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Rheumatoid factor induction in murine models of liver injury

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Summary

Alcoholic liver disease and hepatitis C are associated with the production of autoantibodies such as rheumatoid factors (RF), which bind to IgG and can aid in host defence, but are also associated with pathological conditions such as rheumatoid arthritis. Because little is known about the role of RF in liver disease, we characterized the RF production that either occurred spontaneously in response to alcohol consumption or was induced by injection of an *Escherichia coli* glycolipoprotein in C57Bl/6 mice. Whereas severe liver damage was induced by carbon tetrachloride (CCl_4), minimal damage was caused by chronic alcohol consumption. Liver damage was monitored by measurements of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Circulating RF was induced in response to chronic alcohol consumption; the latter probably involved Toll-like receptor ligation. In contrast, CCl_4-induced damage was not associated with RF induction. However, concurrent treatment with an *E. coli* glycolipoprotein macromolecule that induced RF, protected against CCL_4-induced liver damage as measured by a highly significant decrease ($P = 0{\cdot}008$) at 4 weeks in AST and ALT. RF induced by *E. coli* glycolipoprotein correlated with 'protection' from liver damage, indicating that the RF autoimmune response does not necessarily exacerbate liver disease.

Keywords: alcoholic liver disease, autoimmunity, carbon tetrachloride, rheumatoid factor, Toll-like receptors

Introduction

Despite large amounts of proinflammatory substances (food, bacterial products) in portal venous blood, which supplies 75% of the liver's blood [1], the liver does not normally harbour a constitutive inflammatory response. The liver, although capable of mounting an immune response to pathogens, favours tolerance over immunity [2], and is considered by some to be immune privileged [3]. In pathological conditions, such as hepatitis C virus (HCV) infections or alcoholic liver disease (ALD), tolerance can be broken and one autoantibody detected frequently is rheumatoid factor (RF) [4,5].

RF are autoantibodies that bind IgG in the γ2–γ3 cleft [6]. They can be any isotype, although IgM RF are the isotypes generally measured in clinical laboratories [7]. RF are associated historically with rheumatoid arthritis (RA) where RF are a classification criterion [8]. An essential component of the mixed cryoglobulin (MC) that is detected in 30–40% of patients with HCV is RF. MC appear late in HCV infection, associated with more extensive liver damage [9]. Elevated levels of IgA RF are detected in approximately 90% of patients with alcoholic cirrhosis [5].

The induction of RF requires two signals (see review [10]), one through the B cell receptor (BCR), which is cross-linked by IgG (preferentially part of a complex) [11] and the other through a Toll-like receptor (TLR) [12]. Several TLR ligands induce RF [12–14].

Here, we examine RF in two types of liver damage: chronic alcohol consumption, which perturbs gut flora and impacts in part on liver metabolism by portal vein delivery of bacterial products, and liver disease induced by carbon tetrachloride (CCl_4) [15,16]. We show that chronic alcohol ingestion induced RF, whereas only minimal liver damage occurred. In contrast, CCl_4 caused substantial liver damage but did not induce RF; when RF were induced by an *Escherichia coli* glycolipoprotein (GLP) in the CCl_4 model, significantly less liver damage was observed.

Materials and methods

Mice

C57Bl/6 mice (Charles River, Montreal, Canada) 4–6 weeks old and maintained in a conventional facility were used, except for the 'happy-hour' alcohol study, where C57Bl/6 J (Jackson Laboratory, Bar Harbor, USA) were used. Unless specified, female mice were studied. All studies were approved by the McGill University Animal Care Committee.

