Formation and involution of Mallory bodies ("alcoholic hyalin") in murine and human liver revealed by immunofluorescence microscopy with antibodies to prekeratin

(alkoholic hepatitis/liver pathology/keratin/intermediate filaments/tonofilaments)

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ABSTRACT Antibodies raised against prekeratin intensely and specifically stain, in immunofluorescence microscopy, Mallory bodies ("alcoholic hyalin") present in livers of human alcoholics and griseofulvin-treated mice. The high sensitivity of this method allows the identification of small distinct cytoplasmic structures that are observed during early stages of Mallory body formation, especially frequent in the perinuclear cytoplasm, as well as during stages of Mallory body disintegration and disappearance, such as after withdrawal of the drug. In the latter situation, the prekeratin-containing small particles exhibit a characteristic pattern of arrangement in the hepatocyte periphery. Electron microscopy illustrates that such small bodies are heap-like aggregates of typical Mallory body filaments. Immunofluorescence studies with antibodies to isolated prekeratin polypeptides from bovine hoof or muzzle epidermis show that Mallory body filaments, in particular those in human liver, are immunologically more closely related to prekeratin of tonofilaments from living epidermal cells (stratum spinosum). The data indicate that Mallory bodies contain a pathologic form of prekeratin-like material. They also suggest that disorders of cytoskeletal structures of the intermediate-sized filament class are associated with specific diseases and can be visualized and characterized by immunofluorescence microscopy by using antibodies to constitutive proteins of such filaments.

MATERIALS AND METHODS

Human Liver. Liver tissue from patients with alcoholic hepatitis and cirrhosis was removed at autopsy. For comparison, samples of alcoholic fatty liver and of livers without pathologic alterations were examined.

Animals. Male Swiss albino mice (strain Him:OF 1 SPF; Institute for Laboratory Animal Research, University of Vienna School of Medicine, Himberg, Austria) were fed a powdered standard diet (Altromin, Lippe, West Germany) containing 2.5% griseofulvin (Glaxo, Greenford, England) as described (6). Animals were killed after 1, 2, 3, 4, 6, 8, and 12 weeks of continuous griseofulvin feeding, and 1, 2, 3, and 10 days and 3, 8, and 12 weeks after withdrawal of the drug in order to trace development and involution of MBs. In addition, some mice were kept on the following regimen: continuous griseofulvin treatment for 2.5 months, then 2.5 or 3 months on a drug-free diet, then on the griseofulvin-containing diet again for 4 days prior to sacrifice. In another group of animals final griseofulvin refeeding was replaced by the administration of colchicine for 8 days (10) by using a colchicine dosage of 0.5 μg/g of body weight injected subcutaneously twice a day.

Antibodies. Antibodies were (i) guinea pig antisera to total reconstituted, purified bovine hoof (including stratum corneum) prekeratin (5, 8); (ii) IgG from i made monospecific for prekeratin as described (11); (iii) guinea pig antiserum against electrophoretically separated individual polypeptides or polypeptide fractions of bovine hoof prekeratin and desmosome-associated tonofilaments from bovine muzzle (11-13); (iv) guinea pig IgG from antisera to prekeratin from human epidermal tissue obtained from autopsy skin material that was

The abbreviations used are: MBs, Mallory bodies; DT, indicates desmosome-associated tonofilament prekeratin component; PK, indicates prekeratin component from bovine hoof.

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prepared as described for bovine hoof prekeratin (8); (v) guinea pig IgG from antisera to human MBs isolated from autopsy liver according to French et al. (14) and further purified as described by Franke et al. (5); (vi) guinea pig antisera against murine vimentin (9). Controls included preimmune sera and sera absorbed with the specific antigens.

Immunofluorescence Microscopy. Small pieces of liver were quickly frozen in isopentane cooled with liquid nitrogen. Immunofluorescence microscopy was performed on frozen sections as described (5).

Electron Microscopy. Mice were anesthetized with ether, and the livers were perfused through the portal vein first with Hanks' balanced salt solution and then with 2.5% gluteraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Small cubes of liver tissue were postfixed for at least 4 hr in the same fixative. They were then osmicated and processed for embedding and electron microscopy of ultrathin sections according to conventional procedures.

RESULTS

Reaction of Murine and Human MBs with Antibodies to Diverse Prekeratin Preparations and to Human MBs. Antibodies to prekeratin from total bovine hoof epidermis and from bovine muzzle desmosome-associated tonofilaments strongly and specifically decorated MBs in mouse liver (Fig. 1g). When antisera to individual polypeptide fractions of bovine hoof prekeratin (11) were examined, antibodies to polypeptide fractions PK 1-4—i.e., components with apparent M values of 54,000-63,000—showed stronger reaction with mouse MBs (Figs. 1e, 2a, and 3a) than did antisera to components with lower molecular weights (M, of 48,000-52,000). Antisera to the major polypeptides electrophoretically separated from desmosome-associated tonofilaments of bovine muzzle epidermis also strongly stained MBs, and again antibodies to larger polypeptides (M, values: DT 1, 68,000; DT 2, 60,000; DT 3, 58,000) were more effective (e.g., Fig. 1a) than antisera to the smaller polypeptides (M, values: DT 4, 52,000; DT 5, 51,000) (8, 12). It is noteworthy that some of the antibodies to hoof prekeratin and desmosome-associated tonofilaments (especially PK 1-3 and DT 2, 4, and 5) intensely reacted with tonofilament structures in bile duct epithelial cells (Fig. 1a) (15), whereas other prekeratin antibodies (PK 4 and 5, and DT 1 and 3) exhibited no significant reaction.

