Fate of Mallory Body–containing Hepatocytes: Disappearance of Mallory Bodies and Restoration of the Hepatocytic Intermediate Filament Cytoskeleton After Drug Withdrawal in the Griseofulvin-treated Mouse

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Mallory bodies are characteristic morphological features of alcoholic hepatitis in man and can be produced in the mouse by chronic griseofulvin intoxication. The appearance of Mallory bodies in hepatocytes is associated with derangement of the cytokeratin intermediate filament cytoskeleton, at least as revealed by immunofluorescence and suggested by immunoelectron microscopy. Immunohistochemical studies were performed to answer the question whether Mallory body formation and cytoskeleton alterations finally lead to cell death or are reversible phenomena. Chronically griseofulvin-intoxicated mice killed at different stages of recovery on a normal diet served as experimental animals. It could be shown that (a) Mallory bodies are very durable structures and are found for up to 6 mo after griseofulvin withdrawal as a result of persistence and neoformation; (b) new Mallory bodies can appear even several months after cessation of griseofulvin feeding; (c) Mallory body formation and cytoskeletal changes by themselves do not lead to irreversible cell damage; (d) the cytoskeletal changes are reversible within 7 mo after griseofulvin withdrawal; (e) a dissociation between disappearance of Mallory bodies and restoration of a regularly immunostained cytoplasmic cytokeratin meshwork is observed. (HEPATOLOGY 1990;11: 652-661.)

Intermediate filaments (IF) of mouse hepatocytes contain two major cytokeratin (CK) polypeptides: polypeptide A, corresponding to human polypeptide No. 8; and polypeptide D, corresponding to human polypeptide No. 18. (1). IF form a complex cytoplasmic meshwork that is more densely arranged at the cell periphery and particularly around bile canaliculi (2). Structure and chemical composition of IF (3-6) and their interaction with cellular components (7-9) have been, at least partly, clarified. However, details of their physiological roles are still unclear.

Several pathological situations exist that are accompanied by profound changes of the IF cytoskeleton (10). One of the most striking examples of disease-associated modifications of the IF cytoskeleton occurs in severe alcoholic liver injury, that is, alcoholic hepatitis, which is characterized by the appearance of cytoplasmic inclusions consisting of irregularly arranged filamentous rods with diameters of 10 to 20 nm, termed Mallory bodies (MBs). These filamentous aggregates share several morphological, biochemical and immunological features with CK IF and are now generally regarded to represent abnormal hepatocellular CK material, although the presence of nonkeratin components is not excluded and even likely (2, 11). MBs can be experimentally induced in mice by prolonged feeding of a diet containing griseofulvin (GF) (12). This experimental model provides the opportunity for detailed and controlled studies of the dynamics of MB formation and involution (2).

No unequivocal answer exists to the question whether MB development is only a peculiar morphological expression of liver cell injury or by itself leads to liver cell damage (13-15). GF-intoxicated hepatocytes containing MBs also show alterations of their IF cytoskeleton, that is, derangement, diminution or even disappearance of CK filament bundles, at least as revealed by immunohistochemistry and scanning electron microscopy (2, 16-19).

To examine the fate of MB-containing hepatocytes and of the apparently associated cytoskeletal abnormalities, and, particularly, to answer the question whether MB-containing cells are irreversibly damaged and finally eliminated or able to recover after GF withdrawal, livers of GF-intoxicated mice at different stages of recovery were studied immunohistochemically (and some also immunoelectron-microscopically) using CK antibodies.

Received March 11, 1988; accepted November 10, 1989.

Supported in part by Fonds zur Förderung der wissenschaftlichen Forschung (Grants P 4708 and P 5803 to H.D.).

This work is dedicated to Prof. W. Thiel, Chairman, Department of Anatomy, University of Graz School of Medicine, on the occasion of his birthday.

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MATERIAL AND METHODS

Experimental Design and Immunohistochemistry. Male Swiss albino mice (strain Him OF1 SPF, from the Institute of Laboratory Animal Research, University of Vienna School of Medicine, Himberg, Austria; 35 gm body weight and 3 mo old) received a powdered standard diet (Altromin, Marek, Vienna, Austria) containing 2.5% wt/wt GF (Aldrich-Chemie, Frankfurt am Main, FRG) and water *ad libitum* for 4 mo (12). The experimental protocol was approved by the committee for animal experimentation at the Federal Ministry for Science and Research; GZ 68 205/23-12/89. Thereafter, the mice were fed a GF-free standard diet to allow recovery from GF intoxication. Four mice were killed by decapitation immediately after withdrawal of the GF diet (that is, after continuous GF feeding for 4 mo) to provide "baseline" information of the full-blown stage of GF intoxication, and two to six animals were then killed at intervals of 4 wk for up to 7 mo after drug withdrawal to examine the stages of recovery. The livers were removed and small pieces were quickly frozen in methylbutane precooled with liquid nitrogen. For indirect immunofluorescence microscopy, frozen liver sections, 4 μm thick, were fixed in cold acetone (−20° C) and further processed as previously described (16). The following primary antibodies were used: (a) polyclonal guinea pig antibody prepared against mouse liver CK component D (corresponding to human CK No. 18) reacting in immunoblots with CKs A and D (CKab); in immunofluorescence microscopy, this antibody not only stains a complex CK filament meshwork in epithelial cells, hepatocytes and bile duct epithelia included, but also reacts with MBs in all stages of their development (16); (b) monoclonal antibody K_M 54 and 55 reacting in immunofluorescence microscopy with most large (apparently older) MBs but not with most small (apparently young) MBs and not with normal CK fibrils (20); (c) monoclonal antibody directed against desmoplakins (desmosomal plaque proteins) (21, 22); (d) as secondary antibodies either fluorescein isothiocyanate or Texas red–conjugated IgG to guinea pig immunoglobulins (from rabbit; DAKO, Glostrup, Denmark; Medac, Hamburg, FRG) or to mouse IgG and IgM (from goat; TAGO Inc., Burlingame, CA) were used. Double immunofluorescence microscopy was performed as described previously (20, 22). As negative controls, the liver sections were incubated with preimmune sera and PBS, respectively, instead of primary antibodies as described previously (22). Under these circumstances no specific immunoreaction was observed. Photographs were taken with a Zeiss Photomikroskop III (Carl Zeiss, Ober Kochen, FRG) with epiillumination using Kodak Tri-X Pan (Eastman Kodak Co., Rochester, NY) films.

