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Immunohistochemical detection of tumor necrosis factor-alpha, other cytokines and adhesion molecules in human livers with alcoholic hepatitis

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Abstract. This immunohistochemical study was designed to investigate the possible contribution to and topographical distribution of some important cytokines, such as tumour necrosis factor α (TNFα) and interleukins, in acute alcoholic hepatitis. The well-known inductive capacity of these cytokines with respect to the expression and/or up-regulation of adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1) and endothelial leukocyte adhesion molecule-1 (ELAM-1), was a further point to be studied. Moreover, the proposed induction of adhesion molecules might also be associated with the activation and attraction of a special population of inflammatory cells characteristic for alcoholic hepatitis. Frozen liver samples from patients who died with signs of acute alcoholic hepatitis were evaluated using the alkaline phosphatase anti-alkaline phosphatase immunostaining technique and also single and double indirect immunofluorescence. In acute alcoholic hepatitis TNFα could be detected predominantly in ballooned hepatocytes, which often contained alcoholic hyalin (Mallory bodies). Moreover, TNFα showed a co-distribution with ICAM-1 expressed in the membranes of hepatocytes and with the occurrence of CD11b positive polymorphonuclear leukocytes (neutrophils) suggesting a possible major role of the β_2-integrin Mac-1 as a ligand for ICAM-1. No induction of ELAM-1 could be found. In alcoholic hepatitis cytokines may be responsible for the induction of the adhesion molecule ICAM-1 on hepatocytic membranes and activate a defined population of inflammatory cells, thus contributing to the characteristic histological picture of acute alcoholic hepatitis with its concentration of neutrophils especially in areas with ballooned Mallory body-containing hepatocytes. Our results are in line with clinical findings showing high levels of TNFα and interleukin-1 in sera of patients with alcoholic hepatitis and with the already reported expression of ICAM-1 on hepatocytes.

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Key words: Ethanol – Alcoholic hepatitis – Cytokines – Adhesion molecules – Integrins

Introduction

Although the histological picture of alcoholic hepatitis is not absolutely alcohol-specific, it is still characteristic, with ballooned hepatocytes with or without intracytoplasmic hyaline inclusions (Mallory bodies) and an inflammatory infiltrate with predominance of neutrophils around degenerated hepatocytes, finally leading to fibrosis or cirrhosis (Schaffner and Popper 1970). It is not yet unequivocally established which mechanisms are responsible for these alterations. Perez et al. (1984) isolated a factor chemotactic for neutrophils in cultures of hepatocytes exposed to ethanol. Hultcrantz et al. (1991) regarded this factor as lipo-oxidation product of unsaturated fatty acids induced by acetaldehyde. As in other forms of acute and chronic liver injury (Karck et al. 1988; Muto et al. 1988; Schlayer et al. 1988; Czaja et al. 1989; Andus et al. 1991; Sheron et al. 1991) cytokines may also play a major role in alcoholic hepatitis. Tumour necrosis factor α (TNFα) and interleukin-1 (IL-1) cause an activation of neutrophils by regulating the expression of surface molecules both on neutrophils and vascular endothelial cells (Le and Vilcek 1987). Furthermore, TNFα and IL-1 were shown to upregulate intercellular adhesion molecule-1 (ICAM-1) expression on endothelial cells (Dustin et al. 1986; Le and Vilcek 1987; Osborn 1990) but also on cells of epithelial origin (Lisby et al. 1989; Vogetseder 1989). Two independent ligands for the ICAM-1 molecule exist in the family of leukocyte integrins, namely leukocyte function associated antigen-1 (LFA-1) (CD11a/CD18) (Marlin and Springer 1987) and Mac-1 (CD11b/CD18) (Diamond et al. 1990). The induction of the adhesion molecules ICAM-1 and LFA-3 has already been demonstrated by Volpes et al. (1990) in a diversity of inflammatory liver diseases, including alcoholic hepatitis. Induction of ICAM-1 in human livers

Table 1. List of antibodies used for the immunohistochemical detection of cytokines, adhesion molecules and β_2-integrins

Antibody	Type of antibody	Dilution in IIF	Dilution in APAAP	Source
a-Human ICAM-1	Mouse, monoclonal	1:10	1:100	Bender; Vienna, Austria; CA8
a-Human ELAM-1[1]	Mouse, monoclonal	1:100	1:1000	V. Buurman; Maastricht, Netherlands
a-Mallory body[2] (M_M 120-1)	Mouse, monoclonal	Neat	Not done	H. Denk[2];
a-Human CD18	Mouse, monoclonal	1:50	1:500	Celltech; Berkshire, UK
a-Human CD11a	Mouse, monoclonal	Not done	1:20	Boehringer; Richfield, USA
a-Human CD11b	Mouse, monoclonal	Not done	1:2000	Boehringer; Richfield, USA
a-Human CD11c	Mouse, monoclonal	Not done	1:50	Behring; Vienna, Austria; BMA0310
a-Human IL-1α	Rabbit, polyclonal	Not done	1:20	Genzyme; Cambridge, UK; LP710
a-Human IL-6	Rabbit, polyclonal	Not done	1:20	Genzyme; Cambridge, UK; LP716
a-Human IL-8	Rabbit, polyclonal	Not done	1:400	S.L. Kunkel; Ann Arbor, USA
a-Human TNFα[3]	Rabbit, polyclonal	1:40	1:400	V. Buurman; Maastricht, Netherlands

[1] Leeuwenberg et al. 1989 [2] Zatloukal et al. 1990 [3] Engelberts et al. 1991

with alcoholic hepatitis has also been reported by Burra et al. (1992).

