Clin Exp Immunol 1997; **110**:479–484

Protection from concanavalin A (Con A)-induced T cell-dependent hepatic lesions and modulation of cytokine release in mice by sodium fusidate

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(Accepted for publication 5 August 1997)

SUMMARY

The immunomodulatory effects of the antibiotic sodium fusidate (SF) were tested in a model of T cell-dependent hepatic injury that can be induced in normal mice by a single i.v. injection of Con A. Signs of hepatitis with elevated transaminase activities in plasma, severe infiltration of the liver by neutrophil granulocytes, lymphocytes and monocytes, and necrotic areas were observed in control mice treated intraperitoneally with PBS 24 h and 1 h before Con A challenge. T cell- and macrophage-derived cytokines (IL-2, interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), IL-1β, IL-6) were released with different kinetics in the circulation of these mice. SF, 20, 40 or 80 mg/kg, administered 24 h and 1 h before Con A challenge, protected the mice against the hepatitic effects of Con A. The protective effects of SF were dose-dependent and accompanied by profound modifications of blood levels of cytokines induced by Con A, so that, relative to control mice, SF (80 mg/kg)-treated animals showed markedly diminished plasma levels of IL-2, IFN-γ and TNF-α, along with augmented levels of IL-6. These results suggest that SF might be useful in the treatment of immunoinflammatory liver diseases in humans.

Keywords autoimmune diseases hepatitis fusidic acid immunotherapy T cells

INTRODUCTION

Animal models of human diseases provide suitable tools for the better understanding of pathogenic mechanisms and may lead to insights into novel therapeutic approaches for the clinical setting. Recently, a new model of hepatitis has been described which can be induced in mice by a single i.v. injection of Con A [1–3]. Unlike other commonly used models of immunoinflammatory liver injuries such as lipopolysaccharide (LPS)-induced hepatitis and the autoimmune hepatitis provoked by immunization with syngeneic liver antigens (see [4] for review), which both require the use of hepatotoxic agents (D-galactosamine and Freund's complete adjuvant, respectively) for disease induction [5,6], the sole injection of Con A is sufficient for liver lesions to develop. Within 8–24 h, clinical and histological evidence of hepatitis occurs with elevation of transaminase activities in the plasma and hepatic lesions characterized by massive granulocyte accumulation and hepatic necrosis [1–3]. Con A-induced hepatitis is both T cell- and macrophage-dependent; it can not be induced in nude athymic mice lacking immunocompetent T cells, and it is prevented by anti-T cell immunosuppressants such as cyclosporin A (CsA) and FK506, or by blockade of macrophage functions with silica particles [1–3].

The precise mechanism(s) by which T cells and macrophages exert their hepatogenic potential is not known. Because a massive release of macrophage and T cell-derived cytokines (IL-1, IL-2, IL-6, tumour necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and granulocyte-macrophage colony-stimulating factor (GM-CSF)) occurs with different kinetics in response to Con A, a role has been envisaged for these cytokines in the development of the hepatic lesions. Nonetheless, the role of cytokines in the pathogenesis of this immunoinflammatory condition remains to be defined. For example, the disease is equally prevented by anti-TNF-α antibody [2,3] and IL-6 [3], and the outcome of the disease may therefore depend on a fine balance between pro- and anti-inflammatory cytokines released by Con A-activated cells.

Because of the powerful interference of the sodium salt of fusidic acid, sodium fusidate (SF), with the cytokine network *in vivo* and *in vitro* (see [7] for a review), notably the suppression of the release of TNF-α and enhancement of IL-6, we tested the effects of this drug on the Con A-induced hepatic lesions.

