Induction of Tumor Necrosis Factor α Production by Human Hepatocytes in Chronic Viral Hepatitis

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Summary

Tumor necrosis factor α (TNF-α) is a multifunctional cytokine that has an important role in the pathogenesis of inflammation, cachexia, and septic shock. Although TNF-α is mainly produced by macrophages, there is evidence regarding TNF-α production by cells that are not derived from bone marrow. TNF-α production by normal and inflamed human liver was assessed at both mRNA and protein levels. Using a wide panel of novel anti-TNF-α monoclonal antibodies and a specific polyclonal antiserum, TNF-α immunoreactivity was found in hepatocytes from patients chronically infected with either hepatitis B virus (HBV) or hepatitis C virus. Minimal TNF-α immunoreactivity was detected in the mononuclear cell infiltrate and Kupffer cells. In situ hybridization experiments using a TNF-α RNA probe showed a significant expression of TNF-α mRNA in hepatocytes, Kupffer cells, and some infiltrating mononuclear cells. By contrast, TNF-α was detected at low levels in liver biopsies from normal individuals or patients with alcoholic liver disease and low expression of TNF-α mRNA was observed in these specimens. Transfection of HepG2 hepatoblastoma cells with either HBV genome or HBV X gene resulted in induction of TNF-α expression. Our results demonstrate that viral infection induces, both in vivo and in vitro, TNF-α production in hepatocytes, and indicate that the HBV X protein may regulate the expression of this cytokine. These findings suggest that TNF-α may have an important role in human liver diseases induced by viruses.

TNF-α is a multifunctional cytokine that appears to have an important role in the pathogenesis of tissue damage seen in multiple unrelated conditions such as malaria, chronic heart failure, GVHD, septic shock, and cachexia associated to malignant and infectious diseases (1). The main stimulus for TNF-α production appears to be bacterial LPS. However, other various agents such as parasites, exotoxins, lymphokines, and malignant cells are also capable of inducing the synthesis of this cytokine (1). Although TNF-α is mainly produced by macrophages, this cytokine is also synthesized by NK cells or T lymphocytes stimulated through the CD3, CD2, or CD69 molecular pathways (2–4). TNF-α synthesis has also been observed in certain L929 fibrosarcoma cells and some nonhematopoietic cell lines (5, 6). In addition, TNF-α is produced by thymic mast cells, keratinocytes, as well as myocardial and islet cells (7–9). However, it has not been established whether or not TNF-α is produced by hepatocytes.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are the main causative agents of chronic viral hepatitis in humans. These viruses are capable of infecting hepatocytes, inducing inflammatory and necrotic changes in the liver. Since HBV

is not cytolytic for hepatocytes, the liver damage seen in chronic HBV infection appears to be mainly mediated by the immune response against the virus (10, 11). CTLs appear to play an important role in the liver damage seen in HBV infection (12), and cytokines produced by immune cells, such as TNF-α, might also have a role in the liver damage induced by viruses. The liver appears to be a source of several proinflammatory cytokines such as IL-1, IL-6, and TNF-α (13–14), however, it has not been determined which liver cells are capable of synthesizing these cytokines. In this work, the production of TNF-α by normal and inflamed liver was assessed at protein and mRNA levels. We have found significant TNF-α production in liver from patients with chronic HBV and HCV infections. It is interesting to note that hepatocytes appear to be the main source of TNF-α in these conditions.