The aim of our study was to investigate by immunohistochemistry the possible role of cytokines with respect to the induction of adhesion molecules and their ligands in alcoholic hepatitis. In addition, the immunohistochemical demonstration of cytokines, adhesion molecules and integrins should throw some light on the pathogenesis of morphological alterations associated with alcoholic hepatitis.

Materials and methods

Liver samples from 9 autopsy cases (collected 3–9 h post-mortem), including 3 samples of acute alcoholic hepatitis (group 1: 2 females, 1 male; average age 54 years), 3 samples with massive steatosis of the liver but without alcoholic hepatitis (group 2: 2 males, 1 female; average age 47 years), and 3 samples without alterations of the liver (group 3: 2 males, 1 female; average age 64 years) were collected. The liver samples were first snap frozen in methylpentane precooled in liquid nitrogen and then stored in liquid nitrogen. Additionally, material of these autopsy cases was fixed in 10% buffered formaldehyde solution (pH 7.0) and embedded in paraffin. From the paraffin-embedded material 4 μm thick sections were cut and stained with haematoxylin-eosin and with the chromotrope aniline blue (CAB) stain. All immunohistochemical stainings were performed on frozen material only.

Single and double indirect immunofluorescence (IIF) was performed essentially as described elsewhere (Zatloukal et al. 1990) using the primary antibodies listed in Table 1. The following combinations were made in double IIF (for description and specification of the antibodies see Table 1): (1) anti-ICAM-1 and anti-TNFα (rabbit anti-human recTNFα; Engelberts et al. 1991); (2) M_M 120-1 (this monoclonal antibody reacts with mouse and human Mallory bodies; Zatloukal et al. 1990) and anti-TNFα; (3) anti-CD18 and

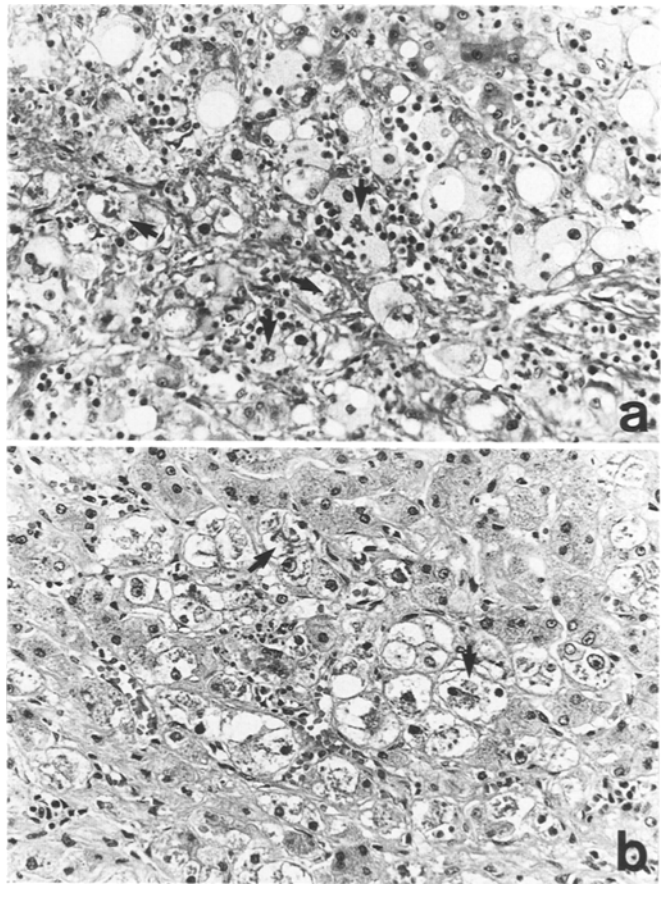

Fig. 1. a Severe alcoholic hepatitis with many Mallory bodies *(arrows)* and a dense inflammatory infiltrate (case 1 of group 1). **b** Moderate alcoholic hepatitis with some Mallory bodies *(arrows)* and a mild inflammatory infiltrate (case 2 of group 1). H&E × 200

