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Activation of Caspases in Lethal Experimental Hepatitis and Prevention by Acute Phase Proteins

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Lethal hepatitis can be induced by an agonistic anti-Fas Ab in normal mice or by TNF in mice sensitized to d-(-)-galactosamine or actinomycin D. In all three models, we found that apoptosis of hepatocytes is an early and necessary step to cause lethality. In the three models, we observed activation of the major executioner caspases-3 and -7. Two acute-phase proteins, \( \alpha_1 \)-acid glycoprotein and \( \alpha_1 \)-antitrypsin, differentially prevent lethality: \( \alpha_1 \)-acid glycoprotein protects in both TNF models and not in the anti-Fas model, while \( \alpha_1 \)-antitrypsin confers protection in the TNF/d-(-)-galactosamine model only. The protection is inversely correlated with activation of caspase-3 and caspase-7. The data suggest that activation of caspase-3 and -7 is essential in the in vivo induction of apoptosis leading to lethal hepatitis and that acute phase proteins are powerful inhibitors of apoptosis and caspase activation. Furthermore, Bcl-2 transgenic mice, expressing Bcl-2 specifically in hepatocytes, are protected against a lethal challenge with anti-Fas or with TNF/d-(-)-galactosamine, but not against TNF/actinomycin D. The acute-phase proteins might constitute an inducible anti-apoptotic protective system, which in pathology or disturbed homeostasis prevents excessive apoptosis. The Journal of Immunology, 1999, 163: 5235–5241.

Hepatitis, either virally or chemically induced, is correlated with increased serum TNF levels (1). As shown by us and others, TNF, when given in combination with d-(-)-galactosamine (GalN) or actinomycin D (ActD), induces lethal hepatitis in mice (2–5), a process that is mediated by TNF-R1 (5). Triggering of CD95/Fas, another member of the TNF-R superfAMILY, also leads to lethal hepatitis (5, 6). Evidence exists that, in contrast to the CD95/Fas model, in the TNF models a simultaneous inflammatory component plays an essential role (7). In all three models, apoptosis of hepatocytes was observed. During recent years, cysteinyl aspartate-specific proteases or caspases have been found to be implicated in the process of apoptosis. These caspases mediate cell death by cleaving essential substrates (8). Caspases operating in the apoptotic process can be divided into two groups, the initiator caspases and the executioner caspases. Initiator caspases are those caspases involved in the activation of the executioner caspases that cleave specific death substrates such as poly(ADP-ribose) polymerase, DNA polymerase kinase, and ICAD, the inhibitor of the caspase-activated DNase (9). Caspase-3 (previously known as CPP32, Yama, or apopain) and caspase-7 (previously known as Mch3, CMH-1, or ICE-LAP3) are two main executioner caspases, and both caspases are activated during apoptosis. Recently, two different pathways of activation of executioner caspases have been reported in the case of Fas-mediated apoptosis, depending on the cell type (10). During Fas-mediated type I apoptosis, there is an efficient recruitment of procaspase-8 in the so-called death-inducing signaling complex (11). Procaspase-8 is activated by oligomerization (12), and consequently caspase-8 (previously known as Mch5, MACH, or FLICE) directly proteolyses procaspase-3 and -7 (13). Release of cytochrome c from the mitochondria is observed, but is not essential for induction of apoptosis (10). During type II apoptosis, for unknown reasons minor procaspase-8 recruitment occurs and low levels of caspase-8 are activated (10). Cytochrome c released from the mitochondria is required for amplification of the caspase cascade. Cytochrome c binds to Apaf-1 and ATP and activates procaspase-9, which in turn activates procaspase-3 and -7 (14). In type II apoptosis, cytochrome c release is absolutely required to activate downstream caspases. Inhibition of cytochrome c release by Bcl-2 prevents type II apoptosis.