Materials and Methods

Antibodies and Epitope Mapping Analysis. The generation of the anti-TNF-α mAb used in this study has previously been described (2, 3). The anti-TNF-α WI-AT-4 mAb was obtained from Pierce Chemical Co. (Rockford, IL). A purified polyclonal anti-TNF-α
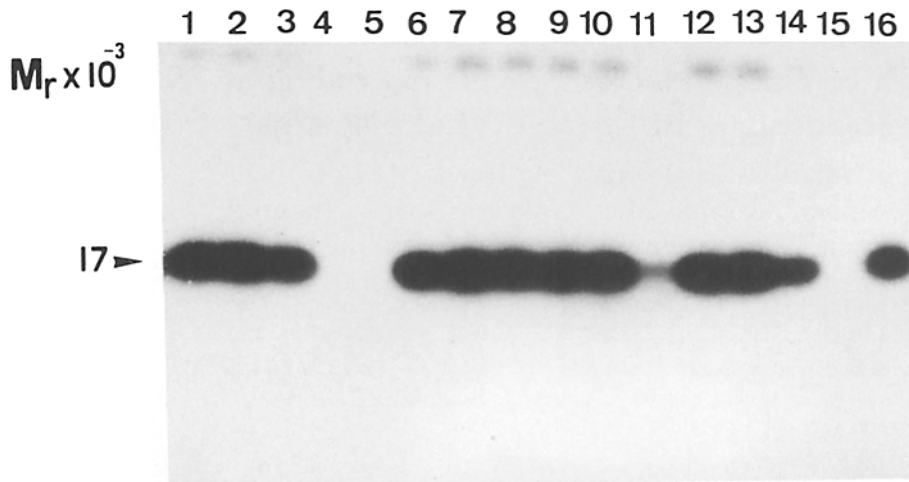
Figure 1. Immunoprecipitation of recombinant ^{125}I-TNF-α by different anti-TNF-α mAbs. Precipitation with the negative (P3X63) and positive (WI-AT-4) control mAb is also shown (lanes *15* and *16*, respectively). Immunocomplexes were isolated, and reduced samples were subjected to SDS-15% PAGE and autoradiography. Lane *1*: AM918 mAb; lane *2*: AM917 mAb; lane *3*: AM915 mAb; lanes *4* and *5*: no mAb; lane *6*: AM933 mAb; lane *7*: AM914 mAb; lane *8*: AM913 mAb; lane *9*: AM912 mAb; lane *10*: AM911 mAb; lane *11*: AM939 mAb; lane *12*: AM909 mAb; lane *13*: AM908 mAb; and lane *14*: AM903 mAb.

antibody developed in sheep was kindly provided by Dr. D. Pascual-Salcedo (Hospital La Paz, Madrid, Spain). Epitopes recognized by the anti-TNF-α mAb were determined by cross-blocking binding assays, as described (15).

Radiolabeling, Immunoprecipitation, and Electrophoresis. The specificity of mAb for TNF-α was confirmed by immunoprecipitation, as described (2). Briefly, rTNF-α was radioiodinated and mixed with mAb-containing culture supernatants. Immunocomplexes were isolated by the addition of 187.1 rat anti–mouse κ chain mAb, followed by protein A from *Staphylococcus aureus* coupled to Sepharose (Pharmacia, Uppsala, Sweden). Immunoprecipitates were processed as previously described (2), and samples were subjected to 15% SDS-PAGE and autoradiography.

Patients and Controls. The study included 20 patients who had liver biopsy findings compatible, by internationally accepted criteria (16), with chronic active hepatitis, 10 type B and 10 type C. Five patients with biopsy-proven alcoholic liver disease and four patients with histologically normal liver were studied as controls. All were negative for HBsAg and anti-HCV antibodies.

Liver Biopsies. After written consent, percutaneous liver biopsies were obtained from all patients. Normal liver biopsies were obtained, after written consent, from patients who underwent a laparotomy for noncomplicated cholecystectomy. A portion of the biopsies was fixed in 10% formaldehyde and embedded in paraffin by routine methods. Another portion of the biopsies was snap frozen in nitrogen-cooled isopentane and stored at $-80°C$ until use.

Immunochemical Staining. Frozen liver sections (4–6 μm) fixed in cold acetone were incubated with the anti-TNF-α mAb. The indirect immunoperoxidase and the APAAP staining techniques were performed as previously described (17–19). In all experiments, P3X63 myeloma culture supernatants, as well as several other mAbs (17–19) were used as controls.