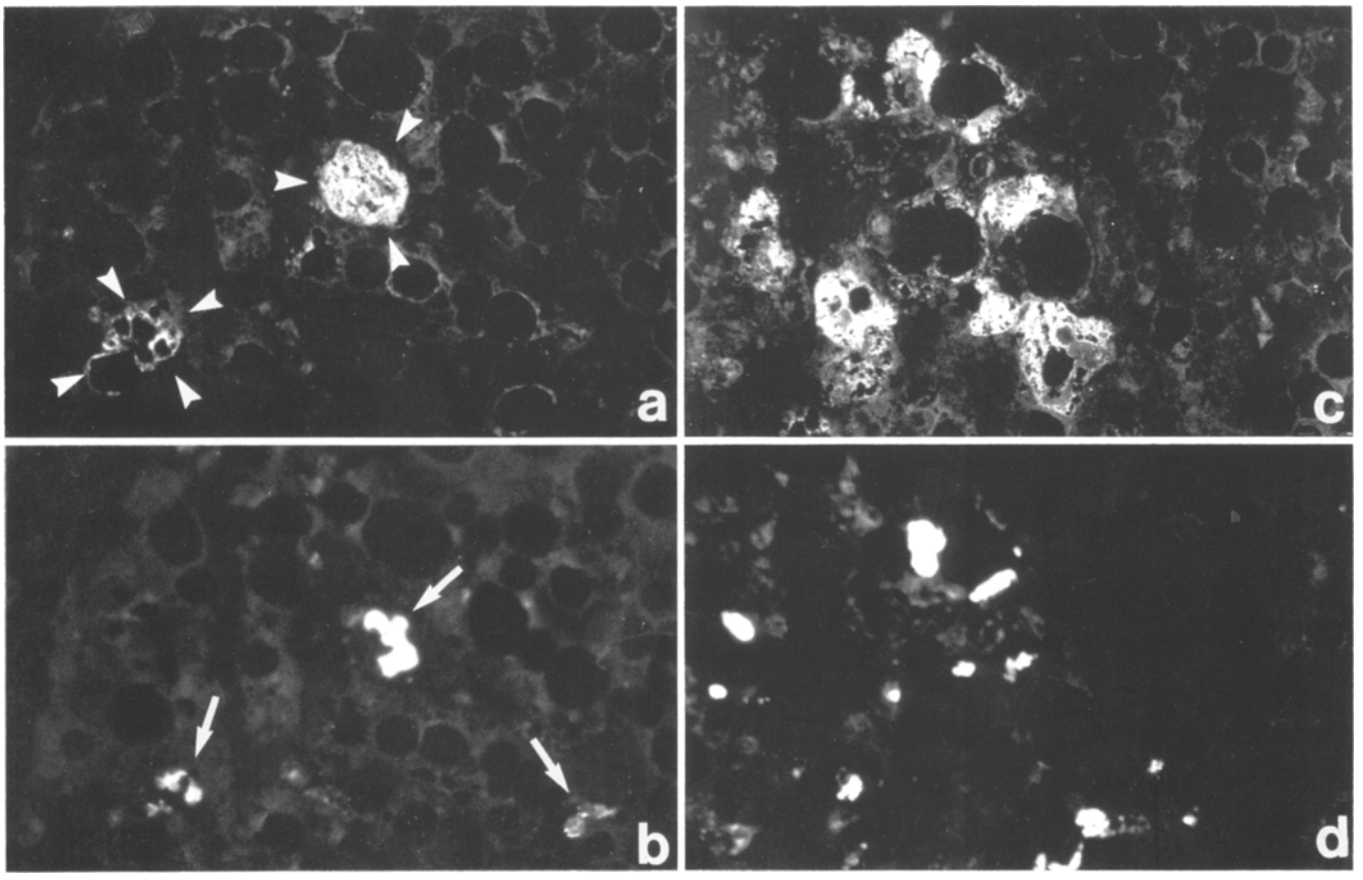

Fig. 2a–d. Alcoholic hepatitis. Double indirect immunofluorescence (IIF) with demonstration of tumour necrosis factor α (TNFα) and Mallory bodies. **a** Two isolated ballooned hepatocytes strongly express TNFα *(arrowheads)*. Within these two hepatocytes Mallory bodies are stained simultaneously (*arrows* in **b**). **c** The left side displays a group of ballooned hepatocytes strongly expressing TNFα. Within these ballooned hepatocytes Mallory bodies are detected (**d**). × 400

anti-TNFα. The monoclonal antibody was used in the first and the polyclonal antibody in the second sequence. The following secondary antibodies were used in single and double IIF: goat anti-mouse IgG + IgM, conjugated with fluorescein isothiocyanate (FITC) or Texas-red (dilution 1:20; Medac; Hamburg, FRG) and swine anti-rabbit Ig conjugated with FITC (Dakopatts; Glostrup, Denmark).

Frozen liver sections 4 µm thick were air-dried and then fixed with cold acetone (−20° C) for 5 minutes for immunohistochemistry (IHC) which was performed using the alkaline phosphatase – anti alkaline phosphatase complex (APAAP) technique with the antibodies and dilutions listed in Table 1. The primary antibodies were diluted in 1% bovine serum albumin, applied to the tissue sections and incubated for 1 h. As secondary antibodies rabbit anti-mouse(pan)Ig (Dakopatts; dilution 1:30) and an affinity purified mouse anti-rabbit-IgG (dilution 1:500; Dakopatts), respectively, were used as linking antibodies and incubated for 30 min in a moist chamber at room temperature. Prior to the addition of APAAP (Dakopatts), the sections were washed twice in PBS. APAAP (diluted 1:100 in PBS) was applied for 30 min and the colour reaction was performed with fast red working solution: 10 mg of naphthol-AS-MX-phosphate (Serva, Heidelberg, FRG) were dissolved in 0.5 ml N,N-dimethylformamide (Serva) and 50 ml of veronal acetate buffer (pH 9.2) to which 50 mg of fast red TR (Serva) and 10 mg of levamisol (Sigma, St. Louis, Mo., USA) were added. The solution was applied after filtration and adjustment of the pH to 9.2–9.8 for 30 min at room temperature. For detection of ICAM-1, liver sections were pre-treated for 15 min either with 0.5 M sodium chloride solution or 0.01% Triton X-100 (Merck, Darmstadt, FRG) was added to the primary antibody.

