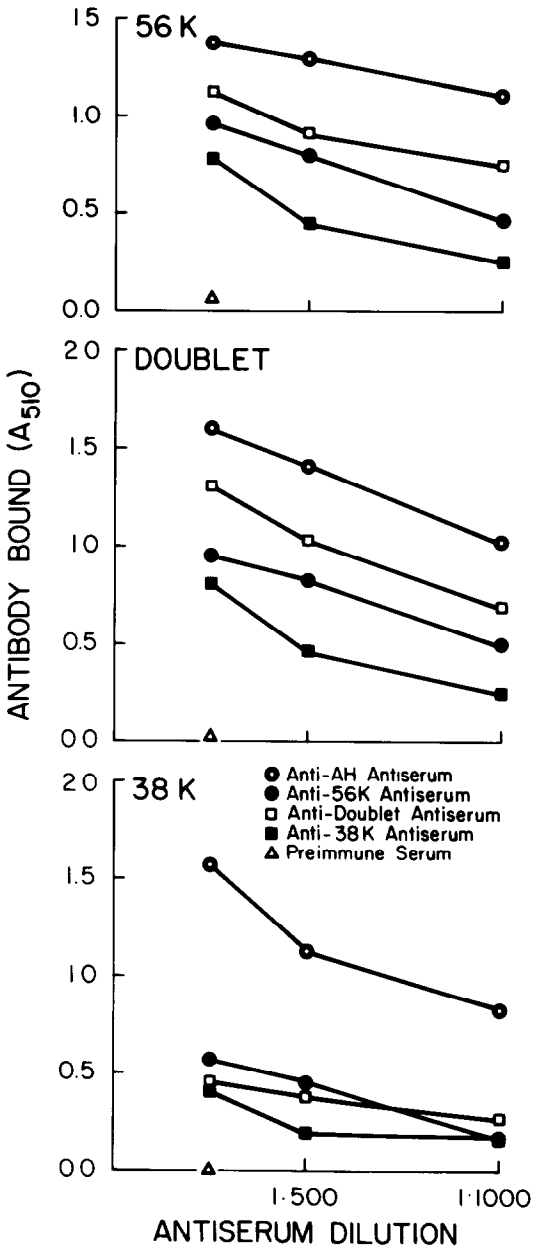

Fig.3. Enzyme immunoassay for antibody. Test tubes containing adsorbed purified AH polypeptides (0.5 μg/tube) were prepared and used in an enzyme immunoassay to determine antibody cross-reactivity.

fication can be controlled or minimized. Furthermore, prekeratin-containing IFs, shown to be related to AH filaments, are also composed of multiple polypeptide components [18]. Although it is tempting to speculate that major AH polypeptides are derived from a single component in the 50 000 M_r range, such a conclusion must await the identification of common sites on the various polypeptides. Such studies are in progress.

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