Immunoelectron Microscopy. Frozen liver sections (four to six sections from each liver), 8 μm thick, derived from four mice fed GF for 4 mo, from three mice recovering from GF intoxication (after 4 mo of GF feeding) for 2 mo and from two untreated controls, respectively, were mounted on cover slides and fixed for 20 min in 4% paraformaldehyde solution in PBS, pH 7.4. They were subsequently treated with PBS containing 50 mmol/L NH_4Cl (3 × 5 min), 1% Triton X-100 in PBS/50 mmol/L NH_4Cl (5 min) and PBS (4 × 5 min) at 0° C. Thereafter, CKab was applied in a dilution of 1:50 in PBS for 1 hr at 0° C and the sections were rinsed with PBS (3 × 5 min) followed by incubation with 5 nm gold particle–conjugated antibodies to guinea pig immunoglobulins (from goat; Auro Probe EM; Janssen Biochimica, Beerse, Belgium; diluted 1:5 with PBS) for 1 hr at 0° C. After washing in PBS (4 × 5 min) the sections were postfixed with 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L, pH 7.3) for 10 min, rinsed in cacodylate buffer (3 × 5 min) and finally treated with 1% osmium tetroxide (30 min at room temperature). In another series of experiments identical treatments (including fixation) were performed with solutions containing 1.2 mmol/L phenylmethylsulfonyl fluoride (Sigma Diagnostics, St. Louis, MO), 0.1 mmol/L iodoacetamide (Sigma Diagnostics) and 5 mmol/L EDTA (Sigma Diagnostics) to inhibit proteolysis. To enhance staining some samples were treated with ruthenium red according to Luft (23). Thereafter, the sections were dehydrated in graded ethanol (50% to 100%) and propylene oxide, embedded in epoxy resin and further processed as described previously (24). From each frozen section two to four areas were selected and embedded. From each area, four sections (0.05 μm thick) were viewed with a Philips EM 400 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ). Controls were performed as described in the previous paragraph with negative results.

Preparation and Gel Electrophoretic Analysis of Cytokeratins. CKs were isolated from mouse livers recovering from GF intoxication for 2 mo on normal diet after a GF-feeding period of 4 mo and from normal mouse livers as described previously (25). For comparison, equal amounts of normal and experimental liver homogenates were treated identically. The protein contents of the homogenates were determined by the method of Bradford (26). Equal aliquots of CK-containing material were electrophoresed on one-dimensional SDS polyacrylamide gels as described previously (25, 27). The electrophoretic results were quantified using a video densitometer (Bio-Rad Laboratories, Richmond, CA). Three independent experiments were performed with the livers of experimental (recovering) and control mice.

RESULTS

Fate of MBs and of the CK Cytoskeleton during Recovery from GF Intoxication. Our studies of the recovery of hepatocytes after GF-induced damage started after continuous GF feeding for 4 mo. The morphological features of long-term GF fed mouse livers have been reported previously (16, 22) and will only be briefly summarized to provide "baseline" information for the recovery studies. GF intoxication of mice for 4 mo led to the appearance of numerous MBs but also to conspicuous changes of the IF cytoskeleton architecture of the hepatocytes as revealed immunohistochemically using CKab, which stained normal CK filaments in addition to MBs. With this antibody, MBs were revealed in different stages of development ranging from small granules, apparently associated with the intersections of the CK filament bundles (early stages of MBs), to larger irregular, cytoplasmic inclusions (fully developed, later stages of MBs). With the monoclonal antibody K_M 54-5 only medium-sized to large (later stages) MBs were stained, whereas early MB granules and normal CK filament bundles were unreactive (20). The process of MB formation was accompanied in most hepatocytes by changes of the arrangement of CK bundles as revealed by CKab: in hepatocytes with early MB stages the CK fibril meshwork was still detectable but often rarefied. In many cells containing large MBs, only a narrow peripheral rim of CK filaments was immunostained (Figs. 1, 2). The hepatocytes were clearly heterogeneous in their reaction to prolonged GF intoxication. Hepatocytes showing various degrees of IF cytoskeleton derangement were either scattered throughout the lobule