For negative controls tissue sections were treated identically, but the primary antibodies were replaced by PBS, normal rabbit serum or normal mouse serum, respectively.

For positive controls sections of lymph nodes from 2 cases of Hodgkin's disease were used for testing antibodies to cytokines and integrins and liver sections of baboons with experimentally induced sepsis were used for testing antibodies to ICAM-1 and endothelial leukocyte adhesion molecule-1 (ELAM-1).

Results

On light microscopy the haematoxylin-eosin-and CAB-stained sections of the livers from group 1 revealed severe alcoholic hepatitis with dense neutrophilic granulocytic infiltration and numerous Mallory bodies in one case and a more focally developed milder form of alcoholic hepatitis in the two other cases (Fig. 1a, b). Two cases were associated with cirrhosis, and one case with mild alcoholic hepatitis showed areas with chicken wire-type fibrosis. All three cases of group 2 showed severe diffuse steatosis with large fat vacuoles within the hepatocytes, but without alcoholic hepatitis. The cases of group 3 (regarded as controls) showed minor lymphocytic infiltrates in the portal tracts and slight portal fibrosis in two cases.

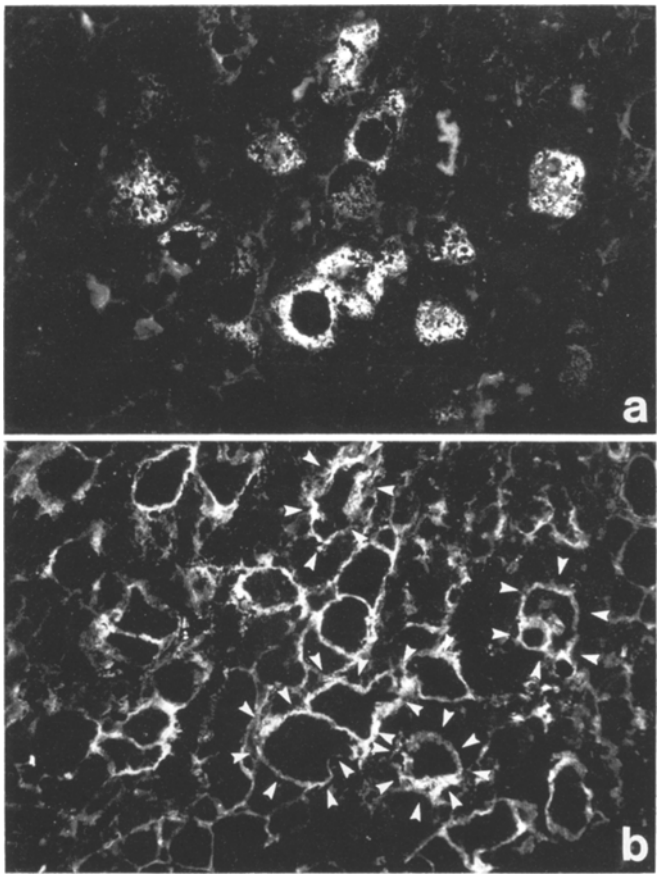

Fig. 3a, b. Alcoholic hepatitis. Double IIF with immunostaining of TNFα and ICAM-1. **a** A group of ballooned hepatocytes express TNFα. These TNFα-positive hepatocytes also show a strong reactivity for ICAM-1 (*arrowheads* in **b**). × 400

