**Protection by α1-acid Glycoprotein against Tumor Necrosis Factor–induced Lethality**

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**Summary**

We here report that α1-acid glycoprotein, a typical acute phase protein, protects mice from lethal shock induced by tumor necrosis factor (TNF) or endotoxin. The protection is observed both in normal and in galactosamine-sensitized mice. Optimal desensitization requires at least 3 mg of α1-acid glycoprotein administered 2 h before the lethal challenge. Under these conditions, complete inhibition of all TNF-induced metabolic changes was observed: fall in body temperature, release of liver transaminases, enhanced clotting time, and mortality. The known platelet aggregation-inhibitory activity of α1-acid glycoprotein provides a possible explanation for this protective capacity.

TNF is considered to be the major mediator of both the shock-inducing and antitumor activities of endotoxins (1–3). But TNF, possibly in combination with IFN, also holds much promise for antitumor therapy, provided the induced systemic toxicity can be suppressed. Because of this, and in view of the key role played by TNF in septic shock, reduction of the generalized, deleterious, inflammatory response is an important objective. We previously reported that a low dose of IL-1 or even TNF can protect mice against a subsequent lethal challenge of TNF plus galactosamine (GalN), LPS plus GalN, or TNF alone, and that a factor produced by hepatocytes is responsible for this IL-1- or TNF-induced protection (4).

In an attempt to identify this factor, we studied the effect of α1-acid glycoprotein (AGP), a typical acute phase protein in humans, rats, mice, and other species (5), the physiological role of which is still poorly understood. AGP is a highly glycosylated, polyanionic protein with an apparent Mr of ~40,000. It is induced mainly in hepatocytes by several cytokines, including TNF and IL-1, and by glucocorticoids (6).

In this report, we demonstrate that AGP is able to very significantly protect animals from lethal shock induced by TNF or LPS.

**Materials and Methods**

*Mice.* Female C57Bl/HeJ mice (Charles River, Sulzfeld, Germany) and female C57Bl/cnb (Studiecentrum voor Kernenergie, Mol, Belgium) were used at the age of 7–9 wk. The animals were kept in 12-h light/dark cycles in a temperature-controlled, air-conditioned room, and received food and water ad libitum. Rectal body temperatures were measured with an electronic thermometer (model 2001; Comark Electronics, Littlehampton, UK).

*Injections and Blood Collections.* Intraperitoneal injections had a volume of 0.5 or 1 ml. The reagents were diluted in pyrogen-free PBS immediately before the injection. Some injections were intravenous in a volume of 0.25 ml. Blood was collected by retroorbital bleeding under ether anesthesia and serum was prepared by incubating blood samples for 30 min at 37°C, separation of the fibrin clot, and centrifugation (15 min; 16,000 g). For preparation of plasma, 450 μl blood was collected by heart puncture with a syringe containing 50 μl sodium citrate (0.1 M). Platelet-free plasma was obtained by two consecutive centrifugations of 15 min at 16,000 g.

*Reagents.* GalN, human AGP, rat AGP, bovine (b) AGP, human transferrin (TF), and BSA were obtained from Sigma Chemical Co. (St. Louis, MO). The bAGP preparations contained <1 ng endotoxin/mg protein and contained no biologically active IL-1, TNF, IL-6, or in vitro TNF-inhibitory activity (as judged on L929 cells). bAGP was >99% pure as mentioned by the manufacturer. On a reducing, denaturing polyacrylamide gel, AGP appeared, as expected, as two bands because of differential glycosylation (5); no contaminating proteins were visible on such a gel. Escherichia coli 0111:B4 LPS and rabbit tissue thromboplastin were from Difco Laboratories (Detroit, MI). Mouse AGP was prepared from turpentine-treated mice as described (7). Recombinant human (rh) TNF and recombinant murine (rm) TNF were made in this laboratory. rhTNF had a sp act of 5 × 10^5 IU/mg and contained <0.11 ng endotoxin/mg protein; rmTNF had a sp act of 1.5 × 10^9 IU/mg and contained <0.96 ng endotoxin/mg protein. Endotoxin levels were assessed by a chromogenic Limulus amoebocyte lysate assay (CoaTest; Kabivitrum, Stockholm, Sweden).

*Quantification of Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST), and Measurement of Modified Prothrombin Time (MPT).* Serum ALT and AST were determined using a Hitachi analyzer and the ALT/AST kit of Sigma Chemical Co. MPT was measured as described (8); in baked, siliconized glass cuvettes, prewarmed at 37°C, 50 μl plasma was added to 175 μl Tris-HCl (10 mM, pH 8, 100 mM NaCl) together with 25 μl of a 1/1,000 dilution of rabbit tissue thromboplastin. After incubation for 3 min, coagulation was started at time zero by the addition of 250 μl Ca^2+ buffer (10 mM Tris-HCl, pH 8, 80 mM NaCl, 20 mM CaCl_2), and fibrin formation was monitored optically.