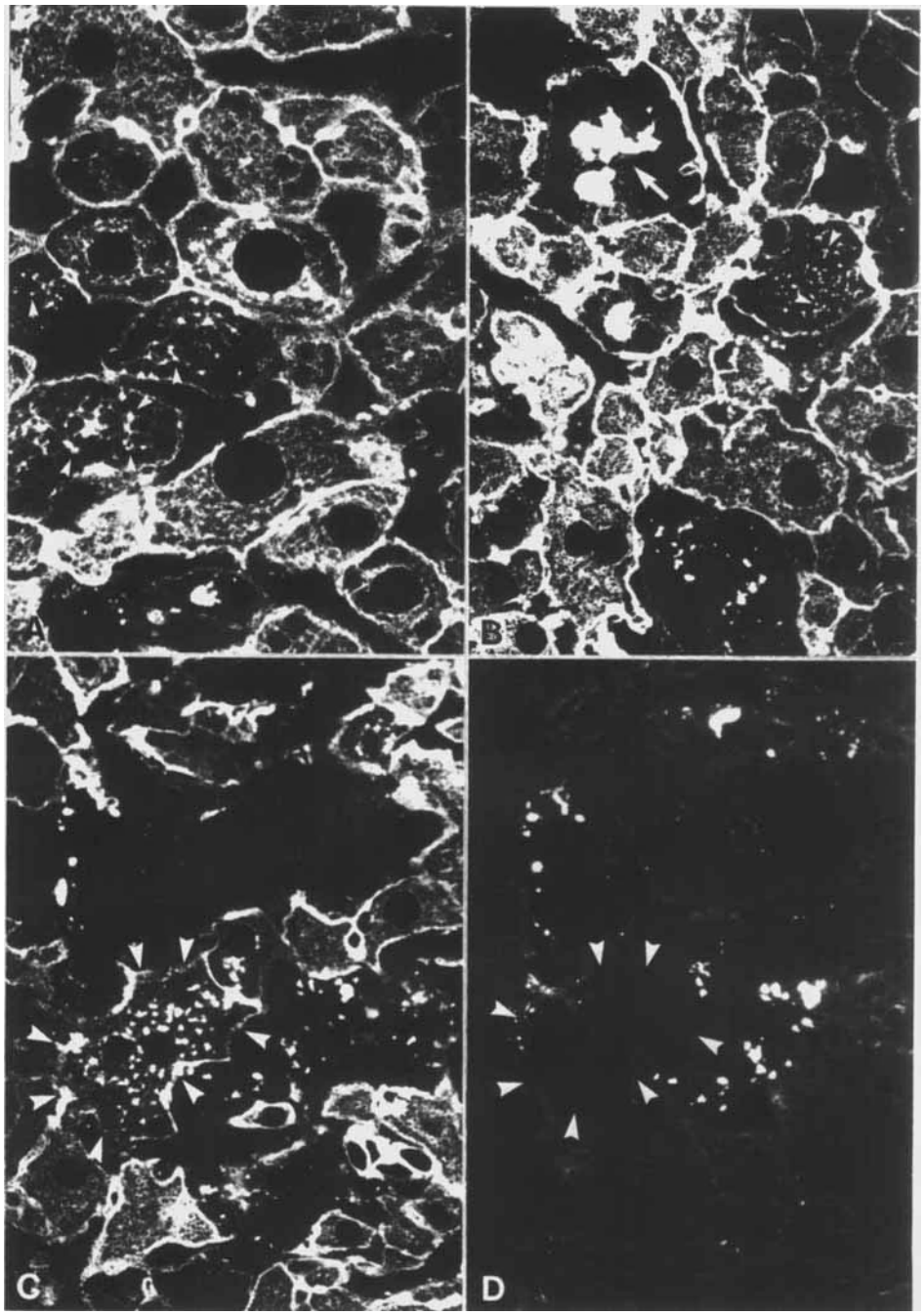

FIG. 1. Indirect immunofluorescence microscopy using the polyclonal CKab (A,B,C) and the monoclonal antibody K_M 54-5 (D) on frozen liver sections of mice fed a GF diet for 4 mo showing MBs in different developmental stages, ranging from small granules, apparently associated with the intersections of the CK fibril meshwork (early MBs; *arrowheads* in A,B), to large irregular inclusions (fully developed MBs; *arrow* in B). In hepatocytes containing larger MBs, only few CK fibrils are demonstrable (cell with arrow in B), whereas hepatocytes devoid of MBs or containing only small MB granules show a complex cytoplasmic meshwork staining (A,B,C). Double immunofluorescence microscopy using CKab (C) and K_M 54-5 (D) reveals that early MBs and CK fibrils are unstained with K_M 54-5 (D; note hepatocyte surrounded by *arrowheads*), whereas CKab decorates the CK filament meshwork and MBs of all stages and sizes (C). The small MBs stained by K_M 54-5 (D) are mostly peripheral and resemble late developmental stages (see also Fig. 2C). (Original magnification ×330.)

or clustered in smaller or larger islands. Severely altered liver cells were often found in proximity to normal-appearing hepatocytes or cells even displaying a more densely arranged IF cytoskeleton.

Cessation of GF intoxication was followed by a time-dependent reduction of hepatocytes with large MBs and a predominance of cells with small MBs located in a peripheral (subcortical) position (Fig. 2A-E). These peripheral MB granules were also stained by K_M 54-5, suggesting their origin from large MBs (Fig. 2B, C). After GF withdrawal, a reduction of cells containing early granular (that is, K_M 54-5 nonreactive) MBs was

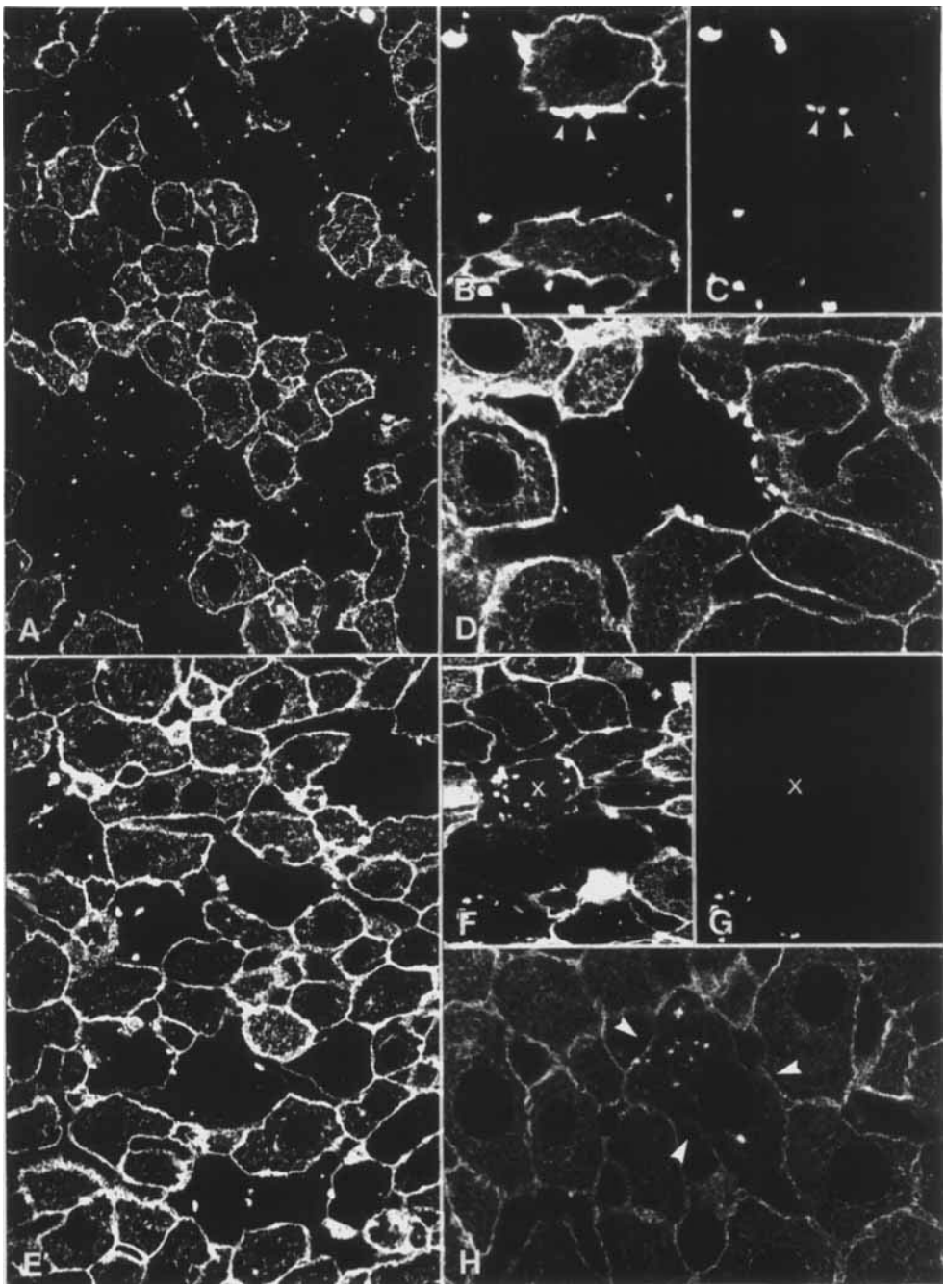

FIG. 2. Indirect immunofluorescence microscopy on frozen liver sections of mice on GF-free diet (after previous intoxication for 4 mo) for 2 (A-C), 4 (D-G), and 6 (H) mo using CKab (A,B,D,E,F,H) and K_M 54-5 (C,G). After a recovery period of 2 mo, numerous CK-negative ("empty") and enlarged hepatocytes contain small, mostly peripherally situated granular MBs (A-C). The peripherally located granular MBs (*arrowheads* in B and C) are stained with K_M 54-5 (B and C show double immunofluorescence microscopy using CKab in B and K_M 54-5 in C), suggesting their derivation from larger (old) MBs. Hepatocytes with a normally appearing cytoskeleton meshwork (after staining with CKab) are in close association with the diseased cells (A,B). Hepatocytes with apparently diminished or missing CK meshwork are reduced in number with prolongation of the GF-free period. Areas occupied by these cells become smaller and often only small groups of "empty" hepatocytes or single cells remain (D, E, GF-free period of 4 mo; H, GF-free period of 6 mo). Note the presence of only few peripheral granular MBs in, or even the absence of MBs from, "empty" hepatocytes (D,E). Despite replacement of GF diet by normal diet for a prolonged period of time (4 to 6 mo), early granular stages of MBs can still occasionally be observed in scattered hepatocytes (*x* in F, *arrowheads* in H). Double staining F (CKab) and G (K_M 54-5) reveals that these MBs are not stained by K_M 54-5 indicating their early nature (*x* in G). (Original magnifications: A,E,F,G ×330; B,C,D,H, ×530.)