In this paper, we intended to study the activation of caspases in three experimental models of apoptosis, viz. TNF/GalN, TNF/ActD, and anti-Fas, all three resembling acute viral hepatitis (15–18). We and others (3–6) have demonstrated that these models cause massive apoptosis of hepatocytes of mice in vivo, followed by the release of liver transaminases. We could also show a differential inhibition of apoptosis by acute-phase proteins in the three models (5). We show here that apoptosis is an early event, associated with activation of caspase-3 and -7, and that pretreatment with the acute-phase proteins \( \alpha_1 \)-acid glycoprotein (\( \alpha_1 \)-AGP) and \( \alpha_1 \)-antitrypsin (\( \alpha_1 \)-AT) prevents caspase activation and lethality. Furthermore, transgenic overexpression of Bcl-2 protected differentially in the three models, which suggests either different mechanisms of induction of apoptosis or involvement of other tissues in inducing lethality in the three models.
Materials and Methods

Animals

Female C57BL/6 mice were obtained from Ifa Credo (Saint Germain-sur-l’Arbresle, France) and were used at the age of 8–10 wk. Heterozygous Bcl-2 transgenic mice were generated as follows. A fragment containing the coding sequence of a human Bcl-2 cDNA, flanked by a rabbit β-globin intron and the rabbit β-globin polyadenylation signal (19), was inserted downstream of the human α2-AT promoter. Transgenic mice were generated as described previously (20) using (C57BL/6 × DBA2)F1 eggs. The highest expressor line, as judged by Western blotting of liver extracts, was used for experiments. Heterozygous Bcl-2 transgenic mice were crossed with C57BL/6 animals. Transgenic offspring were identified by PCR and crossed again with C57BL/6 to obtain 50% transgenic and 50% wild-type mice. Male and female transgenic and nontransgenic mice were used at the age of 7–12 wk. The animals were housed in a temperature-controlled, air-conditioned room with 12-h light/dark cycles and received water and food ad libitum.

Reagents

Recombinant murine TNF-α (TNF) was produced in Escherichia coli and purified to homogeneity in the first author’s laboratory. TNF had a sp. act. of 2.6 × 10^11 IU/mg and an endotoxin contamination of 0.07 ng/mg of protein. Endotoxin levels were assessed by a chromogenic Limulus amebocyte lysate assay (Coastest; Chromogenix, Stockholm, Sweden). Bovine α2-AGP, human α2-AT, ActD, and GalN were obtained from Sigma (St. Louis, MO). Monoclonal hamster anti-mouse Fas Ab 2B2 (IgG) (6) was purchased from PharMingen (San Diego, CA) and had an endotoxin level of 0.05 ng/mg protein according to the manufacturer.

Injections and blood collections

Cytokines and chemicals were dissolved in endotoxin-free PBS before use. Intraperitoneal and i.v. injections were conducted in volumes of 0.5 and 0.2 ml, respectively. Blood was taken from the retro-orbital plexus under light ether anesthesia, and was allowed to clot for 30 min at 37°C and 1 h at 4°C, followed by centrifugation at 16,000 × g. Serum was stored at −20°C.

Determination of serum alanyl aminotransferase (ALT) and body temperature measurement

The ALT content was determined using an enzymatic/colorimetric kit (Sigma). Rectal body temperatures were measured with an electronic thermometer (model 2001; Comark Electronics, Littlehampton, U.K.).

Liver homogenate preparation

Livers were cut to small pieces, washed three times with glycerol buffer (10% glycerol, 5 mM EDTA, 10 mM Tris/Cl, pH 7.4, 200 mM NaCl), and homogenized with a tissue grinder (Wheaton Scientific, Millville, NJ) in the same buffer, supplemented with 1 mM PMSF, 0.3 mM aprotinin, 1 mM leupeptin, and 1 mM oxidized glutathione. All steps were performed on ice. Samples were frozen immediately at −20°C.

Assays for apoptosis

Liver homogenates were centrifuged for 20 min at 13,000 × g, and supernatant was stored at 4°C. Apoptosis was quantified by immunochromatographic determination of histone-complexed DNA fragments in a microtiter plate (21). Briefly, plates were coated with an Ab directed against histon H2B. After blocking, homogenates were added and a biotinylated detection Ab specific for the nucleosome subparticle of histones H2A, H2B, and DNA (22) was administered. Detection was performed with alkaline phosphatase-conjugated streptavidin (Sanvertech, Boechout, Belgium) and substrate I (Sigma). Livers from PBS-treated animals were taken at 100%.

Liver tissue was homogenized, before the release of ALT. Both TNF models have developed by enhanced chemiluminescence (Amersham Pharmacia Biotech, Rainham, U.K.).