Antibodies to human epidermal prekeratin also specifically stained MBs in mouse liver (Fig. 1b), and so did antisera raised against isolated human MBs (Fig. 1d, 7, and 8 and Fig. 3b). However, our guinea pig antisera to human MBs did not significantly decorate fibrils of bile duct epithelia of mouse liver and cultured rat kangaroo (PtK2) cells but did decorate cytoplasmic fibrils present in human (Hela) and bovine (BMGE; cf. ref. 8) epithelial cells as well as in frozen-sectioned bovine muzzle epidermis (data not shown).

Antibodies to bovine hoof prekeratin polypeptides stained human MBs only poorly, if at all, in contrast to intensive decoration of human MBs with the antibodies to the large polypeptides of desmosome-associated tonofilament prekeratin (DT 1-3; Fig. 1e). Hepatocytes of normal human liver and of alcoholic fatty liver exhibited only very poor, if any, reaction with antibodies to prekeratin, as described in rodent liver (15), most probably reflecting the small amounts of tonofilaments present.

MBs of murine and human origin were not stained to a significant extent by antibodies to murine vimentin (Fig. 1e) and to actin.

Patterns of MB Formation and Involution. Mouse liver damaged by griseofulvin administration was examined by using antibodies to bovine prekeratin, to individual prekeratin components, and to MBs on consecutive sections of frozen liver tissue. Agreement of immunolocalization was observed with the different antibody preparations. MB-like material was detected in some mice as early as 4 weeks after commencement of griseofulvin feeding in the form of small granules that were distributed either diffusely in the cytoplasm of scattered liver cells or, more characteristically, in prominent perinuclear arrays. Such perinuclear granules seem to be typical for early stages of MB formation (Fig. 1a, d, and e and Fig. 2). After 6 weeks of continuous griseofulvin administration, sporadic hepatocytes in centrolobular position with irregularly ramified MB-like structures and aggregates of confluent prekeratin-containing granules were observed. At 8 weeks, MB-containing hepatocytes were already numerous and were arranged in groups of 4-10 cells, mostly in the center of the lobules surrounding the central veins. At this stage, MBs in many hepatocytes were large and exhibited irregular, reticular contours or coiled organization. They were often, but not always, in paranuclear position, sometimes forming thick perinuclear rings (Fig. 1e). However, despite the presence of typical fully developed MBs in some cells, adjacent, hepatocytes often still exhibited small granules of MB material or showed arrangements suggestive of stages of confluence of small granules (Fig. 1d and e), indicating continuous development of MBs under sustained griseofulvin administration. Prolonged griseofulvin feeding resulted in only quantitative changes of the pattern in that hepatocytes containing MBs in various stages of development increased in number, and, consequently, the original centrolobular predominance of MB-containing liver cells disappeared. At such later stages, extracellular MBs were sometimes detected by immunofluorescence microscopy in the sinusoidal lumina or were taken up by Kupffer cells. MBs in cells of hyperplastic nodules (6) were identical in their immunoreactivity to those present in hepatocytes.

After discontinuation of griseofulvin feeding, the intensity of decoration of MBs with antibodies to prekeratin and to human MBs progressively decreased, and often a nonfluorescent core portion was visible (Fig. 1f). The number of fluorescing MBs gradually decreased. Concomitant with the disappearance of the typical large MBs, small granules strongly decoratable with antibodies to human MB-material and to prekeratin appeared at the periphery of the hepatocytes (Fig. 3). In some hepatocytes, mostly in centrolobular ones, these granules persisted for up to 2-3 months after drug withdrawal.

When mice, which originally had been fed a griseofulvin-containing diet for 3 months, were kept on a griseofulvin-free diet for 1-3 months and then griseofulvin was readministered for 4 days, large typical MBs reappeared within 4 days (cf. ref. 6). A similar effect of fast reinduction is observed when colchicine is injected for a total of 8 days (10). Such reinduced MBs reacted with antibodies to prekeratin and to human MBs but not with antibodies to vimentin (Fig. 1g-i). Typical MBs can be reinduced with griseofulvin as well as with colchicine in livers that still contain hepatocytes with peripheral granules of MB-like material but also in livers in which no trace of MB-material is detected in the control animals.