also noted. However, in some hepatocytes early stages of MB development could still be demonstrated with CKab for up to 6 mo after drug withdrawal (Fig. 2F-H). Immunoreactivity of CK filaments and MBs in different stages of development is summarized in Table 1.

Reduction of MBs was obviously not due to necrosis of MB-containing hepatocytes because necroses corresponding in extent to the affected cells were not observed. If affected hepatocytes had been destroyed, one would expect extensive spotty and confluent ne-

TABLE 1. Immunoreactivities of polyclonal CKab and monoclonal antibody K_M 54-5 with cytokeratin filaments and Mallory bodies in different stages of development

Antibodies	Cytokeratin filaments	Mallory bodies		
		Early-granular	Fully developed	Late-granular
CKab	+	+	+	+
K_M 54-5	−	−	+	+

CKab = cytokeratin antibody; + = reactive; − = nonreactive.

croses. The normal IF cytoskeletal architecture as revealed by CKab was apparently not restored strictly parallel to MB involution. Two and 3 mo after drug withdrawal the immunohistochemical picture was dominated by the presence of quite large areas of liver parenchyma lacking visible cytoplasmic CK fibril networks but with small granular MBs in their periphery (Fig. 2A). Moreover, hepatocytes devoid both of IF cytoskeleton and MBs were also noted. Hepatocytes with deranged, diminished or lost immunohistochemically stainable IF network were still in contact with the adjacent normal or diseased hepatocytes and remained organized in cell plates (Fig. 2A). A regular distribution of desmosomes was immunohistochemically demonstrable using desmoplakin antibodies in all hepatocytes irrespective of their cytoskeleton alterations as also described previously (not shown) (22). MBs were further reduced in number and size with prolongation of the GF-free period (Figs. 2E and 3A). They had almost disappeared after 6 mo, and the livers were MB-free after 7 mo (Fig. 3B). Five and 6 mo after drug withdrawal, the number of hepatocytes without immunohistochemically demonstrable CK bundles was also greatly reduced (Fig. 3A). Scattered or clustered hepatocytes displayed a loosely but regularly arranged CK meshwork (Fig. 4A-G). Some of these cells still contained small peripheral granular MBs (e.g., Fig. 4A). In some cells, CK fibrils were more concentrated in the perinuclear region (Fig. 4H, I). Seven months after drug withdrawal, only hepatocytes with regular IF cytoskeletal meshwork were seen (Fig. 3B).

Immunoelectron microscopy using CKab confirmed the immunohistochemical findings in normal, long-term GF-fed and recovering mouse livers. In unaltered hepatocytes in all three groups of animals, bundles of IF were coated by gold particles. In contrast, MBs only showed a peripheral decoration by the marker particles, whereas the more centrally located filaments remained unlabeled (Figs. 5, 6). Several types of hepatocytes could be observed in intoxicated and recovering livers: (a) Hepatocytes containing abundant bundles of CK filaments (Fig. 5A, B). At the cell periphery, the bundles were associated with desmosomes. It appeared, however, that some filaments in close proximity to the desmosomal plaque were unlabeled (Fig. 5C). (b) Hepatocytes containing IF bundles surrounding, or in association with, small MBs (Fig. 5D); (c) hepatocytes containing MBs with only few or without bundles of CK filaments attached (Fig. 6). In these cells, immunostained IF bundles were mostly confined to the cell periphery and IF extending through the cytoplasm toward desmosomes were scarce or undetectable (Fig. 7). In some hepatocytes large MBs were in close contact with desmosomes that were devoid of associated IF bundles (not shown). No differences were observed between specimens treated with or without inhibitors of proteolysis or additionally stained with ruthenium red.

CK Content and Composition of Mouse Livers Recovering from GF Intoxication. One-dimensional SDS-PAGE of CKs from the livers of mice recovering from GF intoxication (2 mo on normal diet after a 4-mo GF-feeding period) and showing large areas of liver parenchyma devoid of CK staining revealed a diminution of bands corresponding to CK polypeptides A and D in comparison with CKs isolated from normal mouse livers. The decrease of component A was slightly more pronounced than that of D (a representative experiment is shown in Figure 8).

Moreover, despite identical preparation conditions some differences could be noted between CK material derived from experimental and control livers with respect to minor protein bands and the amount of material not entering the gels and running at the front (compare lanes 1 and 2 in Figure 8).

DISCUSSION

The following observations and conclusions resulted from our experiments:

a. Reversibility of the changes of the cytoplasmic IF meshwork and of MB formation after drug withdrawal.
b. Dissociation between disappearance of MBs and the restoration of the cytoplasmic IF meshwork.
c. Potential of MB neoformation is retained for several months in the absence of GF, indicating that the pathogenic stimulus persists for a rather long time after cessation of the intoxication.

After cessation of GF intoxication, the immunostained IF cytoskeleton gradually reappeared, number and size of MBs were reduced and they finally disappeared after a recovery period of 7 mo, probably by intracellular dispersion and dissolution. Progressive derangement and reduction of the IF cytoskeleton apparently associated with MB formation suggested that MBs may be derived from aggregation or collapse of the IF cytoskeleton (19). However, our results and those of other authors (18) disprove a strict correlation between MB appearance (or disappearance) on one hand and derangement (or restoration) of the CK meshwork on the other: the cytoskeletal architecture in particular, as