Fluorogenic substrate assay for caspase activity

Caspase-like activities were determined by incubation of liver homogenate containing 25 μg of total protein with 50 μM of the fluorogenic substrate acetyl-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me)-aminomethylcoumarin (Ac-DEVD-amc) (Peptide Institute, Osaka, Japan) in 200 μl cell-free system buffer containing 10 mM HEPES, pH 7.4, 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH2PO4, 0.5 mM EGTA, 2 mM MgCl2, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM DTT. The release of fluorescent amc was measured for 1 h at 2-min intervals by fluorometry (Cytofluor; PerSeptive Biosystems, Cambridge, MA). Data are expressed as the maximal rate of increase in fluorescence per min (ΔFmax/min).

Statistics

Significant differences in DNA fragmentation, serum ALT, and body temperature were calculated using a two-tailed t test. Significant differences in final survival time were calculated using Fisher’s exact test.

Results

Induction of apoptosis and transaminase release

We previously reported the conditions needed to induce lethal hepatitis using TNF/GalN, TNF/ActD, or anti-Fas (2, 5); lymphotoxin, TNF alone, even in very high doses, was unable to induce necrosis as a secondary phenomenon, as ALT release follows the presence of histon-complexed DNA fragments in the liver homogenates, before the release of ALT. Both TNF models have comparable kinetics of events; however, induction of apoptosis in anti-Fas-treated mice appears much faster. In these models, apparently necrosis is a secondary phenomenon, as ALT release follows apoptosis. TNF alone, even in very high doses, was unable to

Table 1. Induction of apoptosis and release of ALT as a function of time in several models of hepatitis in mice (n = 2 for all groups)

<table>
<thead>
<tr>
<th>Challenge (h)</th>
<th>DNA Fragmentation</th>
<th>ALT (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF/GalN</td>
<td>1</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>996</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1024</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1167</td>
</tr>
<tr>
<td>TNF/ActD</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>445</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>725</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>0.5</td>
<td>97</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>206</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>824</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>902</td>
</tr>
<tr>
<td>TNF</td>
<td>3</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>130</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>122</td>
</tr>
<tr>
<td>GalN</td>
<td>2</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>110</td>
</tr>
<tr>
<td>ActD</td>
<td>2</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>80</td>
</tr>
</tbody>
</table>

a PBS-treated mice were taken as 100% DNA fragmentation.
b The value for PBS-treated mice was 133 U/L.
c TNF/GalN challenge: 0.5 μg TNF + 20 mg GalN i.p. (LD100).
d TNF/ActD challenge: 0.5 μg TNF + 20 μg ActD i.p. (LD100).
e Anti-Fas challenge: 15 μg i.v. (LD100).
f TNF challenge: 20 μg i.v. (LD100).
g GalN challenge: 20 mg i.p.
h ActD challenge: 20 μg i.p.
induce apoptosis, although a clear ALT increase was observed. Neither GalN nor ActD alone had any effect on the parameters examined (Table I).

**In vivo correlation between induction of apoptosis and activation of caspases**

To verify the induction of apoptosis during lethal hepatitis, mice were treated with a lethal dose of TNF/GalN, TNF/ActD, or anti-Fas, livers were collected at several time points, and the extracted DNA was analyzed on agarose gels to visualize DNA ladders, indicative of apoptosis. We found that DNA ladders appear rather suddenly and coinciding with the immunochemical detection of DNA fragmentation (Figs. 1–3 and Table I). By Western blotting and immunodetection with specific antisera, we found that activation of procaspase-3 as well as procaspase-7 coincided with the appearance of DNA ladder patterns. TNF, GalN, or ActD alone had no effect (results not shown). The samples in which caspase-3 and caspase-7 fragments were detected cleaved fluorogenic Ac-DEVD-amc but not acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin, which confirms that the detected caspase-3 and caspase-7 fragments represent active caspases (Fig. 4).