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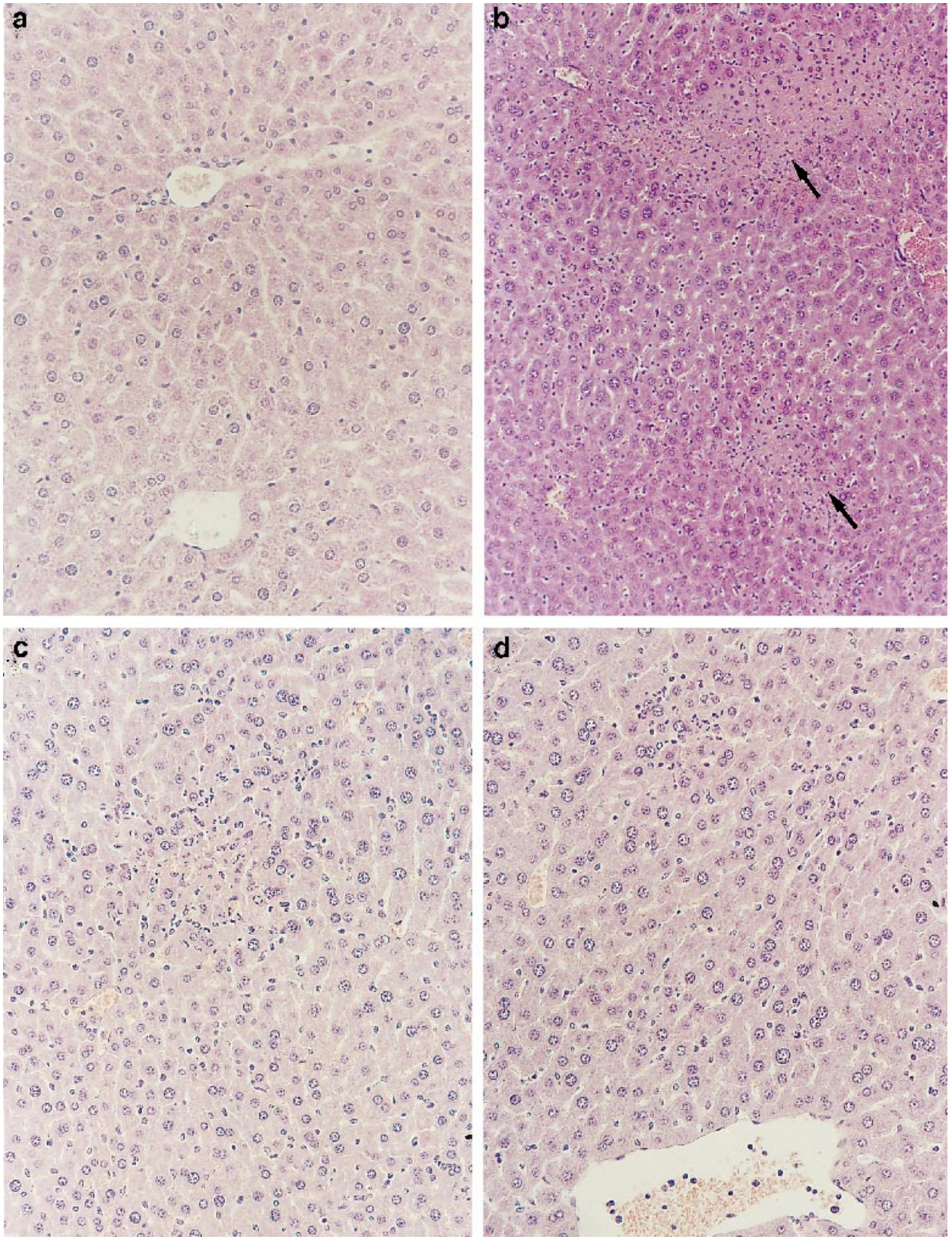
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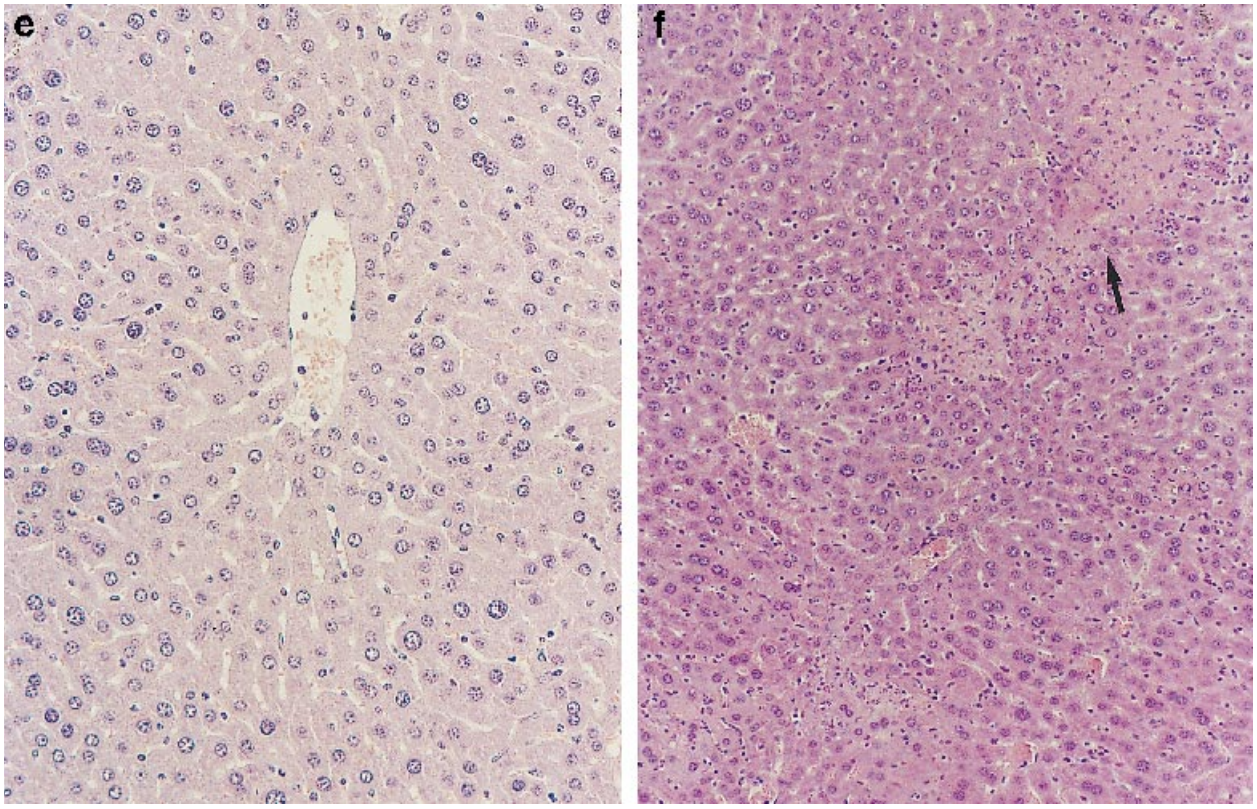