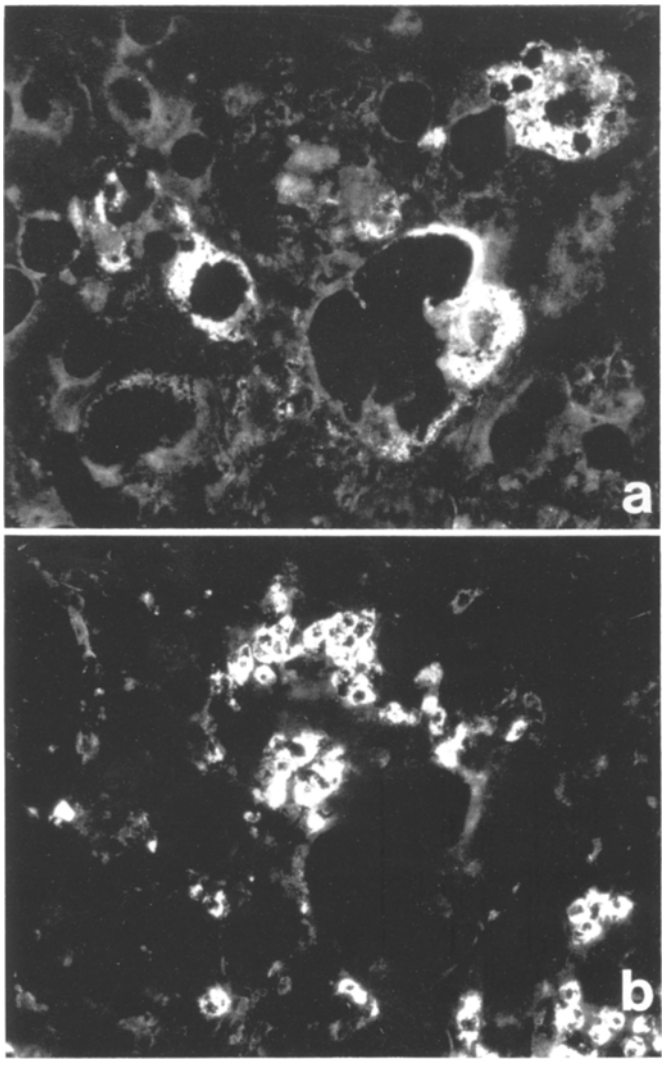

Fig. 4a, b. Alcoholic hepatitis. Double IIF with demonstration of TNFα and CD18. **a** Some ballooned hepatocytes strongly express TNFα. In this area, sometimes in close association with TNFα-positive hepatocytes, clusters of CD18 positive inflammatory cells (neutrophils) are decorated (**b**). × 400

On immunofluorescence microscopy TNFα was detected in ballooned hepatocytes with or without Mallory bodies, sometimes in single cells (Fig. 2a), but predominantly in cell clusters in alcoholic hepatitis (Fig. 2c). In some areas most of the degenerated hepatocytes positive for TNFα contained Mallory bodies as shown in double IIF (Fig. 2b, d). Furthermore, double IIF disclosed a co-distribution of TNFα-positive hepatocytes with ICAM-1-positive hepatocytes (Fig. 3a, b). The antibody to ICAM-1 reacted with the periphery of hepatocytes (Fig. 3b). CD18-positive inflammatory cells could be seen preferentially in areas of ballooned TNFα- and ICAM-1-positive hepatocytes, but there was no strict co-distribution on a cell to cell basis (Fig. 4a, b).

In the livers of groups 2 and 3 only some scattered hepatocytes and sinusoidal lining cells showed TNFα positivity. Some CD18-positive leukocytes were dispersed in liver sections of these groups.

Using IHC alcoholic hepatitis cases revealed TNFα in groups of ballooned hepatocytes, often containing Mallory bodies (Fig. 5a, b). Other cells, like Kupffer cells, endothelial cells and bile duct epithelium displayed TNFα focally and weakly. With the antibody directed against IL-1α no clear-cut positive reaction could be found in hepatocytes and non-parenchymal cells. The antibody to IL-6 reacted predominantly with Kupffer cells but not with hepatocytes. The antibody to IL-8 reacted with all leukocytes, macrophages, Kupffer cells and probably other sinusoidal lining cells. Furthermore, endothelial cells and some degenerating hepatocytes were decorated by this antibody, but there was no strict association with Mallory body-containing hepatocytes. Within the three groups no differences were seen with respect to the reaction profiles of IL-6 and IL-8, although the number of positive cells was much greater in group 1.

TNFα positivity was correlated with ICAM-1 staining of hepatocytes, as shown in sequential sections, but a clear-cut membraneous reaction pattern could be demonstrated only after addition of a detergent to the primary antibody solution (Fig. 6). No significant reactivity for ELAM-1 could be detected in all groups investigated (data not shown). After suppression of background staining by addition of detergent to the ICAM-1 anti-