**Inhibitory effects of protective acute-phase proteins**

We described earlier that α1-AGP protects mice against lethality induced by TNF/GalN or TNF/ActD, or anti-Fas, livers were collected at several time points, and the extracted DNA was analyzed on agarose gels to visualize DNA ladders, indicative of apoptosis. We found that DNA ladders appear rather suddenly and coinciding with the immunochemical detection of DNA fragmentation (Figs. 1–3 and Table I). By Western blotting and immunodetection with specific antisera, we found that activation of procaspase-3 as well as procaspase-7 coincided with the appearance of DNA ladder patterns. TNF, GalN, or ActD alone had no effect (results not shown). The samples in which caspase-3 and caspase-7 fragments were detected cleaved fluorogenic Ac-DEVD-amc but not acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin, which confirms that the detected caspase-3 and caspase-7 fragments represent active caspases (Fig. 4).

**Endogenous protection by Bcl-2 overexpression**

It has been described that transgenic overexpression of Bcl-2 in hepatocytes of mice prevents anti-Fas-induced apoptosis of hepatocytes (27, 28). We were interested to study the effect of Bcl-2 overexpression on induction of apoptosis and lethality by the three models. In Tables II and III, we demonstrate that Bcl-2 transgenic mice are protected very well against a lethal injection of anti-Fas: hypothermia, apoptosis, transaminase release, and lethality are completely prevented. Against TNF/GalN, transgenic Bcl-2 mice were also able to protect, but clearly over a less broad dose range, while no protection was observed in the TNF/ActD model.

**Discussion**

TNF and related members of the TNF family are very pleotropic cytokines. Many of them show an interesting antitumor activity, in
vivo as well as in vitro. However, application of e.g. TNF in therapy is currently limited due to the toxicity, which is basically the result of the proinflammatory nature of TNF (29). It has been demonstrated that TNF, but also Abs triggering CD95/Fas, cause extreme toxicity in the liver (2–6). TNF alone is not hepatotoxic (our unpublished observations), but requires the presence of a liver-specific or general transcription-blocking agent (GalN and ActD, respectively). GalN is a liver-specific transcription-blocking agent, which depletes hepatocytes from UTP, UDP, and UMP by interfering in the galactose pathways (15). Moreover, it was suggested that toxicity in the TNF/GalN, TNF/ActD, and anti-Fas model resemble viral forms of acute hepatic failure (15–18). We are interested to identify on the one hand the factors that mediate the toxicity induced in these models and on the other hand potentially inhibitory factors. We demonstrated earlier that two acute-phase proteins, α1-AGP and α1-AT, are able to protect in a differential fashion, in the sense that α1-AGP protects against the lethality induced by TNF/GalN and TNF/ActD, while α1-AT confers protection only in the former model (2, 5, 24). We had hoped to shed more light on the mechanism of induction of hepatitis and on the basis of the differential protection by 1) identifying the induction of active caspases in the hepatitis models, 2) studying the effect of the acute-phase reactants on the activation of caspases, and 3) looking at the effect of overproduction of Bcl-2 on the induction of acute hepatitis.

In the three hepatitis models, we first studied the kinetics of apoptotic events by determining the appearance of fragmented DNA in an ELISA system as well as on agarose gels and found that, as compared with the release of transaminases, apoptosis is an early event, followed by necrosis (which is also macroscopically

FIGURE 3. DNA laddering and activation of caspases occurs from 2 h after anti-Fas challenge. Mice were injected i.v. with 15 μg anti-Fas. Then, 0.5, 1, 2, or 3 h after challenge, livers were removed and homogenized. A, Agarose gel (1.8%) from DNA extracted from liver homogenates after ethidium bromide staining. B, Western blot of liver homogenates for caspase-3 and -7. Positive controls are caspase-3- and caspase-7-transfected HEK cells. Samples represent pooled homogenates of two mice. Shown is a representative example of at least three independent experiments.