Fig. 1. The effects of sodium fusidate (SF) on the development of Con A-induced hepatitis in NMRI mice. (a) Liver from a PBS-treated mouse not injected with Con A. No histological abnormalities are detectable. (b) Liver from control (PBS-treated) mice injected with Con A. Infiltration from neutrophil granulocytes and cellular necrosis (arrows) are seen. These histological features, rarely observable in the mice treated with 20 mg/kg SF (c), become less evident in mice treated with 40 (d) and 80 (e) mg/kg SF. SF treatment (80 mg/kg) 1 h after Con A challenge was no longer effective (f).

MATERIALS AND METHODS

Mice and hepatitis induction

Male Naval Medical Research Institute (NMRI) albino mice, 6–8 weeks old, were purchased from Charles River (Calco, Italy). The mice were kept under standard laboratory conditions (non-specific pathogen-free) at 24°C with free access to food and water. The food was withdrawn 16 h before the experiments. The mice were divided into five experimental groups and challenged each with 20 mg/kg Con A (Sigma Chemical Co., St Louis, MO). Con A was dissolved in sterile PBS and injected to mice via the tail vein. The groups were treated intraperitoneally either with PBS or SF (Sigma) according to the experimental design shown in Table 1. An additional control group consisted of mice challenged only with PBS (Table 1). Because marked increases of transaminase activity along with severe histological signs of hepatic injuries have been reported to develop 8 h after Con A injection in these mice [1,2], the animals were killed after 8 h, and blood and livers were collected.

Table 1. Experimental design and effects of sodium fusidate (SF) on Con A-induced hepatitis in mice

Groups	No.	Hepatitis induction (Con A)	SF (mg/kg)			ALT (U/ml)	*P*
			−24 h	−1 h	+1 h		
A	7	−	−	−	−	42 ± 9	< 0·0001
B	12	+	−	−	−	6524 ± 2123	Control
C	11	+	20	20	−	107 ± 59	< 0·0001
D	12	+	40	40	−	57 ± 43	< 0·0001
E	14	+	80	80	−	47 ± 14	< 0·0001
F	12	+	−	−	80	4512 ± 4016	NS

Eight hours after Con A application the mice were killed and blood samples collected from individual mice for alanine aminotransferase (ALT) measurement.