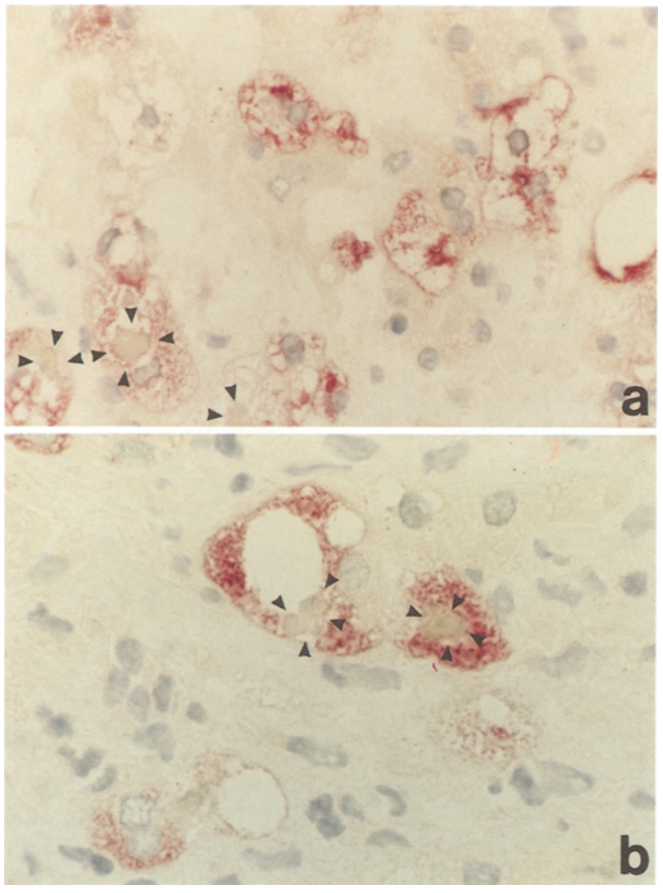

Fig. 5a, b. Alcoholic hepatitis. Demonstration of TNFα by alkaline phosphatase anti-alkaline phosphatase (APAAP). Most of the ballooned hepatocytes show a positive immunoreaction with the antibody to TNFα. Moreover, at least in some of these hepatocytes Mallory bodies can be seen *(arrowheads)* in **a** and **b**. The Mallory bodies are better seen at higher magnification (**b**). **a** × 440; **b** × 630

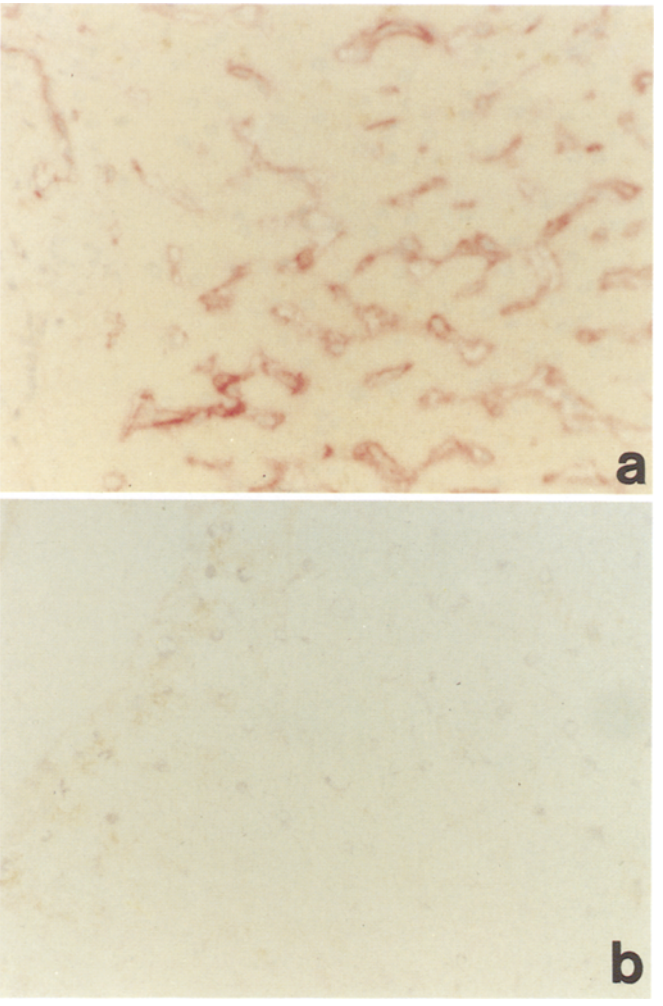

Fig. 7a, b. Normal liver (control). Demonstration of ICAM-1 by APAAP before (**a**) and after (**b**) addition of a detergent (Triton-X 100) to the primary antibody solution. Without addition of detergent the hepatic sinusoids show a moderately positive reaction (**a**) which is abolished after addition of detergent (**b**). × 250

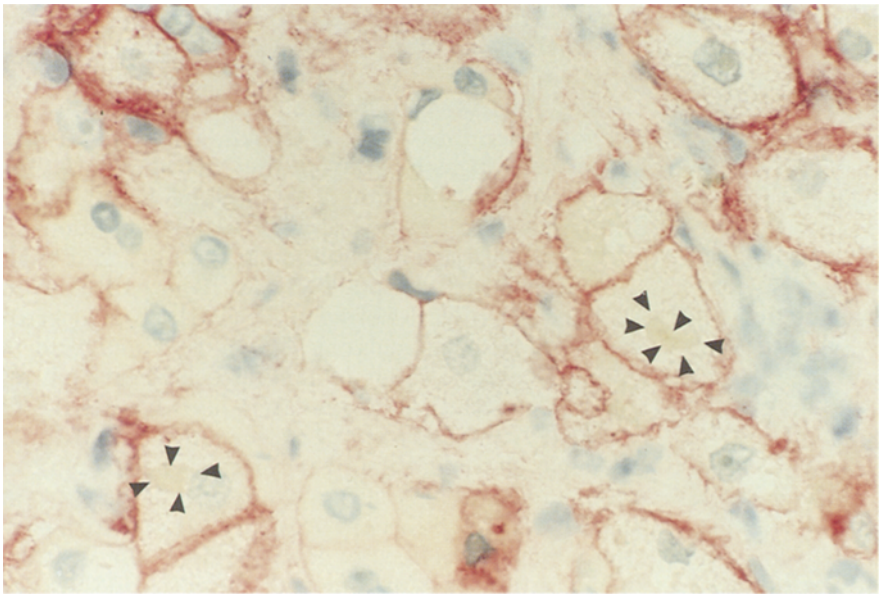

Fig. 6. Alcoholic hepatitis. Demonstration by APAAP of ICAM-1 after addition of a detergent to the primary antibody. The ballooned hepatocytes show an especially strong positive and distinct membranous reaction pattern. Some of the ballooned hepatocytes also contain Mallory bodies *(arrowheads)*. × 400

