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INTERMEDIATE FILAMENTS: ANALYSIS OF FILAMENTOUS AGGREGATES INDUCED BY GRISEOFULVIN, AN ANTITUBULIN AGENT

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SUMMARY: Mice fed griseofulvin, an antibiotic with antimicrotubular activity, formed hepatocellular aggregates of intermediate filaments, which resembled those associated with human alcoholic liver disease. These aggregates, termed Mallory bodies, were isolated from both human and mouse liver and the composition of these structures compared. Electrophoretic analysis indicated that the mouse filaments were composed of four major polypeptides (51,000, 47,000, 37,000, and 36,000 daltons). Human Mallory bodies possessed a similar number of components but of different molecular weights (56,000, 51,000, 50,000, and 38,000 daltons). Guinea pig antisera prepared against both whole human Mallory bodies and the major human polypeptide (56,000 daltons) crossreacted with mouse Mallory body material in both immunochemical and immunocytochemical systems. Our findings suggest that the two filament systems possess similar biochemical and immunological properties.

Intermediate filaments (10 nm) are cytoskeletal components with wide distribution in nature (1). On the basis of recent biochemical and immunological evidence, IFs can be grouped into at least five tissue and cell-specific categories (2). IFs are also associated with a number of pathological conditions. One such condition is human alcoholic liver disease, where IFs accumulate as hepatocellular aggregates known as Mallory bodies (3). Recent findings have suggested that MBs are related to prekeratin-containing IFs found in cells of epithelial origin (4,5).

An elucidation of the mechanism of MB biogenesis would be of considerable value in assessing the role of these structures in the pathogenesis of alcoholic liver disease. However, it is apparent that the kinds of studies required to accomplish this goal are not feasible in humans. Recently, a potential model system for MBs was reported by Denk et al. (6). These workers demonstrated that

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Abbreviations: IFs, intermediate filaments; MBs, Mallory bodies; Gris-MBs, griseofulvin-induced Mallory bodies; SDS, sodium dodecylsulfate.

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mice fed the antibiotic, griseofulvin, for extended periods developed hepatocellular lesions essentially indistinguishable from human MBs. Furthermore, since griseofulvin possesses antimicrotubular activity (7), the involvement of the microtubule system in MB biogenesis was suggested (8). Such conclusions are premature and must await a direct comparison of human and mouse MBs to determine whether the two MB systems represent similar entities. The present report describes the isolation of MBs from mice and a preliminary biochemical and immunological comparison of human and mouse aggregates.

METHODS

Induction of Gris-MBs. Swiss albino mice (25g) were fed griseofulvin (2.5% w/w: kindly provided by Ayerst Laboratories, McNeil Laboratories, and Schering Corp.) in standard laboratory chow for 120-140 days. Diets were supplemented with vitamin C (10g Tang per kg dry weight) to avoid excess mortality. At the end of the feeding period, the mice were sacrificed by cervical dislocation, and small pieces of liver tissue processed for light and electron microscopy.

Isolation of MBs. Human MBs were isolated by the Ficoll viscosity barrier procedure of Tinberg et al. (9). Gris-MBs were prepared using the same procedure except that the following modifications were added. Low speed centrifugations were increased to 1100Xg from 365Xg to accommodate the smaller Gris-MBs. Prior to sedimentation through the Ficoll barrier, the crude MB sample was centrifuged through a barrier composed of 60% (w/w) sucrose. This step was added to remove the heme pigment present in liver tissue of mice fed the antibiotic. Human and Gris-MBs were suspended in deionized water and stored for no more than 24 hours at 4^{0}C.

SDS-gel electrophoresis. Electrophoresis was carried out in the discontinuous gel system of Laemmli(10) (10% acrylamide). Human MB polypeptides were isolated using preparative gel electrophoresis as described by Lindstrom et al. (11). The effectiveness of the preparative procedures was assessed by re-electrophoresis of eluted polypeptides.

Preparation of Anti-MB Antisera. SDS-solubilized human MBs and isolated 56,000 dalton (56K) polypeptide were injected intramuscularly into guinea pigs (1:1 with Freund's complete adjuvant). Approximately 100 µg protein were used for each injection. Boosters (incomplete adjuvant) were administered at days 14 and 28 and the animals bled by cardiac puncture (ether anesthesia) at day 38. Sera were stored at -20^{0}C.