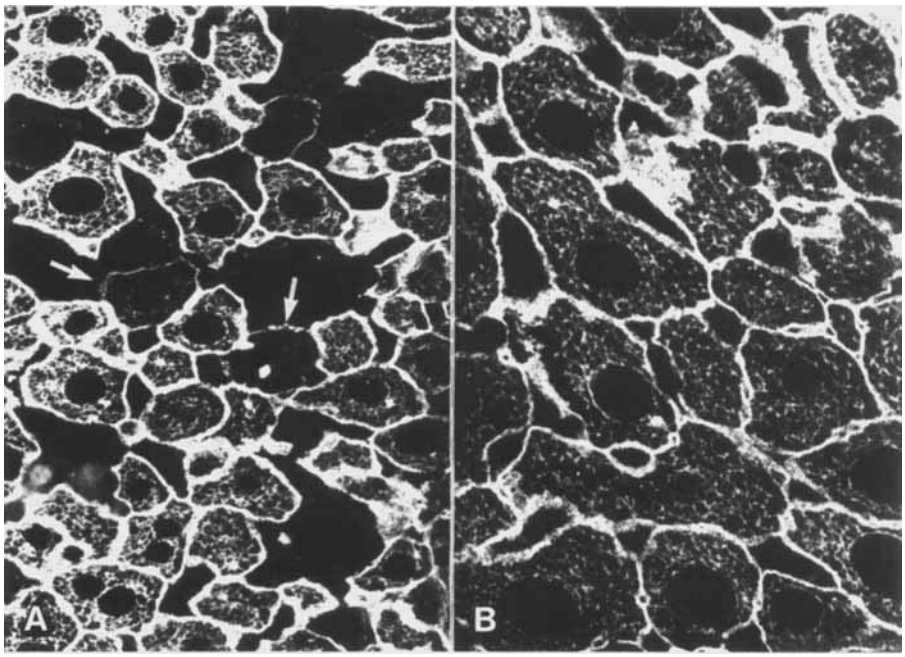

FIG. 3. A. Six months after GF withdrawal, cells with a loosely arranged fibrillar meshwork are demonstrable with CKab (*arrows* in A), indicating regeneration of the cytoskeleton. Some of these cells still contain MB granules. B. Seven months after cessation of GF intoxication, the restoration process is completed and hepatocytes again display a regular immunostained IF meshwork. (Original magnifications: A, ×330; B, ×530.)

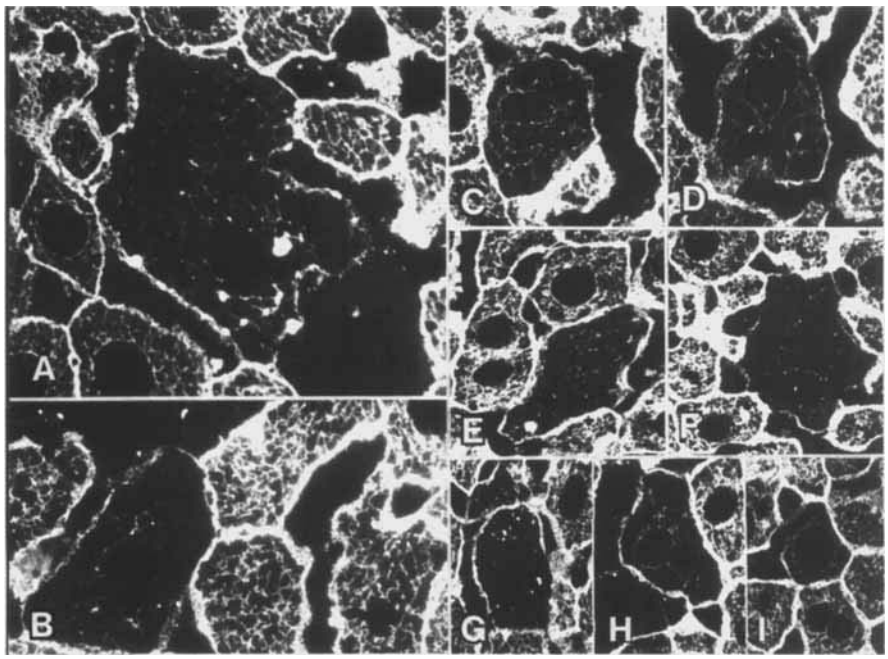

FIG. 4. During regeneration, a loose network of CK fibrils is demonstrable with CKab as shown in this composite figure. Some hepatocytes are still enlarged and contain small MBs (A). Other hepatocytes resemble in size surrounding cells with intact cytoskeleton but still contain a loose CK network (B-I). Cytokeratin fibrils are often more concentrated around the nucleus (for example, H and I), and this perinuclear position seems to indicate earlier stages of cytoskeleton regeneration. (Original magnifications: A,B,C,D, ×530; E,F,G,H,I, ×330.)

revealed by immunohistochemistry, was not readily restored with disappearance of MBs, and hepatocytes without MBs and fluorescent CK fibrils were observed during the earlier stages of recovery. Therefore a direct transition between MBs and normal CK filaments, that is, by collapse and dispersal, is unlikely.

The structural background of the impairment of CK-specific staining of diseased hepatocytes is still controversial. Several observations suggest, but certainly do not prove, diminution of hepatocellular CK