For statistical analysis each group is compared with group B.

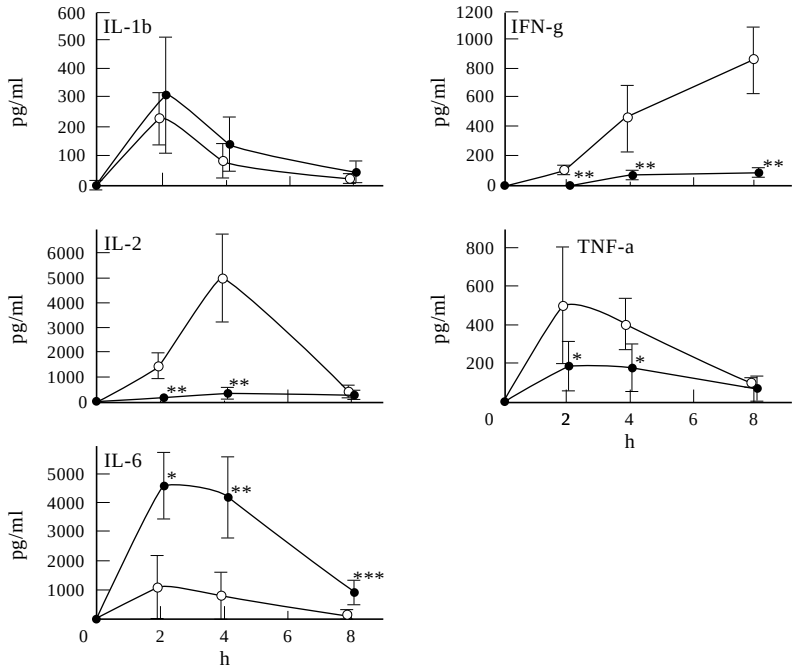

Fig. 2. Modulation by sodium fusidate (SF) of Con A-induced cytokine release. Seven mice from each group were killed before (T0) and 2, 4 and 8 h after injection of Con A, and plasma samples from individual mice were used for cytokine measurements. Except for the mice killed at T0, receiving no treatment, all the other animals were injected (intraperitonealluy) with either PBS or SF (80 mg./kg) 24 h and 1 h before Con A challenge. Data are shown as mean ± s.d. *$P < 0{\cdot}006$; **P < 0·0001; ***$P < 0{\cdot}001$; and IFN-γ; **$P < 0{\cdot}003$ by ANOVA.

Assay for plasma transaminase activities

Plasma alanine aminotransferase (ALT) activity was determined by a standard photometric assay using a bichromatic analyser.

Cytokine measurements

Plasma samples from individual mice were collected for measurement of IL-1β, IL-2, IL-6, IFN-γ and TNF-α. Solid-phase ELISA kits for detection of mouse IL-1β and IL-2 were from Genzyme (Cambridge, MA), for IFN-γ from Biosource (Camarillo, CA) and for IL-6 and TNF-α from Endogen Inc. (Boston, MA). Samples were run in duplicates according to the manufacturer's instructions. The lower limit of sensitivities of the assays were 2 pg/ml for IFN-γ, 10 pg/ml for IL-1β and 15 pg/ml for IL-2, IL-6 and TNF-α. To calculate mean values, samples with cytokine values below the level of detection were assigned the limit of sensitivity of the assay as theoretical values.

Histological examinations

Eight hours after Con A injection, livers were removed, fixed in 10% formalin, embedded in paraffin, sliced in 5-μm sections and stained with haematoxylin and eosin (H–E) for histological examinations at 125-fold magnification. This was performed by two observers unaware of the treatment of the mice.

Calculation of data

Results are expressed as mean values ± s.d. Statistical analysis was performed by ANOVA.