For immunofluorescence studies, frozen liver sections were incubated with a biotinylated polyclonal sheep anti-TNF-α antiserum. After washing three times with Tris-buffered saline, the sections were incubated with an FITC-labeled avidin (Vector Laboratories,

Inc., Burlingame, CA). Subsequently, sections were washed and cell nuclei were counterstained with 4'-6-diamino-2-phenylindole (DAPI). Negative controls included both preimmune serum and an irrelevant sheep anti–digoxigenin antiserum (Boehringer Mannheim, Mannheim, Germany).

RNA Probes. Plasmid pGEM-hTNF-α (20) were used as templates to generate digoxigenin-labeled sense and antisense RNA probes (21). A 550-bp fragment of the rat serum albumin cDNA was used as positive control for hybridization on hepatocytes.

In Situ Hybridization. Paraffin-embedded liver sections were dewaxed with bio-clear (Bio-Optica, Milan, Italy), digested 12 min with 20 μg/ml proteinase K (Sigma Chemical Co., St. Louis, MO), and hybridized overnight at 45°C with a preheated (75°C, 5 min) hybridization mixture (100 ng/ml of RNA probe, 2× SSC, 0.5 mg/ml yeast tRNA, 10 mM dithiothreitol, 50% formamide, 10% SDS, and 20 mM Tris HCl, pH 7.5), as described (21). After posthybridization washings in 50% formamide, 5× SSC, and 10% SDS at 55°C, sections were incubated for 30 min at room temperature with a mouse anti–digoxigenin mAb (Boehringer Mannheim). Sections were then washed and stained by the APAAP technique, as described above. Negative (TNF-α sense RNA probe) and positive (antisense albumin RNA probe) controls were run simultaneously with the TNF-α antisense RNA probe.

Transfection of HepG2 Cells. HepG2 cells were transfected by the calcium phosphate precipitation method. Transient transfections were carried out employing the recircularized whole genome of HBV (22), as well as the plasmid pSBDR-X, which contains the open reading frame of HBV X gene under the transcription control of its own promoter and enhancer (kindly provided by Dr. A. Siddiqui, University of Colorado, Denver, CO). An irrelevant plasmid (pAIMI-neo containing the open reading frame of CD69/AIM) was used as negative control. Transfected HepG2 cells were grown on glass coverslips and cells fixed in methanol/acetone. Immunodetection of TNF-α was carried out by indirect immunofluorescence staining using both anti-TNF-α mAb and a specific anti-TNF-α polyclonal antiserum. An anti vascular cell adhe-