Voluntary alcohol consumption

Two alcohol consumption methods were studied. For continuous exposure, mice were housed individually in cages equipped with two 75-ml dripper-style bottles (Rolf C. Hagen, Montreal, Canada). Mice were given 4 days' forced exposure to 8% alcohol only (made in tap water from anhydrous ethanol), followed by 12 weeks of free choice between water and alcohol. As control, mice were provided access to water only. Alcohol and water bottles were switched from side to side at each measurement to avoid position bias. In the limited access 'happy-hour' group, eight operant self-administration chambers (modular mouse test chambers; Medical Associates Inc., St Albans, Vermont, USA) were used. Each chamber was equipped with two liquid dipper devices, which delivered 0·01 ml of fluid. Mice were habituated to operant chambers (one mouse/chamber) and their behaviour shaped initially with sweetened milk rewards. Milk solution and water were contingent upon activation of a lever. Sessions were 2 h in duration, 5 days/week for up to 2 months. Results obtained using limited access protocols have been reported for a decade by several groups [17,18]. Alcohol acquisition was instituted where alcohol concentrations (starting with 2% ethanol) were increased and milk was decreased, leaving a final 8% alcohol solution. The amounts of alcohol and water ingested at each session were recorded. Mice were weighed and bled weekly from the saphenous vein and blood was collected by cardiac puncture, following euthanasia.

Carbon tetrachloride (CCl_4)-induced liver damage

Mice ($n = 5$) were injected intraperitoneally (i.p.) with 0·4 ml/kg CCl_4 (MP Biomedicals Inc., Montreal, QC, Canada) in 200 µl mineral oil (Sigma-Aldrich, St Louis, MO, USA) or mineral oil alone twice weekly for 4 weeks. Mice were bled weekly from the saphenous vein, killed 24 h after the last injection and blood was collected by cardiac puncture. Groups of mice were injected with CCl_4 or mineral oil as above, with and without 5 µg *E. coli* glycolipoprotein (GLP) in 200 µl phosphate-buffered saline (PBS) weekly for 4 weeks. GLP was used as a positive control, as it is known to induce RF. For these studies GLP was semipurified from 24-h cultures of *E. coli* by precipitation in 40% ammonium sulphate and gel filtration in an S300 column and screened for RF induction *in vitro*. Peak 1 consistently induced RF and was used in the experiments. Purity of GLP as determined by silver staining revealed two to three bands of approximately 300–400 kDa. Carbohydrate staining indicated a broad band.

Detection of RF

IgM and IgA RF were detected as described previously [19]. For the IgA RF assay the bound RF was detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgA (Southern Biotech, Birmingham, AL, USA), diluted 1/4000 in PBS-Tween. All samples were tested in duplicate in two independent experiments with appropriate positive and negative controls. Interassay variability was corrected for by using the positive control. Care was taken to analyse data of age-matched controls, as RF is induced as the mice age as a normal part of ageing.

Measurement of total IgM

Total IgM was measured by enzyme-linked immunosorbent assay (ELISA) as described previously [19], with slight modifications. ELISA plates were coated with 100 µl/well of a 3-µg goat anti-mouse IgM + IgG (Jackson ImmunoResearch)/ml 0·05 M carbonate–bicarbonate (pH 9·6) overnight at 4°C. Bound IgM was detected using 100 µl/well HRP-conjugated goat anti-mouse IgM (Jackson ImmunoResearch) diluted 1/8000 in PBS-Tween. A standard curve was generated using mouse IgM (BD Biosciences, Mississauga, ON, Canada). All samples were tested in duplicate in two independent experiments with appropriate positive and negative controls.

Blood urea nitrogen (BUN)

BUN was measured by a colorimetric assay (ThermoTrace, Trace Scientific Ltd, Melbourne, Australia) following the manufacturer's method. Assays were performed in duplicate and the concentration was calculated based on a known standard (Sigma-Aldrich).

Liver enzymes

AST and ALT levels were analysed by a multi-channel autoanalyser in the clinical chemistry department of the Montreal General Hospital on only the blood collected by cardiac puncture, due to the requirement for large volumes of serum.

Histology

Standard haematoxylin and eosin, as well as Sirius red staining for collagen, were performed by the Bone Centre at McGill University on liver specimens from selected mice collected at euthanasia.