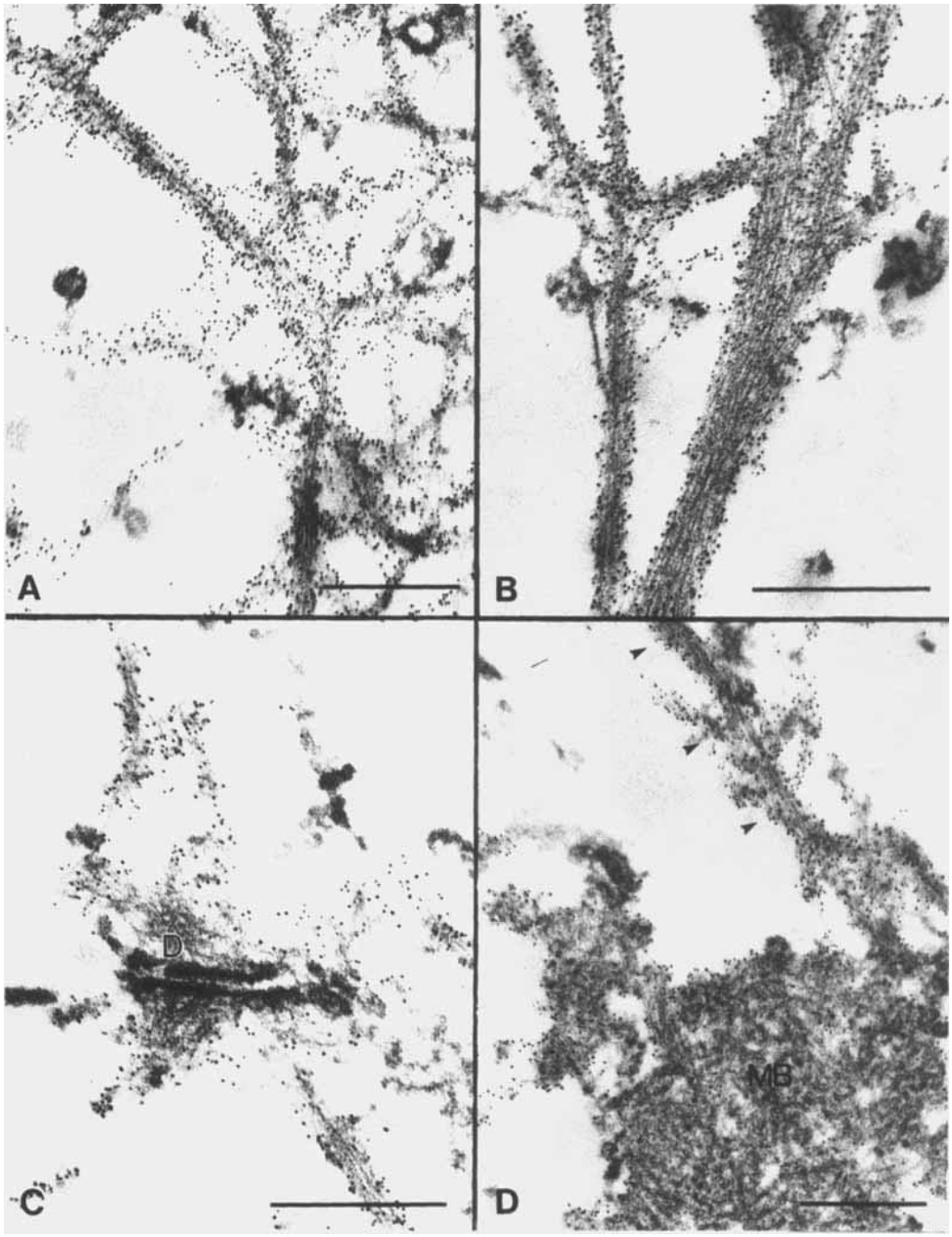

FIG. 5. Immunoelectron microscopy of a mouse liver after 4 mo of GF treatment using CKab. Bundles of CK filaments are decorated by gold particles. In unaltered hepatocytes the CK filament bundles form a rather dense cytoplasmic meshwork (A,B). At the cellular periphery they are associated with desmosomes (*D* in C). Note that some filaments in proximity to the desmosomal plaque are unlabeled by gold particles. In hepatocytes containing small MBs *(MB)* the CK meshwork is largely unaltered and CK bundles *(arrowheads)* are in proximity to MBs (D). MBs are only decorated by gold particles at their periphery, whereas the more centrally located MB filaments remain unlabeled (D). Bars denote 0.5 μm.

meshwork: (a) In immunofluorescence microscopy of frozen sections of GF-intoxicated mouse livers identical results were obtained with a panel of polyclonal and monoclonal antibodies to CKs, including the antibody Lu-5 (Preisegger KH, et al., Unpublished observations). At present, Lu-5 is one of the most general CK probes. The epitope recognized by this antibody is present in most CK polypeptides of both the acidic and basic subfamily (28). Because immunohistochemical staining is based on antigenicity, masking of the specific CK epitopes by conformational changes or because of interaction with other cellular components could account for negative results and thus simulate lack of antigenic structures (29). However, it is highly unlikely that all

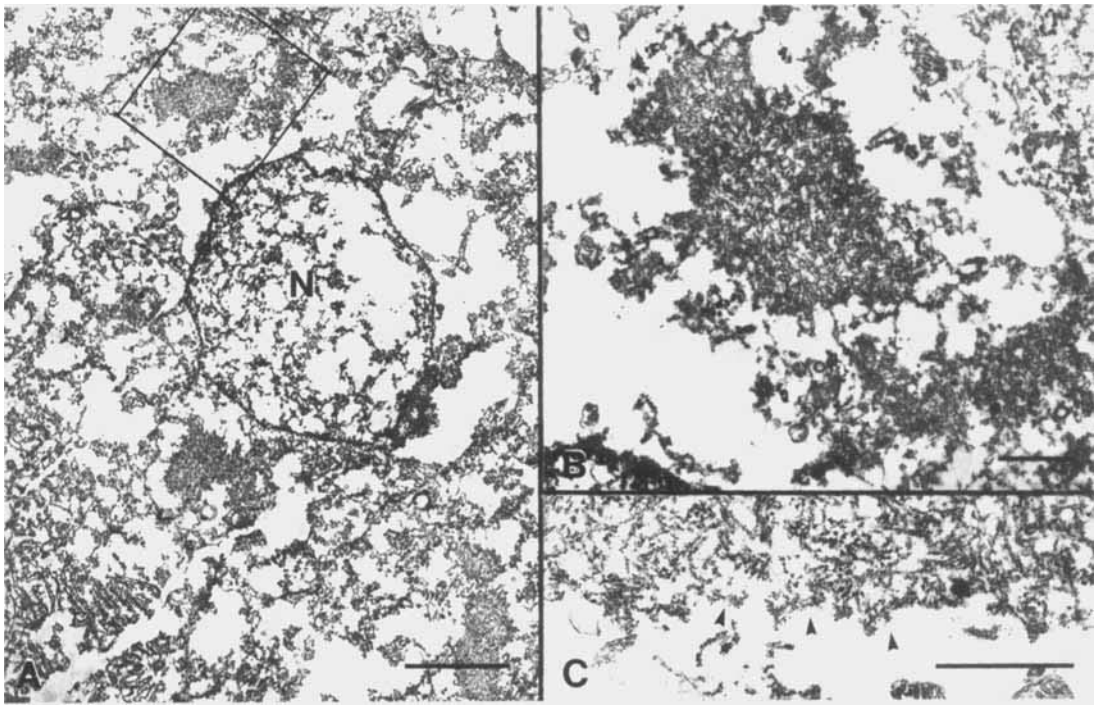

FIG. 6. Immunoelectron microscopy of a mouse liver after 4 mo of GF treatment using CKab. (A). The hepatocyte contains several MBs. (Higher magnification of area indicated in A is shown in B.) MBs are peripherally decorated by gold particles (higher magnification in C; *arrowheads*). Bundles of (labeled or unlabeled) CK filaments are not demonstrable (see higher magnifications in B and C). Cell organelles, including endoplasmic reticulum, are visible within the cytoplasm (*N* = nucleus). Ruthenium-red–treated material. Bars denote 5 μm (A), 1 μm (B,C).

the epitopes recognized by these polyclonal and monoclonal CK antibodies are modified simultaneously to account for the negative immunofluorescence results. (b) The immunofluorescence results were also confirmed by immunoelectron microscopy. Desmosomes free of attached IF bundles were frequently found in affected hepatocytes (24). An artifact is unlikely because Fey, Wan and Penman (30) have demonstrated that IF-desmosome associations are stable and preserved both in Triton-extracted, Epon-Araldite–embedded thin sections and in whole mount preparations. Moreover, our results clearly demonstrate that larger IF bundles can be visualized by the immunoelectron microscopic technique used. However, the method may not be sensitive enough to detect all single IF. Masking of CK antigenic determinants may also be involved, that is, it may account for the lack of CK-specific staining of filaments in proximity to the desmosomal plaque (Fig. 5D). (c) Biochemical analyses of the CK-IF skeleton isolated from recovering and control livers revealed diminution of CK polypeptide bands in the livers during the early recovery stage in which large numbers of hepatocytes with undetectable or reduced cytoskeleton were present.