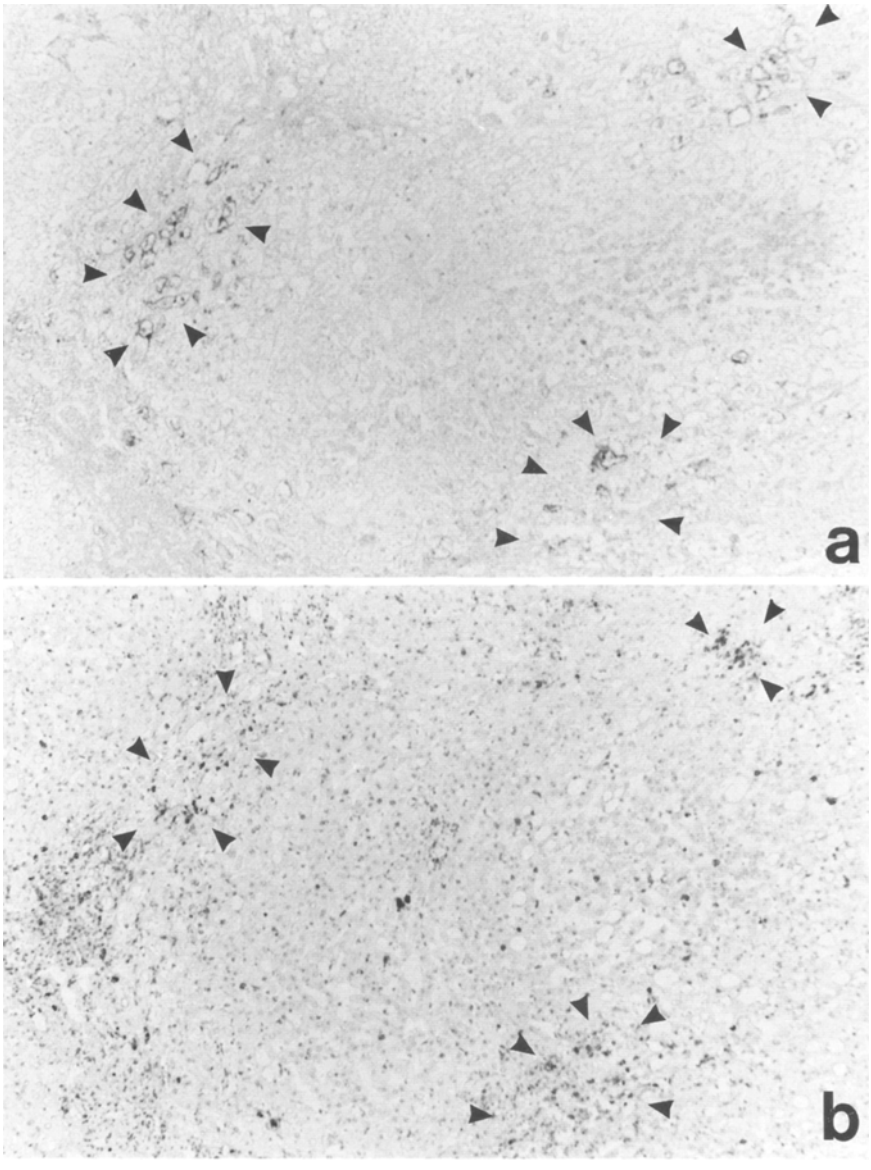

Fig. 8a, b. Alcoholic hepatitis. Serial sections with demonstration of ICAM-1 in **a** and CD11b-positive inflammatory cells in **b.** In areas with ICAM-1 expression on hepatocytes a co-distribution of CD11b-positive inflammatory cells (neutrophils) is shown (*arrowheads* in **a** and **b**). × 60

body, no immunoreaction was found on hepatocytes and sinusoidal lining cells in the livers of groups 2 and 3 (compare Fig. 7a with 7b).

In alcoholic hepatitis the reaction with the antibodies to the integrins reflected the inflammatory process. Almost all leukocytes and also a larger proportion of Kupffer cells were stained with CD11a and CD18 (LFA-1) antibodies. With the CD11c antibody a positive reaction could only be found on some Kupffer cells and macrophages. The CD11b (Mac-1) antibody, which exclusively reacted with neutrophils, revealed an accumulation of the latter in areas with ICAM-1 positivity, as shown in sequential sections (Fig. 8a, b).