Figure 2. Immunohistochemical detection of TNF-α in liver sections. (*A–D*) Immunoperoxidase staining of liver sections from a patient with chronic active hepatitis C (*A*), alcoholic liver damage (*C*), and a control individual (*B*). The negative control (P3X63 myeloma supernatant) of immunoperoxidase staining is also shown (*D*). All images correspond to liver sections run at the same time. Sections were counterstained with Carazzi's hematoxylin. Original magnifications at 400 (*A, C, D*) and 200 (*B*). (*E–F*) Immunofluorescence staining of liver sections from a patient with chronic active hepatitis C with a polyclonal anti-TNF-α antiserum. To show the presence of cellular infiltrate, cell nuclei were counterstained with DAPI (blue fluorescence) in *F*. Cell infiltrate shows minimal TNF-α immunoreactivity (green fluorescence). Original magnification at 600.
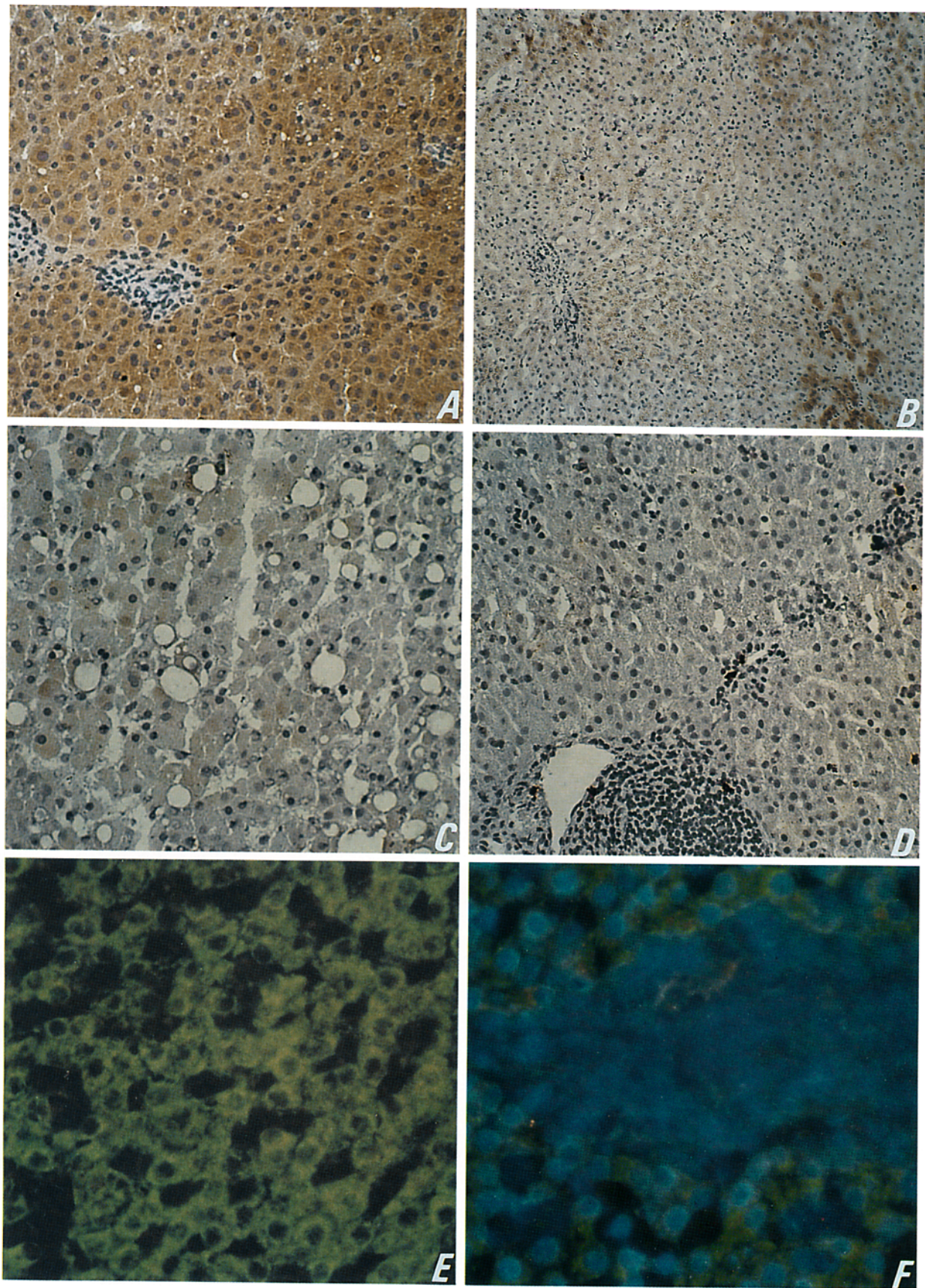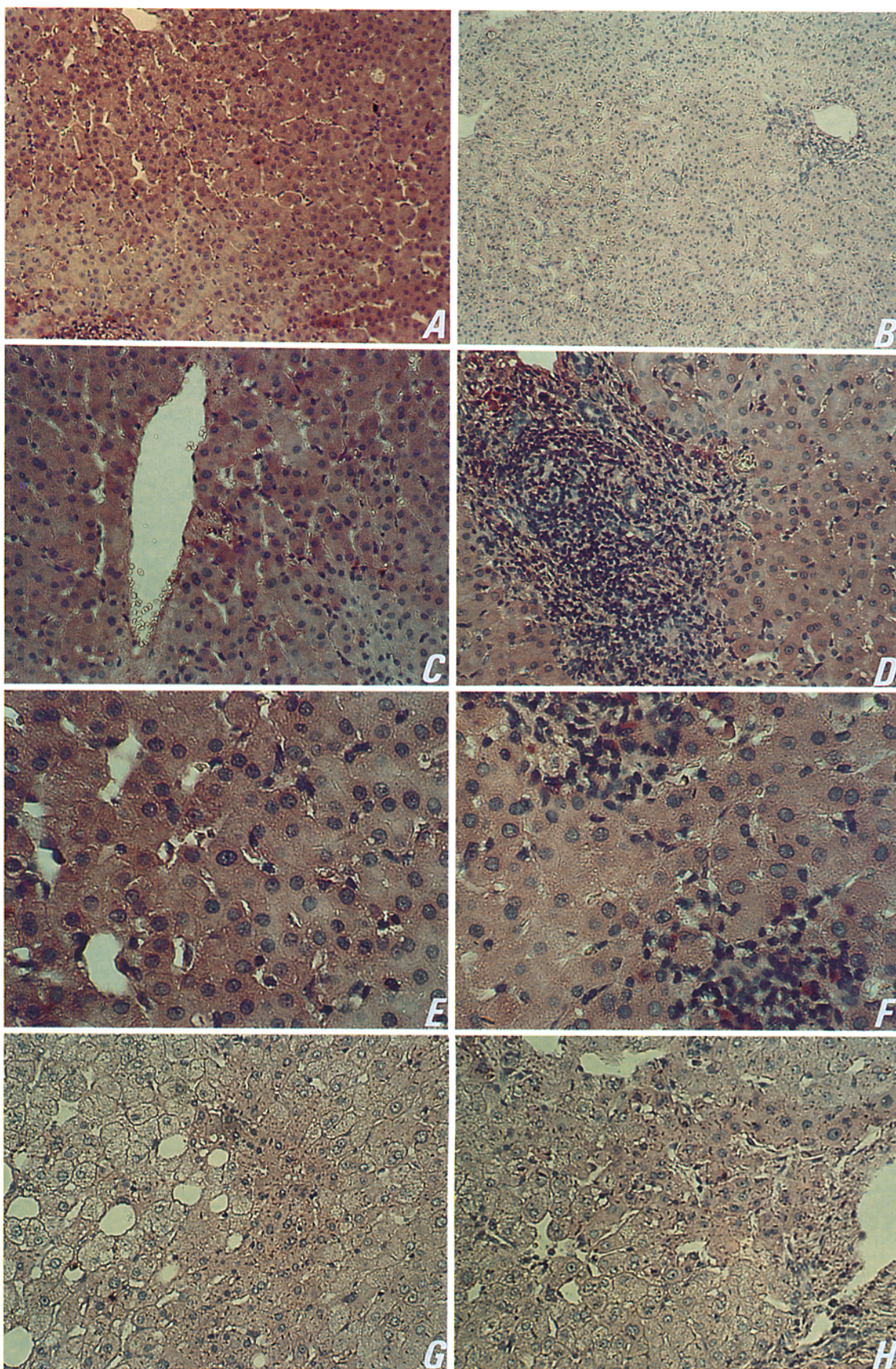
sion molecule 1 mAb was used as negative control, and an anti intercellular adhesion molecule 1 (ICAM-1) mAb was used as a positive control (23). The 2.2.15 cell line, which is a stably transfected derivative of HepG2 cells developed by transfection with a dimerized HBV genome (22), was also tested for TNF-α immunoreactivity.