Our results and interpretations are in contrast to the electron microscopic studies of Irie, Benson and French (31), performed on detergent-extracted normal and GF-mouse livers and supported by morphometry, that revealed that GF feeding did not significantly alter the amount of IF and microtubules. However, according to the figures presented by these authors, hepatocytes with small MBs were studied that, at least under our experimental conditions, are usually still associated with CK filament bundles. Diminution of CK-IF bundles was also not confirmed by Katsuma et al. (32), who studied detergent-extracted frozen sections of GF-intoxicated mouse livers and cultured cells by immunofluorescence and electron microscopy. They demonstrated persistence of an impressively complex cytoplasmic filamentous network and explained the phenomenon of "empty" hepatocytes, as revealed by immunofluorescence microscopy, by changes in antigenicity caused by appearance of IF with a different set of acidic and basic CK polypeptides rather than by diminution or even disappearance of filaments. In confirmation, Ohta, Marceau and French (33) demonstrated by immunoelectron microscopy of hepatocytes of cytochalasin B–infused rat liver after detergent extraction that many filaments of intermediate dimensions remained unstained with CK antibodies, suggesting that changes of antigenic determinants occur in diverse pathological situations. Moreover, Lai et al. (34) recently stated that, in paraffin sections of human alcoholic hepatitis, monoclonal and polyclonal CK antibodies (35β H11 and antiprekeratin antibodies) stained all hepatocytes with or without MBs. These results, however, are not

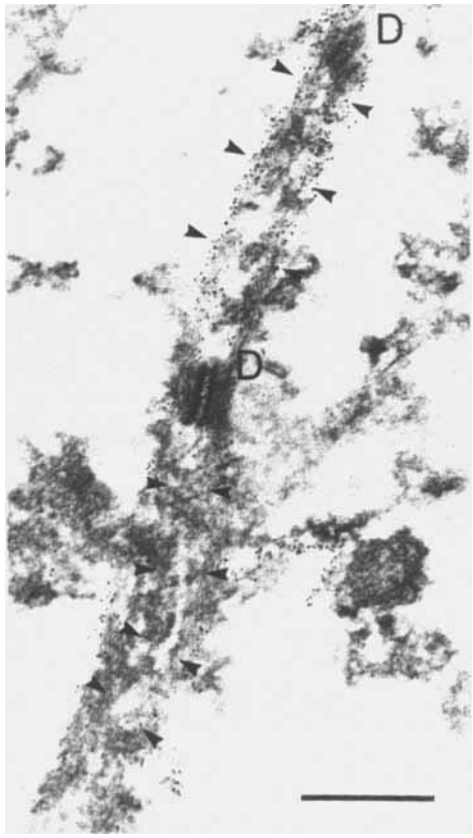

FIG. 7. Immunoelectron microscopy of a mouse liver after 4 mo of GF treatment using CKab. Desmosomes *(D)* are associated with only few CK bundles that are aligned in parallel to the cell borders *(arrowheads)*. CK bundles extending through the cytoplasm and abutting on desmosomes are not demonstrable. Bar denotes 0.5 μm.

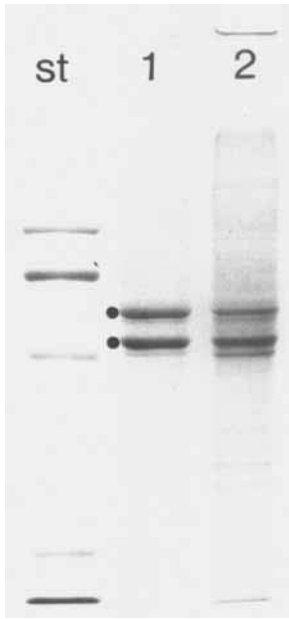

FIG. 8. One-dimensional SDS-PAGE of CK proteins from normal (control; *lane 1*) and recovering (2 mo of recovery after a GF-feeding period of 4 mo containing large areas of liver parenchyma devoid of CK staining; *lane 2*) liver. Amounts of extracted liver tissue, preparation conditions and aliquots analyzed on the gel were identical. Dots denote CK polypeptides A *(upper band)* and D *(lower band)*. Note that CKs isolated from recovering liver *(lane 2)* are diminished as compared with the control *(lane 1)*. There are, in addition, slight differences with respect to material not entering the gels (present in the well and at the interphase between stacking and resolving gels) and material running at the front. CK-enriched residue derived from recovered liver also differs from control by the presence of minor protein bands with molecular weights higher and lower than those of typical liver CKs. Some of the bands with lower molecular weights may reflect proteolytic degradation products. Molecular mass standards *(st)* are (from top to bottom) 92.5, 66.2, 45, 30 and 21.5 kD.

corroborated by our experience with these antibodies, at least on frozen mouse liver sections (Preisegger KH, et al., Unpublished observations).

A unifying concept could be proposed to account for the apparently conflicting observations reported in the literature: GF intoxication leads to dissociation and rarefication of the thicker IF bundles, but the more delicate single IF meshwork may persist. Changes in antigenicity of CK filaments could also play a role.

Alterations of the IF cytoskeleton could result from imbalance between synthesis and degradation, postsynthetic modifications of CK polypeptides and interaction with associated proteins (35). For example, in cultured rat mammary cells inhibition of protein synthesis leads to loss of IF within 7 days (36). Moreover, degradation mediated by a cytoplasmic Ca^{++}–activated neutral protease could also play a role (37). The observation that a submembranous, more densely arranged felt of fibrils was often preserved in hepatocytes with an altered cytoplasmic IF network is also compatible with the idea of proteolysis because it has been found (in epidermal cells) that IF close to the cell membrane were more resistant to proteolytic degradation than more centrally located ones (38). Rarefication of the cytoplasmic IF meshwork could also result from disturbance of filament assembly accompanied by an increase of the soluble subunit (e.g., heterotetramer) pool (39). These possibilities are presently under investigation.

Acknowledgments: The excellent secretarial assistance of Ms. R. Spindler and Ms. E. Ogriseg and the photographical work of I. Georgiev are gratefully acknowledged.