Immunological Methods. Immunodiffusion was carried out in 1% agarose, 0.1% SDS, 0.5% Triton X-100, and 0.9% NaCl as described by Yen et al.(12).

RESULTS

Isolation of Gris-MBs. Griseofulvin feeding resulted in the formation of MBs in approximately 80% of the mice in the colony. In cryostat sections,

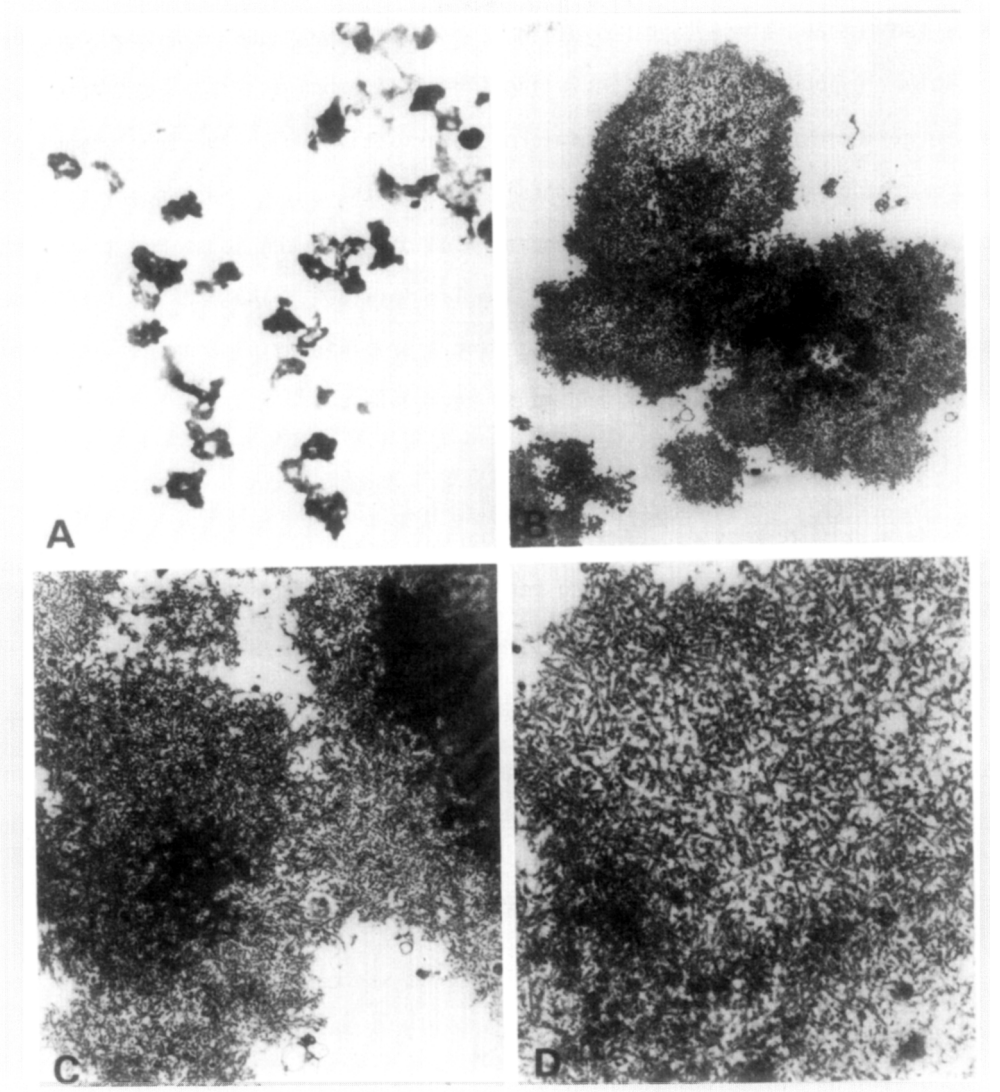

Figure 1. Light and electron microscopy of Gris-MB fractions. A. Light micrograph. The typical clumped, ropelike appearance of MBs is evident. Hematoxylin and eosin. X300. B. Low magnification electron micrograph showing both Type II and Type III MBs. Contaminating membrane vesicles are also visible. X4,200. C. Type III Gris-MB. X10,800. D. Type II Gris-MB. Some of the filamentous material appears to be coalescing into the amorphous substance indicative of the Type III MB. X15,600. Uranyl acetate and lead citrate.