FIGURE 4. Effect of α1-AGP and α1-AT on TNF and anti-Fas-induced apoptosis, ALT release, and caspase activation. Mice were pretreated i.p. with PBS, 5 mg α1-AGP, or 1 mg α1-AT 2 h before a challenge with 0.5 μg TNF/20 mg GalN (i.p.), 0.3 μg TNF/20 μg ActD (i.p.), or 10 μg anti-Fas (i.v.). Then, 3 h (anti-Fas) or 6 h (TNF/GalN and TNF/ActD) after the challenge, blood was withdrawn and livers were excised. Open bars represent DNA fragmentation, closed bars represent ALT levels, and hatched bars represent Ac-DEVD-amc cleavage activity. Samples represent pooled homogenates of two mice. Shown is a representative example of at least three independent experiments.
visible as the livers look entirely black). The anti-Fas-induced hepatitis develops more rapidly than the TNF-induced ones. We also found that active executioner caspases-3 and -7 appear in close time relation with the appearance of ladder patterns and fragmented DNA. Clearly the strongest signals were found after anti-Fas compared with TNF/ActD and TNF/GalN, which, together with the kinetics of events, illustrates the aggressive nature of the anti-Fas model. In this latter model, similar observations were reported recently (30). We could find no active forms of other important caspases, like caspase-1, -6, or -8, perhaps because their concentrations are below detection limits. From these studies, we believe that the results obtained with the Bcl-2 transgenic mice illustrate the interesting observation that liver-specific overexpression of Bcl-2 confers complete protection against anti-Fas-induced lethality, as already seen by one of us (27) in inflammatory bowel disease, etc.

Although the protection by acute-phase proteins against apoptosis per se is very interesting, we are still left with the question why this prevention is seen only in certain models and not in others. In the anti-Fas-triggered model, for instance, we were unable to see any effect of these protective proteins. We believe that the differential effect of the acute-phase proteins reflects a different mechanism of induction of apoptosis (although the executor last-stage caspase-3 and -7 are present in all three models). We believe that the results obtained with the Bcl-2 transgenic mice illustrate this hypothesis: we described the interesting observation that liver-specific overexpression of Bcl-2 confers complete protection against anti-Fas-induced lethality, as already seen by one of us (27) and another group (28). Others were not able to find protective effects of Bcl-2 against anti-Fas, but this was in a cell-free system

### Table II. Bcl-2 transgenic mice are partially protected from a lethal challenge with TNF/GalN and fully protected from a lethal challenge with anti-Fas

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Control mice</th>
<th>Transgenic mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 μg TNF/20 mg GalN</td>
<td>0/5</td>
<td>0/5</td>
</tr>
<tr>
<td>0.3 μg TNF/20 mg GalN</td>
<td>4/5</td>
<td>0/5*</td>
</tr>
<tr>
<td>0.5 μg TNF/20 μg ActD</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>0.1 μg TNF/20 μg ActD</td>
<td>4/5</td>
<td>5/5</td>
</tr>
<tr>
<td>0.3 μg TNF/20 μg ActD</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>0.5 μg TNF/20 μg ActD</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>10 μg anti-Fas</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>15 μg anti-Fas</td>
<td>5/5</td>
<td>0/5*</td>
</tr>
<tr>
<td>20 μg anti-Fas</td>
<td>ND</td>
<td>0/5</td>
</tr>
</tbody>
</table>

* TNF/GalN and TNF/ActD were administered i.p., anti-Fas i.v. All doses were adjusted to 20 g of body weight.
†, p = 0.0476 for transgenic vs wild-type mice.
*, p = 0.0079 for transgenic vs wild-type mice.

### Table III. DNA fragmentation, release of ALT, and drop in body temperature induced by TNF/GalN and anti-Fas are prevented in Bcl-2 transgenic mice

<table>
<thead>
<tr>
<th>Challenge</th>
<th>DNA Fragmentation (%)</th>
<th>Serum ALT (U/L)</th>
<th>Body Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>Bcl-2 controls 100 ± 32</td>
<td>60 ± 20</td>
<td>36.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Bcl-2 transgensics 100 ± 8</td>
<td>57 ± 21</td>
<td>36.9 ± 0.5</td>
</tr>
<tr>
<td>TNF/GalN</td>
<td>Bcl-2 controls 938 ± 225</td>
<td>4004 ± 1255</td>
<td>28.9 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>Bcl-2 transgensics 106 ± 14*</td>
<td>169 ± 24*</td>
<td>36.9 ± 0.5*</td>
</tr>
<tr>
<td>TNF/ActD</td>
<td>Bcl-2 controls 533 ± 46</td>
<td>6534 ± 1495</td>
<td>24.7 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Bcl-2 transgensics 495 ± 83</td>
<td>7953 ± 5661</td>
<td>24.3 ± 2.2</td>
</tr>
<tr>
<td>Anti-Fas</td>
<td>Bcl-2 controls 988 ± 111</td>
<td>3432 ± 1186</td>
<td>25.1 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Bcl-2 transgensics 154 ± 22*</td>
<td>182 ± 22*</td>
<td>36.8 ± 0.2*</td>
</tr>
</tbody>
</table>