RESULTS

SF-induced protection against serological and histological signs of Con A-induced hepatic injury

Two of 14 (14%) Con A/PBS-treated control mice, and one of 12 (8%) of those challenged with Con A and treated with 20 mg/kg SF died before sacrifice. These mice were not considered for serological and histological analyses. As expected, and in agreement with previous studies [1,2], acute signs of liver damage were found at sacrifice in all mice of the control group injected with Con A and treated only with PBS. These consisted of marked elevations of ALT in the plasma and severe lobular infiltration with neutrophil granulocytes, lymphocytes and monocytes, and the inflammatory process was also detected both in the portal areas and around the central veins. Moreover, diffuse hepatocytic necrosis which contained neutrophil granulocytes was also observed (Fig. 1b). Pretreatment with SF offered a significant protection against the biochemical and histological signs of hepatic injury. Whilst even the lowest dose of SF offered a protective effect, a trend towards higher degrees of protection could be noticed with the 40 mg/kg and, in particular, 80 mg/kg (Table 1 and Fig. 1a–e). In contrast, when treatment with 80 mg/kg SF was delayed until 1 h after Con A injection, the drug was no longer effective, and these mice showed clinical and histological parameters not significantly different from those of the control group (Table 1 and Fig. 1f).

SF-induced modulation of blood cytokines in Con A-challenged mice

Because Con A-induced hepatitis develops with simultaneous elevation in the circulation of several macrophage- and T cell-derived cytokines [1–3], and since this may control or reflect the progression of the immunoinflammatory syndrome, we next examined if SF interfered with the systemic levels of these molecules. Two groups of mice, treated with either PBS or SF (80 mg/kg) 24 h and 1 h before Con A application, were killed before Con A injection (T0) and 2, 4 and 8 h thereafter. As shown in Fig. 2a–e, IL-1β, IL-2, IL-6, IFN-γ and TNF-α, which were undetectable in the circulation of all the mice at T0, were present in large amounts in the blood of the PBS/Con A-treated mice. In agreement with

previous studies, the cytokines appeared with different kinetics in response to Con A, rapidly (+ 2 h to + 4 h) in case of IL-1β, IL-2, IL-6 and TNF-α and slower in the case of IFN-γ. This pattern was modified by SF, in that the SF-treated mice showed significantly reduced circulating levels of TNF-α, IL-2 and IFN-γ, and augmented levels of IL-6 (Fig. 2a–d). There were no significant differences in plasma levels of IL-1β between the experimental and control groups of mice, although SF-treated mice showed a trend toward higher levels of the cytokine (Fig. 2e).

DISCUSSION

Our present observation that immunoinflammatory hepatitis can be induced in mice by a single i.v injection of Con A further confirms the original data by Tiegs' group [1,2], which were independently reproduced by others [3,8]. Most histological and immunopathogenic pathways described in those studies were also observed here. The infiltrative hepatic lesions were primarily composed of neutrophil granulocytes, lymphocytes and monocytes, and diffuse hepatocytic necrosis was seen, and hepatitis development was accompanied by a marked increase in blood levels of IL-1β, IL-2, IL-6, IFN-γ and TNF-α. However, unlike Gantner *et al*., who reported 'intracellular DNA-containing apoptotic bodies, karyorrhexis and diffuse cloudy swelling in the hepatocellular cytoplasma' [2], these phenomena were only rarely visible. The reason for this discrepancy is not known, although environmental factors might be involved.

Besides confirming the previous studies [1–3], we show here for the first time that prophylactic treatment with SF protects mice against the hepatitis-inducing effects of Con A. The antibiotic fusidic acid and its sodium salt SF were previously found to possess immunomodulatory properties *in vitro* and *in vivo* ([9–12,16,17], reviewed in [7]). The drug down-regulates IL-2, IFN-γ, and, though to a lower extent, IL-1 secretion *in vitro* and also inhibits the lymphocyte costimulatory activity of IL-1α/β and IL-6 [9]. The interference of SF with the cytokine network *in vivo* may, however, be more pronounced and more complex than anticipated from *in vitro* studies, and administering SF to endotoxin-challenged rodents markedly suppresses the blood levels of TNF-α and augments those of IL-6 [10–12]. IL-6 has recently been shown to exert anti-inflammatory activities. These might in part be mediated by a double antagonistic effect of IL-6 on IL-1 and TNF, e.g. by inhibition of IL-1 and TNF production on the one hand along with up-regulated secretion of two of their naturally occurring inhibitors, the IL-1 receptor antagonist and sTNF receptor p55. Thus, the ability of SF to increase IL-6 levels while at the same time suppressing those of proinflammatory cytokines such as IL-2, IFN-γ and TNF-α may explain its beneficial effects in the prevention/treatment of immunoinflammatory conditions such as type 1 diabetes [10,16,17], chronic endogenous uveitis [18], Bechet's colitis [19], Crohn's disease [20] and septic shock [10] in humans and/or rodent models.