Discussion

Hepatotoxicity of ethanol involves several metabolic pathways. Ethanol is hepatotoxic through redox changes which, among other effects, result in lipid peroxidation (Lieber 1990; Lieber and DeCarli 1991) and possibly also in the generation of leukotactic substances (Perez et al. 1984; Hultcrantz et al. 1991). An altered cytokine homeostasis has also been implicated in the pathogenesis of alcoholic liver disease, but to our knowledge this aspect has been investigated only clinically (McClain et al. 1986; Bird et al. 1990; Khoruts et al. 1991). McClain et al. (1986) described elevated serum IL-1 concentrations in acute alcoholic hepatitis and, more recently, Bird et al. (1990) found high plasma TNFα levels in patients with alcoholic hepatitis. Khoruts et al. (1991) also reported elevated levels of circulating TNFα, IL-1 and IL-6 in chronic alcoholics. In an immunohistochemical study on adhesion molecules in inflammatory liver diseases, Volpes et al. (1990) found a strong expression of ICAM-1 and LFA-3 in sinusoidal lining cells and in hepatocytes in alcoholic hepatitis. A strong expression of ICAM-1 in hepatocytes and the occurrence of LFA-1 positive leukocytes was also described by Burra et al. (1992). We were able to confirm these finding and, moreover, to show for

the first time co-distribution of degenerated hepatocytes expressing ICAM-1 with TNFα-containing hepatocytes and with Mac-1-positive granulocytes.

The goal of our immunohistochemical study was to investigate the distribution and consequently the possible role of some cytokines in acute alcoholic hepatitis and to verify the proposed possibility of an induction of adhesion molecules by these cytokines. We found TNFα especially in degenerating ballooned hepatocytes often in association with Mallory body formation (Figs. 2, 5). The occurrence of TNFα and ICAM-1 and their co-distribution in areas of degenerating hepatocytes was most striking in case 1, but to a minor degree also in the other two cases with alcoholic hepatitis. Since production of TNFα in the liver is mainly a function of non-parenchymal cells, predominantly Kupffer cells (Decker 1990; Andus et al. 1991), the demonstration of TNFα in hepatocytes might reflect the uptake of TNFα via TNFα-receptors on hepatocytes, although the generation of TNFα by hepatocytes cannot be exluded. Further immunohistochemical investigations combined with in situ hybridization for the demonstration of TNFα-mRNA could solve this problem. TNFα directs various leukocytes to sites of inflammation through induction of adhesion molecules, like ICAM-1 and ELAM-1, and activates them (Manoque et al. 1991). Therefore, the observed co-localization of TNFα and ICAM-1 is not surprising. Interestingly, TNFα could not be detected in neutrophils (Fig. 4) – also a possible source of TNFα – indicating that a transfer of TNFα to hepatocytes via neutrophils is very unlikely. Moreover, we found no induction of ELAM-1 in sinusoidal lining cells in alcoholic hepatitis, which has been reported by Redl et al. (1991) in the livers of baboons with experimentally induced *E. coli* sepsis (Redl et al. 1991). In sepsis and endotoxinaemia Kupffer cells are the major source of TNFα and IL-1 (Chensue et al. 1991), which induce the strong ELAM-1 expression on sinusoidal endothelial cells (Redl et al. 1991). Therefore, the lack of ELAM-1 expression in alcoholic hepatitis may point to hepatocytes as the primary source of TNFα. As shown by Volpes et al. (1990), sinusoidal cells seem to express ICAM-1 in normal livers. This could, however, be a non-specific phenomenon, since pretreatment of the tissue sections with high salt solutions diminished and the addition of a detergent to the antibody abolished antibody binding to sinusoidal lining cells (Fig. 7a, b) whereas the membranous immunoreaction on hepatocytes persisted (Fig. 6a, b). The β_2-integrins LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) are both natural ligands for ICAM-1 (Marlin and Springer 1987; Diamond et al. 1990). Whereas LFA-1 is expressed virtually on all leukocytes, Mac-1 is preferentially expressed on neutrophils (Diamond et al. 1990). In sequential sections we have seen that in alcoholic hepatitis granulocytes react exclusively and very strongly with the CD11b antibody and that CD11b-positive granulocytes accumulate in areas with ICAM-1 expression on hepatocytes (Fig. 8). This suggests a major role of ICAM-1 as a ligand for Mac-1 during the inflammatory processes in alcoholic hepatitis. While the reaction between Mac-1 and ICAM-1 might be responsible for the adherence of neutrophils to hepatocellular membranes, chemotactic lipid peroxidation products (Hultcrantz et al. 1991) or the potent chemotactic factor IL-8; which can be produced in high amounts also by TNFα- or IL-1-stimulated hepatocytes (Thornton et al. 1990) are probably the reason for emigration of neutrophils from the liver sinusoids and accumulation around degenerating hepatocytes. Exposure of neutrophils to TNFα is followed by activation, reflected by increased phagocytosis, release of oxygen radicals and degranulation (Manoque et al. 1991). Subsequently, activated neutrophils might be responsible for, or contribute to, liver cell degeneration and necrosis (Uchida et al. 1984). In alcoholic hepatitis neutrophils may even invade ballooned, Mallory body-containing hepatocytes and then release their lysosomal enzymes within the cytoplasm of the hepatocytes with subsequent deleterious effects (Takahashi et al. 1987).

The induction of ICAM-1 and the activation of leukocytes together with the multipotent function of TNFα in alcoholic hepatitis may all contribute to Mallory body formation in hepatocytes. This notion is supported by the association of TNFα-positive ballooned hepatocytes with Mallory bodies.

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