Results and Discussion

TNF-α is mainly produced by macrophages; however, there is evidence regarding TNF-α production by cells that are not derived from bone marrow (5–8). The constitutive activity of the TNF-α gene promoter is canceled in nonmacrophage cell lines, but an as yet unidentified cell factor appears to be capable of overcoming this suppressive state (24). This appears to be the mechanism that accounts for the TNF-α synthesis by some nonhematopoietic cells (5–7). In this study, we addressed the production of TNF-α by normal and inflamed human liver, specifically, the synthesis of this cytokine by virally infected hepatocytes.

To investigate the possible production of TNF-α in normal and inflamed liver, we generated a panel of TNF-α-reactive mAbs. These mAbs were found to be specific for three different antigenic determinants of TNF-α: epitopes A (two mAbs), B (seven mAbs), and C (three mAbs). All these mAbs selectively immunoprecipitated the same 17-kD form of TNF-α recognized by control anti-TNF-α mAb (Fig. 1). With this panel of mAbs, a high level of TNF-α immunoreactivity was detected on liver sections from patients with chronic HBV and HCV infections. Such reactivity was observed using anti-TNF-α mAbs specific for epitope B, but not with those specific for A or C epitopes. TNF-α immunoreactivity was mainly observed in hepatocytes, showing a predominant cytoplasmic staining (Fig. 2 A). In most cases, all hepatocytes were diffusely stained, but in some cases, the strong reactivity was mainly located to the periportal areas, specifically where inflammation and piecemeal necrosis was more evident. A weak TNF-α immunoreactivity was observed in some infiltrating cells, with no staining of Kupffer cells. These results were confirmed using a specific polyclonal anti-TNF-α antiserum (Fig. 2, E and F). The staining pattern obtained in liver sections using anti-TNF-α mAb was clearly different from those obtained with other mAbs (11, 18, 19, and data not shown). In addition, no staining was obtained with the P3X63 myeloma supernatant (Fig. 2 D).

A low level of TNF-α immunoreactivity was observed in cells from normal liver sections (Fig. 2 B). In these cases, a significant staining was detected in some hepatocytes located to perivenular areas, whereas in other sites, only scattered positive hepatocytes were found. No apparent TNF-α expression was detected on Kupffer cells. Liver sections from alcoholic hepatitis showed a pattern of TNF-α expression

similar to that found in normal liver (Fig. 2 C). TNF-α immunoreactivity was mainly located in the cytoplasm, suggesting accumulation of the protein into the cells. It is possible that TNF-α is processed by a different pathway in hepatocytes than in macrophages or T lymphocytes (2, 4, 25). Thus, the hepatocyte-derived TNF-α might not be actively secreted, exerting their actions predominantly in the same cell that is producing, or in the neighboring cells. On these conditions, no endocrine effects of hepatocyte-derived TNF-α should be expected.

Immunohistochemical studies were confirmed by immunoblotting analysis of liver homogenates using a specific polyclonal antiserum. These analyses revealed the presence of a specific band of 17 kD in immunoprecipitates from livers with HBV infection (data not shown). The above results demonstrated the presence of immunoreactive TNF-α molecules in hepatocytes, with a remarkable upregulated expression in chronic viral hepatitis.

In situ hybridization experiments using a specific RNA probe revealed the presence of high TNF-α mRNA levels in liver sections from both chronic HBV and HCV infections. A strong expression of TNF-α mRNA was observed in hepatocytes (Fig. 3 A), with a pattern that resembled that obtained in immunostaining experiments using anti-TNF-α mAb. Positive cells for TNF-α mRNA were also found in the mononuclear cell infiltrate (Fig. 3, D and F), a finding that contrasted with the very low TNF-α immunoreactivity of these cells (Fig. 2, A and F). Positive cells with a location and morphology of Kupffer cells were found in all cases (Fig. 3, E and F). Low levels of TNF-α mRNA were detected in liver sections from alcoholic liver disease (Fig. 3 G). In these specimens, the TNF-α mRNA-expressing cells were also mainly hepatocytes. In normal liver sections, scattered positive hepatocytes were found (Fig. 3 H), whereas Kupffer cells were usually negative.