Gris-MBs bound horseradish peroxidase, a property previously reported for human MBs (13) and apparently due to the presence of lectin-like activity (14). To determine whether the two MB systems shared additional properties, purified

preparations of each were obtained using similar isolation procedures. Human MBs were routinely 90-95% pure. The purities of Gris-MB preparations (6 attempts), however, averaged between 70-90%, with heme-containing pigment globules (a consequence of griseofulvin feeding) representing the major contaminant (Fig. 1). Both Type II (totally composed of filaments) and Type III (filaments surrounding an amorphous core) MBs (3) were present in our preparations. Normal mouse liver did not yield a final pellet indicating the high degree of selectivity of the isolation procedure.

Gel Electrophoresis. Gris and human MBs were co-electrophoresed to determine the existence of common components (Fig. 2). Gris-MBs were resolved into four major polypeptides (51,000, 47,000, 37,000, and 36,000 daltons) and a number of minor components. These minor bands varied in intensity from preparation to preparation, while the distribution of major bands remained constant. Similar electrophoretograms were obtained from Gris-MB isolates treated with Triton X-100 and KCl to remove contaminating pigment material (15). Four major components were also detected in purified human MBs. However, the molecular weight distribution of these polypeptides (56,000 [56K], 51,000, 50,000 [doublet], and 38,000

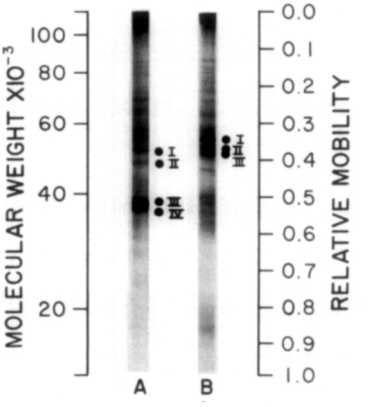

Figure 2. SDS-polyacrylamide gel electrophoresis of MBs. Freshly prepared human and Gris-MBs were solubilized in SDS and mercaptoethanol and electrophoresed on 10% acrylamide gels as described in the text. Approximately 20 µg protein were loaded on each channel. Proteins were stained with coomassie brilliant blue. A. Gris-MB fraction. B. Human MB preparation. Major components are designated by dots. A component of 38,000 daltons was also detected on most human MB preparations.

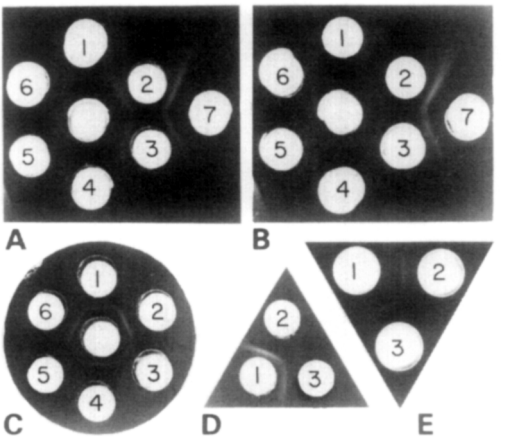

Figure 3. Immunodiffusion. Analysis was carried out in 1% agarose, 0.1% SDS, 0.5% Triton X-100, and 0.9% NaCl. Antigens were solubilized in 0.1-0.5% SDS. All wells contained 20 µl. Sera were tested undiluted. Antigen wells contained approximately 10 µg protein. Diffusion was carried out for 24-48 hours at room temperature. A. Center well: 56K polypeptide; 1: anti-MB antiserum; 2: anti-MB antiserum (different source); 3: anti-56K antiserum; 4-6: Preimmune sera from animals supplying antisera. 7: human MBs. B. Same as 3A except that center well contained doublet polypeptides. C. Center well: anti-MB antiserum; 1: 56K polypeptide; 2: doublet polypeptides; 3: 38K polypeptide; 4: human MBs; 5: Gris-MBs; 6: human MBs. D. Well 1: anti-MB antiserum; 2: human MBs; 3: Gris-MBs. E. Immunodiffusion in the absence of detergents. The analysis was carried out as described above except that PBs was used as the buffer in the agarose medium. Well 1: human MBs; 2: anti-MB antiserum; 3: anti-56K antiserum. Differences in the intensity of the precipitin lines in the various experiments shown are due to varying titres of the sera employed.