* TNF/GalN and TNF/ActD were administered i.p., and anti-Fas was administered i.v. (n = 5 in all groups). All doses were adjusted to 20 g of body weight.
† Data are 4 h (anti-Fas), 5 h (TNF/ActD), or 6 h (TNF/GalN) after the challenge.
*, p < 0.05 for transgenic mice vs controls.
AGP and/or published observations). Up-regulation of Bcl-2 or Bcl-xL by recombinant caspases and in TNF cytotoxicity assays (our unpublished results) contain any caspase-inhibitory activity on the proteolytic activity of several mediators, such as PAF and Tx-A2 (7). The involvement of TNF/GalN model inflammation is present in all 18 different tissues sampled lack of protection by Bcl-2 against anti-Fas-induced apoptosis. There are indeed reports, using the former two types. Unfortunately, no further experimental data can be collected to underbuild this hypothesis. However, one should be cautious in interpreting data on Bcl-2-mediated inhibition of anti-Fas-induced apoptosis. There are indeed reports, using Bcl-2-transfected cell lines and hepatocytes in Bcl-2-transgenic mice, that describe protection by Bcl-2 against anti-Fas-induced apoptosis (27, 34–36); in contrast, data have been published showing lack of protection by Bcl-2 against anti-Fas-induced apoptosis (33, 37–39). Additional differences between the three models of hepatitis come from unpublished work (C. Libert) showing that in the TNF/GalN model inflammation is present in all 18 different tissues sampled, while in the TNF/ActD model inflammation was observed in the lung and in the anti-Fas model only apoptosis was observed (in the liver, but also slightly in the lung). Because acute-phase proteins such as α1-AGP are suspected to have antiinflammatory properties, we believe that in the TNF/GalN model apoptosis is prevented because inflammatory molecules are inhibited, some of which contribute to the induction of apoptosis.

The mechanisms by which α1-AGP and α1-AT prevent caspase activation are unclear. We have reported earlier that α1-AGP and α1-AT do not block TNF-induced gene expression in hepatocytes (5). Direct inhibition of caspases upstream of procaspase-3 and procaspase-7 is also excluded because α1-AGP and α1-AT do not contain any caspase-inhibitory activity on the proteolytic activity of recombinant caspases and in TNF cytotoxicity assays (our unpublished observations). Up-regulation of Bcl-2 or Bcl-s1a by α1-AGP and/or α1-AT, is very unlikely, because α1-AGP nor α1-AT protect in the anti-Fas model, while Bcl-2 does (our unpublished observations). In the TNF/GalN model, we have demonstrated the involvement of several mediators, such as PAF and Tx-A2 (7). The induced apoptosis of hepatocytes could be the result of a combined action of TNF, GalN, and TNF-mediated mediators. The mechanism of protection conferred by α1-AGP and α1-AT might be the prevention of activity or release of these mediators. Anti-Fas-induced apoptosis results from direct binding on the receptor, without involvement of sensitizing mediators and therefore no targets for α1-AGP or α1-AT. The TNF/ActD model appears to behave intermediately and to involve a mediator that can be inhibited by α1-AGP. The inhibition of apoptosis by α1-AGP and α1-AT appears to be an early inhibition of apoptotic mediators, and in that respect is distinct from late interventions, such as the inhibition of apoptotic events by Hsp70, which occurs even in the presence of active caspase-3 (40). However, the fact that acute-phase proteins are able to prevent apoptosis in vivo, whatever the mechanism, is interesting in view of their potential use as therapeutic agents in disorders involving unwanted apoptosis. The acute-phase proteins might also constitute an inducible anti-apoptotic, protective system that in pathology or disturbed homeostasis prevents excessive apoptosis.

Acknowledgments
We thank L. Van Geert and E. Spruyt for animal care, and F. Duerinck for purifying TNF. We also thank Dr. M. Monestier (Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France) for providing the hybridoma producing mAbs necessary for the immunochemical determination of histone-complexed DNA fragments.

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