Electron Microscopy. When early stages of MB formation—i.e., when only a few hepatocytes show granules positively reacting with antibodies to prekeratin or human MB-material—were examined by electron microscopy, small heaps (diameters ranging from 0.1 to 1 µm) of randomly oriented typical fimbriated filaments were seen in locations, often around the nucleus (Fig. 2a and b), corresponding to those
described by immunofluorescence microscopy (Fig. 2c). A conspicuous association of these small aggregates of MB filaments with tufts of typical tonofilament bundles was seen (Fig. 2b). MB material arranged in the hepatocyte periphery after withdrawal of the drug showed an identical filamentous ultrastructure.
DISCUSSION

MBs experimentally produced in mice by griseofulvin feeding strictly conform to those arising in alcoholic hepatitis and various non-alcohol-associated liver disorders in their light and electron microscopic appearance (5, 6). Moreover, human and murine MBs are closely related immunologically because both react with antibodies to tonofilament prekeratin of bovine muzle epidermis and to isolated human MBs. This substantiates our concept (5) that MB formation, also in human liver, represents a special form of abnormal hyperkeratosis. The finding that material contained in MBs of different species crossreacts, which agrees with data of French et al. (16), appears to reflect the immunologic relatedness observed with prekeratins from various vertebrates (8, 11, 17). Whether the observed difference of reaction of antibodies to bovine hoof prekeratin between murine and human MBs reflects different composition of the prekeratin or the MBs in these species is not known. In addition, it remains to be clarified whether prekeratin-like material accumulated in the filamentous components of MBs represents an abnormal assembly of normal hepatocyte prekeratin filaments or is composed of prekeratin-like proteins specifically synthesized during treatment with griseofulvin in mice or during excessive chronic alcohol ingestion in humans. In this context it is, however, worth stating that MBs can contain, in addition to the prekeratin-like proteins, other materials, possibly enriched in the granular portions of MBs (cf. ref. 3) and in small membranous inclusions often associated with MBs (3, 5). Therefore, although our data show that MBs in general contain prekeratin-like proteins in their filamentous components, they do not exclude the occurrence of other materials (i.e., non-

FIG. 2. (a) Electron micrograph of a hepatocyte showing small (precursor) paranuclear aggregates of MB filaments (outlined area and arrowheads). Bar denotes 1 μm. (b) Higher magnification from the area outlined in a. Note the occurrence of typical tonofilament bundles in the immediate vicinity of small MBs (arrows). Bar denotes 1 μm. (c) Immunofluorescence microscopy of a corresponding stage of MB formation with antibodies to bovine hoof stratum corneum prekeratin fraction PK 1, showing perinuclear MB granules. Bar denotes 20 μm.
prekeratinous substances) in some MBs or in MBs in general.

In several other epithelial cells, abnormal hyperkeratosis has been correlated with vitamin A deficiency (cf. ref. 18). In preliminary biochemical determinations of vitamin A content of mouse liver under griseofulvin treatment and during subsequent drug withdrawal, a correlation of appearance and size of MBs with lowered hepatic vitamin A levels has been observed (unpublished results). For example, we have found hepatic vitamin A contents of 30% and 13% of that present in control animals after 12 and 60 days, respectively, of griseofulvin treatment. These findings, together with clinical and pathological observations of reduced vitamin A contents in livers and blood sera of patients with various forms of liver damage, alcoholic hepatitis included (refs. 19 and 20; see also ref. 21 for review), as well as in other conditions known to result in MB formation (22), are compatible with the notion that vitamin A deficiency may be involved in the initiation of MBs as it is in hyperkeratotic deviations in other epithelia.

Immunofluorescence microscopy with antibodies to purified prekeratin is a very sensitive and specific method for studying the formation and involution of MBs. It is evident that MBs develop by coalescence of small precursor granules of prekeratin-rich filamentous material. Such a mode of development from small aggregates of MB material has already been suggested by Mallory (1) and Christoffersen (23), on the basis of light microscopic observations, to occur in livers of human alcoholics.

The lack of decoration of MB aggregates, reinduced in the griseofulvin-pretreated liver by griseofulvin and by colchicine, by antibodies to murine vimentin refutes the possibility that these reinduced MBs are aggregates of filaments of the vimentin-type, which are well known to form perinuclear whorls in various cells growing in vitro under the influence of colchicine (9, 15, 24).

Recently, antibodies to constitutive proteins of cytoskeletal intermediate-sized filaments have been produced in various laboratories and have been used for identifying, differentiating, and localizing different types of intermediate-sized filaments (9, 17, 24–26). With this method the following major filament types have been distinguished: (i) the type containing desmin, specific for myogenic cells; (ii) the type containing prekeratin-like proteins characteristic of epithelial cells; (iii) neurofilaments specific for neuronal cells; and (iv) the filament type containing vimentin, which is characteristic of all mesenchymal cells but has also been observed in epithelial cells and myogenic cells grown in vitro. Our study on MB formation in liver presents an example of the application of such antibodies to the examination of possible pathologic Disorders of component proteins of intermediate-sized filaments, in this case of the prekeratin type.

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