The *in vivo* relevance of this mechanism of action for the immunosuppressive properties of SF is in accordance with the impact of the drug in Con A-induced T cell-dependent hepatic injury studied here. Hence, the histological and serological protection afforded by SF was accompanied by modifications of cytokine plasma levels, including reduced levels of IL-2, IFN-γ and TNF-α, and augmented levels of IL-6. In this model TNF-α and IL-6 play a pathogenic and protective role [1–3], and the inhibition of TNF-α, with the simultaneous increase of IL-6 levels, may thus be central to the beneficial effects of SF. The ability of SF to reduce the blood levels of IL-2 and IFN-γ may also be related to the protection afforded in this model by CsA and FK506, two immunosuppressants which selectively block IL-2 and IFN-γ production [21]. Moreover development of chronic active hepatitis has been reported to occur in transgenic mice expressing IFN-γ in the liver [22].

Although IL-1α/β ([3] and present study) are rapidly released in the circulation following Con A challenge, the failure of *in vivo* treatment with neutralizing anti-IL-1α antibody to prevent clinical and histological markers of the hepatic lesions [3] argues against the pathogenic involvement of at least this IL-1 species in this hepatitis model. Whilst it is thus not entirely unexpected that SF afforded its preventive effects without decreasing IL-1β plasma levels, the trend toward higher values of this cytokine in Con A-challenged mice treated with SF compared with control mice contrasts with the capacity of this drug to suppress IL-1α/β secretion from phytohaemagglutinin/LPS-activated human peripheral blood mononuclear cells *in vitro* [9]. The reason for this discrepancy is not known.

Chronic hepatic diseases such as chronic hepatitis and liver cirrhosis are major causes of morbidity and mortality. It is known that hepatitis B virus (HBV) and hepatitis C infection may favour the development of both chronic hepatitis and hepatocellular carcinoma (HCC) [23,24], and there is evidence that HCC occurs in cirrhotic liver, and that chronic hepatitis is a prerequisite for HCC development. There is also evidence that cell-mediated immune phenomena directed against viral determinants on hepatocytes [25], and probably driven by cytotoxic T lymphocytes [26], may be implicated in HBV-induced hepatocellular injury.

That SF successfully prevents immunoinflammatory hepatic lesions induced by Con A in mice should therefore warrant further studies for its use in the treatment of immunoinflammatory liver diseases. In this regard, the failure of SF to reverse the inflammation once induced by Con A does not necessarily minimize the potential use of SF in the treatment of human immunoinflammatory diseases, including inflammatory hepatitis. Pathogenic differences exist between human diseases and experimental animal diseases, and this should be taken into account when transferring these studies to the clinical setting. For example, CsA also fails to reverse Con A-induced hepatitis once initiated [8], and CsA has nevertheless beneficial effects in humans with immunoinflammatory diseases, including primary biliary cirrhosis [21]. However, because treatment with SF of humans has been shown to reversibly increase transaminase activities [7,20], this could indicate a potential hepatotoxicity of the drug which might limit its use in patients with hepatic impairment. Nonetheless, increase in transaminase activity usually occurs at a dose of 1·5 g SF/day, which roughly corresponds to 20 mg/kg in the mouse. This dosage still exerted clear anti-inflammatory effects in the present study. More importantly, rodents metabolize SF more rapidly than humans [27]. It is thus possible that SF has anti-inflammatory effects in humans at doses substantially lower than 1·5 g/day, which would reduce the risk of liver toxicity. Other strategies might be considered aiming at synergistic effects of SF with other immunosuppressants which might allow the combined use of drugs at lower and less toxic doses than when each is given alone.

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