REFERENCES

1. Moll R, Franke WW, Schiller DL, Geiger B, Krepler R. The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 1982;31:11-24.
2. Denk H, Lackinger E, Vennigerholz F. Pathology of the cytoskeleton of hepatocytes. In: Popper H, Schaffner F, eds. Progress in liver diseases. Vol VIII. New York: Grune & Stratton, 1986: 237-251.
3. Magin TM, Jorcano JL, Franke WW. Cytokeratin expression in simple epithelia. II. cDNA cloning and sequence characteristics of bovine cytokeratin A (no. 8). Differentiation 1986;30:254-264.
4. Romano V, Hatzfeld M, Magin TM, Zimbelmann R, Franke WW, Maier G, Ponstingl H. Cytokeratin expression in simple epithelia. I. Identification of mRNA coding for human cytokeratin no. 18 by a cDNA clone. Differentiation 1986;30:244-253.
5. Steinert PM, Rice RH, Roop DR, Trus BL, Steven AC. Complete

amino acid sequence of a mouse epidermal keratin subunit and implications for the structure of intermediate filaments. Nature 1983;302:794-800.
6. Steinert PM, Steven AC, Roop DR. The molecular biology of intermediate filaments. Cell 1985;42:411-419.
7. Schliwa M, Van Blerkom J. Structural interaction of cytoskeletal components. J Cell Biol 1981;90:222-235.
8. Traub P, ed. Intermediate filaments: a review. New York: Springer-Verlag, 1985.
9. Drochmans P, Freudenstein C, Wanson J-C, Laurent L, Keenan TW, Stadler J, Leloup R, et al. Structure and biochemical composition of desmosomes and tonofilaments isolated from calf muzzle epidermis. J Cell Biol 1978;79:427-443.
10. Rungger-Brändle D, Gabbiani G. The role of cytoskeletal and cytocontractile elements in pathologic processes. Am J Pathol 1983;110:361-392.
11. Denk H, Krepler R, Lackinger E, Artlieb U, Franke WW. Immunological and biochemical characterization of the keratin-related component of Mallory bodies: a pathological pattern of hepatocytic cytokeratins. Liver 1982;2:165-175.
12. Denk H, Gschnait F, Wolff K. Hepatocellular hyalin (Mallory bodies) in long term griseofulvin-treated mice: a new experimental model for the study of hyalin formation. Lab Invest 1975; 32:773-776.
13. French SW, Burbige EJ. Alcoholic hepatitis: clinical, morphologic, pathogenic, and therapeutic aspects. In: Popper H, Schaffner F, eds. Progress in liver disease. Vol VI. New York: Grune & Stratton, 1979:557-579.
14. MacSween RNM, Burt AD. Histologic spectrum of alcoholic liver disease. Semin Liver Dis 1986;6:221-232.
15. Orrego H, Blake JE, Blendis LM, Medline A. Prognosis of alcoholic cirrhosis in the presence and absence of alcoholic hepatitis. Gastroenterology 1987;92:208-214.
16. Denk H, Franke WW, Dragosics B, Zeiler I. Pathology of cytoskeleton of liver cells: demonstration of Mallory bodies (alcoholic hyalin) in murine and human hepatocytes by immunofluorescence microscopy using antibodies to cytokeratin polypeptides from hepatocytes. Hepatology 1981;1:9-20.
17. Kimoff RJ, Huang S. Immunocytochemical and immunoelectron microscopic studies on Mallory bodies. Lab Invest 1981; 45:491-503.
18. Barbatis C, Morton J, Woods JC, Burns J, Bradley J, McGee JO'D. Disorganization of intermediate filament structure in alcoholic and other liver diseases. Gut 1986;27:765-770.
19. Okanoue T, Ohta M, Ou O, Kachi K, Kagawa K, Yuki T, Okuno T, et al. Relationship of Mallory bodies to intermediate filaments in hepatocytes: a scanning electron microscopy study. Lab Invest 1985;53:534-540.
20. Hazan R, Denk H, Franke WW, Lackinger E, Schiller DL. Change of cytokeratin organization during development of Mallory bodies as revealed by a monoclonal antibody. Lab Invest 1986; 54:543-553.
21. Cowin P, Kapprell H-P, Franke WW. The complement of desmosomal plaque proteins in different cell types. J Cell Biol 1985; 101:1442-1454.
22. Denk H, Lackinger E, Cowin P, Franke WW. Maintenance of desmosomes in mouse hepatocytes after drug-induced rearrangement of cytokeratin filament material. Exp Cell Res 1985; 161:161-171.
23. Luft JH. Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anat Rec 1971;171:347-368.
24. Denk H, Franke WW. Rearrangement of the hepatocyte cytoskeleton after toxic damage: involution, dispersal and peripheral accumulation of Mallory body material after drug withdrawal. Eur J Cell Biol 1981;23:241-249.
25. Franke WW, Denk H, Kalt R, Schmid E. Biochemical and immunological identification of cytokeratin proteins present in hepatocytes of mammalian liver tissue. Exp Cell Res 1981; 131:299-318.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248-254.
27. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.
28. Franke WW, Winter S, von Overbeck J, Gudat F, Heitz PU, Stähli C. Identification of the conserved, conformation-dependent cytokeratin epitope recognized by monoclonal antibody (Lu-5). Virchows Arch [A] 1987;411:137-147.
29. Bosch FX, Leube RE, Achtstätter T, Moll R, Franke WW. Expression of simple epithelial type cytokeratins in stratified epithelia as detected by immunolocalization and hybridization in situ. J Cell Biol 1988;106:1635-1648.
30. Fey EG, Wan KM, Penman S. Epithelial cytoskeletal framework and nuclear matrix-intermediate filament scaffold: three-dimensional organization and protein composition. J Cell Biol 1984;98:1973-1984.
31. Irie T, Benson NC, French SW. Relationship of Mallory bodies to the cytoskeleton of hepatocytes in griseofulvin-treated mice. Lab Invest 1982;47:336-345.
32. Katsuma Y, Swierenga SHH, Khettry V, Marceau N, French SW. Changes in the cytokeratin intermediate filament cytoskeleton associated with Mallory body formation in mouse and human liver. Hepatology 1987;7:1215-1223.
33. Ohta M, Marceau N, French SW. Changes in the organization and antigenic determinants of intermediate filaments of rat hepatocytes after infusion of cytochalasin B *in vivo*. Am J Pathol 1988;133:578-588.
34. Lai YS, Thung SN, Gerber MA, Chen ML, Schaffner F. Expression of cytokeratins in normal and diseased livers and in primary liver carcinomas. Arch Pathol Lab Med 1989;113:134-138.
35. Steinert PM, Roop DR. Molecular and cellular biology of intermediate filaments. Annu Rev Biochem 1988;57:593-625.
36. Bologna M, Allen R, Dulbecco R. Organization of cytokeratin bundles by desmosomes in rat mammary cells. J Cell Biol 1986;102:560-567.
37. Schiller DL, Franke WW. Limited proteolysis of cytokeratin A by an endogeneous protease: removal of positively charged terminal sequences. Cell Biol Int Rep 1983;7:3.
38. Wolff K, Schreiner E. Differential enzymatic digestion of cytoplasmic components of keratinocytes: electron microscopic observations. J Ultrastruct Mol Struct Res 1971;36:437-454.
39. Soellner P, Quinlan RA, Franke WW. Identification of a distinct soluble subunit of an intermediate filament protein: tetrameric vimentin from living cells. Proc Natl Acad Sci USA 1985; 82:7929-7933.