[38K] daltons) differed from that obtained for Gris-MBs. Some stained material, reflecting unsolubilized components of unknown composition, remained at the gel origin.

Immunological Studies. Immunodiffusion was used to determine the extent of crossreactivity between human and Gris-MBs. Diffusion of SDS-solubilized human and Gris-MBs against anti-human MB antiserum resulted in the formation of precipitin lines (Fig. 3D). The apparent fusion of these lines indicated that, with respect to this particular antiserum, the two MB preparations contain identical antigenic determinants. Similar results were obtained with antisera obtained from animals immunized with electrophoretically pure 56K human MB polypeptides (not shown). The lines formed were considerably weaker than those

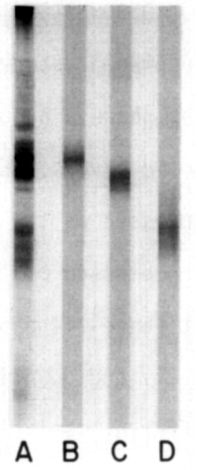

Figure 4. SDS-gel electrophoresis of isolated human MB polypeptides. Major human MB components were eluted from preparative gels as described in the text and re-electrophoresed on the standard analytical system. A. Human MBs; B. 56K; C. Doublet; D. 38K.

obtained with anti-MB antiserum, possibly indicating differences in titre. Although these results indicated a promising start into assessing the nature of crossreacting species in the two MB systems, a further analysis of antiserum reactivity suggested a much more complicated situation. Anti-MB antiserum formed precipitin lines when diffused against all major human MB polypeptides obtained by preparative electrophoresis (Fig. 4,3C). Anti-56K antiserum, however, reacted not only with its respective antigen, but also strongly with doublet material (Fig. 3A,3B) and weakly with 38K polypeptides (not shown). Anti-MB antiserum did not react positively with actin, tubulin, or human immunoglobulin (thought to be present on MBs). Furthermore, the lines observed were not due to any detergent-induced artifact since reactivities were also obtained in native systems (Fig. 3E). In addition, preimmune guinea pig serum did not form precipitin lines against any of the antigens tested.

The results obtained with immunodiffusion were confirmed by immunocytochemical labeling (indirect immunoperoxidase) of cryostat sections of both human and mouse liver. Absorption of sera with MBs resulted in either abolition or reduction of labeling.

DISCUSSION

The results obtained in the present study indicate that MBs formed in mice fed the antibiotic, griseofulvin, share a number of properties with similar structures found in human alcoholic livers. For example, both MBs appear to possess lectin-like activity expressed as the capacity to bind glycoproteins. Furthermore, both MB systems are composed of four major polypeptides in the 30,000 - 60,000 molecular weight range. The polypeptide profile obtained for Gris-MBs differs both qualitatively and quantitatively from that reported by Franke et al. (15). Using a different isolation procedure, they demonstrated the presence of six polypeptides (66,000, 64,000, 57,000, 54,000, 51,000 and 48,000 daltons). It is difficult to rationalize these differences since they did not report the purities of their preparations. Polypeptide profiles obtained from both human and Gris-MBs resemble that reported for prekeratin-containing IFs (15) thereby supporting the notion that MBs are related, at least, in part to this IF subclass.

Our results also suggest an immunological relationship between human and Gris-MBs. However, attempts to determine the nature of crossreacting species were complicated by the fact that human MB polypeptides appear to exhibit significant intracomponent immunocrossreactivity. At present, we do not know whether major human MB polypeptides are essentially different molecules which share some common immunogenic region or whether they are actually modified versions of a single major component. The second possibility is suggested by earlier results which indicated that all three major human MB polypeptides possess lectin activity (14). We are currently investigating whether Gris-MB polypeptides display a similar kind of crossreactivity.

Our findings therefore suggest that experimentally-induced MBs and MBs found in human alcoholic liver disease possess common biochemical and immunological properties. These results, taken together with evidence presented earlier by others (4,5,15), strongly support the potential employment of the griseofulvin mouse system as a model for certain features of alcoholic liver disease. This

system will undoubtedly be most effective when used to study MB biogenesis with special emphasis on the factors involved in the aggregation of normal IFs into these pathological aggregates.

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