*Statistics.* Significant differences in body temperature, AST,
ALT, and MTP were calculated using a two-tailed t test. Significant differences in final survival were calculated using a chi-square test, while significant differences in survival time (Kaplan Meier plots) were calculated using the Mantel modified Gehan's Generalized Wilcoxon Test.

Results

We investigated whether AGP had protective effects against different lethal challenges with TNF or LPS. As shown in Table 1, bAGP could fully protect C3H/HeJ mice against a lethal combination of mTNF and GalN. In these mice, the LD_{50} for mTNF in combination with GalN is 0.1 μg.

Table 1. Protection by bAGP against the Lethal Effect of mTNF/GalN Injection

| Pretreatment* | bAGP (3 mg) | bAGP (10 mg) | TF (10 mg) | BSA (10 mg) |
|---------------|-------------|--------------|------------|-------------|
| Challenge     |             |              |            |             |
| 0.01 μg mTNF/20 mg GalN | 0/6 | 0/6 | 0/6 | ND | ND |
| 0.05 μg mTNF/20 mg GalN | 2/6 | 0/6 | 0/6 | ND | ND |
| 0.1 μg mTNF/20 mg GalN | 6/6 | 0/6 | 6/6 | ND | ND |
| 0.5 μg mTNF/20 mg GalN | 6/6 | 0/6 | 6/6 | 6/6 | 6/6 |
| 1.0 μg mTNF/20 mg GalN | 6/6 | 3/6 | 0/6 | 6/6 | ND |

* C3H/HeJ mice were pretreated 2 h before the lethal challenge. Lethality (number of dead mice vs. total number of mice) was monitored 24 h after the challenge (no further deaths occurred).

bAGP was capable of protecting against at least 5-10 such lethal doses. Not only bAGP protected against mTNF/GalN lethality; human, rat, or mouse AGP also protected mice against mTNF/GalN lethality (results not shown). Furthermore, TF and BSA showed no protective activity. We used these two preparations (glycosylated and unglycosylated, respectively) as protein controls.

As demonstrated in Fig. 1, statistically significant (p <0.001) protection was observed when bAGP was administered 2 or 4 h, or even (p <0.05) 8 h before the lethal challenge. The animals of the latter group, however, finally all died. As can be seen in Fig. 1A, after injection of mTNF/GalN, the body temperature virtually drops to room temperature within 6 h. This is not the case in the animals pretreated with bAGP 2 or 4 h before the challenge.

When studying the minimal dose necessary to confer protection against mTNF/GalN, we observed a very sharp dose/response relationship (Fig. 2): 3 mg bAGP still fully protected, whereas 1 mg was no longer effective.

5-6 h after injection of mTNF/GalN, very high levels of ALT and AST were found in circulation as a result of massive necrosis of the liver (Table 2). Also the enhanced clotting time (MPT) is a reflection of this necrosis. Mice pretreated with bAGP no longer exhibited these important metabolic changes.

In Table 3 we demonstrate that bAGP not only protects against TNF in GalN-sensitized mice, but also in normal mice, for which the LD_{50} (intravenous) of mTNF amounts to about 20 μg. hTNF, which only interacts with the murine receptor p55, is far less toxic than mTNF for mice (9); in the presence of GalN, however, it becomes almost as toxic. But bAGP protected against hTNF/GalN lethality (Table 3), and against the local Shwartzman reaction (results not shown). Furthermore, bAGP desensitizes towards a potentially lethal LPS injection in GalN-sensitized mice.

Discussion

GalN drastically sensitizes animals to the shock-inducing properties of LPS or TNF (10-12). Because GalN is a specific
Figure 2. Dose dependency of bAGP-induced protection against mTNF/GalN lethality. C3H/HeJ mice (n = 10) were treated at -2 h with 10 mg bAGP (■), 3 mg bAGP (▲), 1 mg bAGP (●), 0.3 mg bAGP (●) or PBS (▼). At t = 0 h, mice were challenged with 0.5 μg mTNF/20 mg GalN. (A) body temperature (°C) after challenge. (B) Kaplan Meier plot of survival after challenge.

Table 2. Effect of bAGP on the Change in Metabolic Parameters after Injection of a Lethal Dose of mTNF/GalN

| Pretreatment* | PBS       | bAGP (3 mg) |
|---------------|-----------|-------------|
| AST1          | 10,240 ± 640 | 600 ± 400   |
| ALT1          | 10,480 ± 400 | 360 ± 240   |
| MPT5          | 1,500 ± 139  | 164 ± 16    |
| Body temperature1 | 30.4 ± 2.2  | 38.0 ± 0.5  |
| Lethality1    | 8/8        | 0/8         |

* C3H/HeJ mice were pretreated 2 h before the lethal challenge (0.5 μg mTNF/GalN).
1 Mean and SD (n = 8) in U/liter, 5 h after challenge; bAGP-pretreated mice were significantly different (p < 0.05) from PBS-pretreated mice (t test). Basal levels were 93 ± 31 U/liter (AST) and 97 ± 22 U/liter (ALT).
5 Mean and SD (n = 6) relative to untreated mice taken as 100, 5 h after challenge (100% value for control mice was 61 ± 2 s); bAGP-pretreated mice were significantly different (p < 0.05) from PBS-pretreated mice (t test).
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1 Number of dead mice vs. total number of mice, 24 h after challenge (no further deaths occurred); bAGP-pretreated mice were significantly different (p < 0.05) from PBS-pretreated mice (chi-square test).