It has been described that HBV is capable of upregulating the expression of ICAM-1 and HLA-DR on hepatocytes (23, 26). This effect appears to be mediated by the HBV protein X that transactivate gene promoters containing nuclear factor (NF) κB and other sequence motifs (23, 26). It is conceivable that TNF-α synthesis by hepatocytes may also be modulated by viral proteins, such as HBV protein X. The presence of NF-κB responsive sequences in the TNF-α gene (27), further supports this point. To test this possibility, we performed transfection experiments on HepG2 cells using both the whole HBV genome and the HBV X gene. HepG2 cells transiently transfected with either HBV whole genome or HBV X gene, showed strong TNF-α immunoreactivity (Fig. 4 A, and data not shown). TNF-α staining was mainly cytoplasmic, but cell membrane immunoreactivity was also seen. Untransfected HepG2 cells or those transfected with the negative control plasmid (pAIMI-neo), did not show TNF-α immunoreac-

Figure 3. In situ detection of TNF-α mRNA using a nonradioactive RNA probe. An enzyme-linked color reaction (fast red) identifies cells hybridizing to the digoxigenin-11-UTP-labeled TNF-α probe. All images correspond to the same experiment and were run simultaneously. Antisense probe in a liver section from a patient with chronic active hepatitis C (A, C, D, E, and F). Negative control (sense probe) in a liver section from the same biopsy (B). Original magnifications at 200 (A and B), 400 (C and D), and 600 (E and F). (G–H) Antisense probe in a liver section from a patient with alcoholic liver disease (G) and in a normal liver section (H). Original magnification at 400.
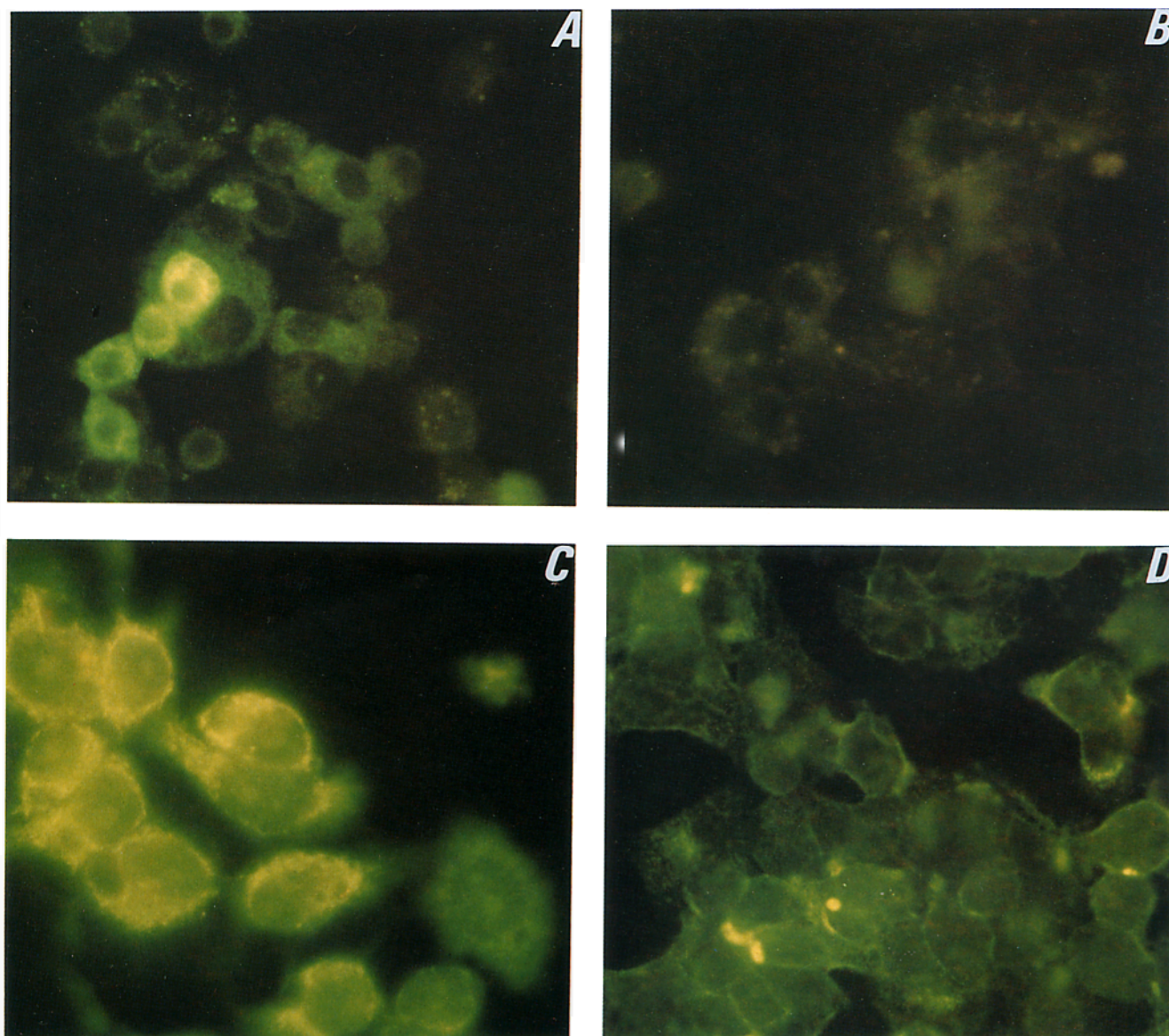
Figure 4. Detection of TNF-α in hepatoblastoma HepG2 cells by indirect immunofluorescence. (*A*) Cells transiently transfected with whole HBV genome and stained with the anti-TNF AM918 mAb. (*B*) Cells transiently transfected with a negative control plasmid (pAIMI-neo) and stained with the anti-TNF AM918 mAb. (*C*) Hep.G2 cells stably transfected with dimerized HBV genome (cell line 2.2.15) stained with the anti-TNF-α AM918 mAb. (*D*) Cells stably transfected with dimerized HBV genome and stained with an anti-ICAM-1 mAb.

tivity (Fig. 4 *B*, and data not shown). These results were confirmed with the 2.2.15 cell line, stably transfected with dimerized HBV genome. These cells also showed TNF-α immunoreactivity with both anti-TNF-α mAb and a specific polyclonal antiserum (Fig. 4 *C*, and data not shown). As has been described (23), HepG2 cells transfected with HBV genome or HBV X gene, showed a significant ICAM-1 immunoreactivity (Fig. 4 *D*). ICAM-1 was mainly located to the cell membrane (Fig. 4 *D*), with a staining pattern that contrasted with that obtained with anti-TNF-α mAb.

Since TNF-α production was detected, at low levels, in hepatocytes from individuals with no evidence of hepatic viral infection, stimulus for hepatocytes TNF-α production, other than viral proteins, must exist.

TNF-α appears to play a role in the tissue damage seen in conditions in which inflammation and liver necrosis occur, such as GVHD and liver allograft rejection (28, 29). Large quantities of TNF-α may have a deleterious local effect in HBV and HCV liver infection through several mechanisms. TNF-α may participate in the increased liver expression of ICAM-1 and other adhesion receptors in both HBV and HCV liver infection (18, 19), contributing to the inflammatory and cytotoxic phenomena seen in these conditions. TNF-α might be cytotoxic itself, and in addition, it might participate in

the activation of cytotoxic immune cells. TNF-α also might induce HBV and HCV viral replication, as in the case of HIV (30). The possible autocrine induction (via TNF-α) of large quantities of nitric oxide synthesis by hepatocytes (31) is another interesting possibility.

Our results demonstrate for the first time the in vivo production of TNF-α by human hepatocytes, with a remarkable upregulation in HCV and HBV infection. The constitutive

production of TNF-α by nonhemopoietic cells may have an important role in the normal physiology of several tissues, including the liver. Under abnormal circumstances, such as viral infections, an increased production of TNF-α by nonhemopoietic cells might be deleterious and mediate tissue damage. Local production of TNF-α by nonimmune cells may have an important role in tissue inflammation and necrosis seen in infectious and autoimmune diseases.

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