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hepatotoxin (it inhibits transcription and translation specifically in hepatocytes [13]) the liver is believed to play a central role in the defence against injury or systemic shock.

In response to several cytokines and other factors released after injury, hepatocytes augment the expression of a set of proteins, called the acute phase proteins (reviewed in 14). Some of these proteins, such as C-reactive protein, serum amyloid A and α1-antitrypsin, have been reported to protect animals from lethal endotoxemia or other inflammatory challenges (15-18). Protection against the lethal effects of TNF by acute phase proteins has, however, never been described.

In this report we demonstrate that AGP significantly protects mice from TNF- or LPS-induced lethality in GalN-sensitized as well as in normal mice. bAGP protects against the enhanced plasma clotting time, the release of liver transaminases, the reduction of body temperature and mortality, when given at least 2 h before the challenge. The bAGP dose required in a bolus injection for protection is 3 mg, resulting in a serum concentration of about 1.5 mg/ml, and resembles the peak serum concentration of AGP during an acute phase response, namely ~1.2 mg/ml (19, 20).

Since the mechanism by which TNF leads to lethality in GalN-sensitized animals is still largely unknown, the exact target of AGP remains unclear. However, Piguet et al. (21) recently demonstrated that platelet aggregation and adhesion to the endothelium plays a critical role in LPS/GalN toxicity. After aggregation, platelets may produce thromboxanes, possible mediators of the toxic TNF activities in GalN-sensitized animals (22). It is quite possible, therefore, that the potent platelet aggregation-inhibitory activity of AGP (23) provides the explanation for its protective capacity.

AGP is also capable of augmenting the LPS-induced cytokine production of macrophages (24), and some cytokines (IL-1 and TNF) can desensitize against TNF/GalN-induced lethality (4, 25). However, optimal IL-1-induced protection requires that IL-1 is injected 12 h before TNF/GalN. Furthermore, we demonstrated that the liver mediates the IL-1 effect. Hence, AGP may well be the IL-1-induced molecule in the liver responsible for desensitization (4). It may also be noted that we were unable to observe IL-1 or TNF in circulation in C3H/HeJ mice after AGP injection (data not shown). Whether AGP is the only factor responsible for IL-1/TNF-induced desensitization will only become clear,
Table 3. bAGP Protects against TNF- or LPS-induced Lethality in Normal and GaIN-sensitized Mice

| Challenge          | PBS                  | bAGP (3 mg)          |
|--------------------|----------------------|----------------------|
|                    | Body temperature\(^1\) | Lethality\(^3\)     | Body temperature\(^1\) | Lethality\(^3\)     |
| 0.5 μg mTNF/20 mg GaIN | 30.4 ± 2.2           | 8/8                  | 38.0 ± 0.5\(^1\) | 0/8\(^1\)          |
| 0.5 μg hTNF/20 mg GaIN | 28.9 ± 2.5           | 8/8                  | 36.9 ± 0.8\(^1\) | 1/8\(^1\)          |
| 20 μg mTNF         | 27.9 ± 3.0           | 5/5                  | 36.4 ± 0.8\(^1\) | 0/5\(^1\)          |
| 0.1 μg LPS/20 mg GaIN | 26.6 ± 2.7           | 5/5                  | 37.2 ± 0.5\(^1\) | 0/5\(^1\)          |

\(^{*}\) Mice, namely C3H/HeJ (TNF) or C57Bl/CNB (LPS), were pretreated 2 h before the lethal challenge.

\(^{1}\) Mean and SD (°C) measured 6 h (GaIN model) or 18 h (normal model) after the challenge; \(^{1}\) means significantly different (p <0.05) from PBS-pretreated mice (t test).

\(^{3}\) Number of dead mice vs. total number of mice, 24 h (GaIN model) or 48 h (normal model) after challenge (no further deaths occurred); \(^{1}\) means significantly different from PBS-pretreated mice (chi-square test).

when mice with a targeted disruption of the AGP genes will be available.

It has been reported that AGP induces an IL-1-inhibiting factor in macrophages (26), most probably the IL-1 receptor antagonist (27). However, based on earlier studies with rIL-1 receptor antagonist, we can conclude that this molecule is not sufficiently potent to explain the protection observed against TNF/GaIN (28).

Our findings provide evidence that AGP is part of a feedback system induced in the liver and capable of significantly inhibiting the deleterious effects of TNF.

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