Table 1. Serum IgM and IgA rheumatoid factor (RF) as measured by enzyme-linked immunosorbent assay increased with alcohol exposure, in the 'happy hour' group.

Strain	Sex/days alcohol	IgM RF†	IgA RF†
B6	F, 25 days	0·411 ± 0·570	0·242 ± 0·072
B6	F, 60 days	0·587 ± 0·128	0·602 ± 0·483
B6	M, 5 days	0·163 ± 0·82	0·135 ± 0·066
B6	M, 60 days	0·434 ± 0·108	0·281 ± 0·089

†Values are mean O.D. ± s.d., with n = 4. (Females and males consumed 7.9 ± 2.5 and 3.98 ± 2.76 g alcohol/kg/day respectively).

Statistics

Data were analysed using InStat2 (GraphPad, San Diego, CA, USA). Student's *t*-test or the Mann–Whitney *U*-test was used as appropriate.

Results

For both alcohol models no significant differences in weight gains were observed between mice drinking alcohol compared to water-drinking controls. For the continuous-exposure mice, the mean daily ethanol consumed was 4·35 ± 2·06 g absolute ethanol/kg body weight/day. No evidence of hepatic damage was observed in haematoxylin and eosin-stained liver sections (data not shown). For the limited-exposure, happy-hour group 7·9 ± 2·5 g alcohol were consumed/kg/day. This is slightly more than a binge-drinking episode in man (6 g/kg/day).

IgM and IgA RF increased with length of exposure to alcohol in the happy hour group (Table 1). Serum IgM RF correlated significantly with the amount of alcohol consumed ($r = 0{\cdot}65$, $P = 0{\cdot}0006$). No correlation was found between AST, ALT and blood alcohol or average daily intake of alcohol at the 2-month time-point, although there was an approximately two- to threefold increase in both enzymes in B6 female mice consuming alcohol compared to the water-drinking mice (AST: alcohol 243 ± 93, water 101 ± 48; ALT: alcohol 77 ± 55; water 22 ± 2) with slightly smaller differences in males (AST: alcohol 220 ± 92, water 150 ± 95; ALT 69 ± 30, 39 ± 7), which might reflect the lower amounts of alcohol consumed. There was a negative correlation between IgM RF and AST ($r = -0{\cdot}615$) and with ALT ($r = -0{\cdot}517$), suggesting that the RF response was associated with a beneficial host response. We cannot comment on the histopathology as no liver was harvested. In studies where 3·8 g/alcohol kg/day was consumed, pathological changes in the liver were evident and ALT levels increased threefold over non-alcohol controls in C3H/HeN mice [20].

Mice continuously consuming alcohol had a significant 3·5-fold increase in IgM RF compared to water-drinking controls, where IgM RF increased 0·8-fold ($P = 0{\cdot}0095$) (Fig. 1a), whereas the total IgM increased similarly in both groups (Fig. 1b). Over time, alcohol consumption increased ($r = 0{\cdot}65$). There was a strong correlation between the duration of alcohol consumption and the amount of RF produced ($r = 0{\cdot}89$, $P < 0{\cdot}0001$) (Fig. 1c). In mice consuming water only, there was a correlation between time and IgM RF production, although the *r*-value ($r = 0{\cdot}78$) was lower than for alcohol-consuming mice (Fig. 1c). Thus IgM RF was induced as the mice aged. There was a 22% and 28% increase in mean total serum IgM over time in the ethanol- and water-consuming mice, respectively. No significant increase in IgA RF production (data not shown) was seen.

No physical abnormalities were observed in mice treated with CCl_4 with or without *E. coli* GLP (used as a positive control to induce the RF response *in vivo*) or the vehicle control over the 4-week study. No abnormalities in liver histology when stained with haematoxylin and eosin were observed at the termination of the study (data not shown). Similarly there were no differences in the amount of fibrosis seen in these specimens when stained with Sirius red. In mice injected with CCl_4, AST and ALT were significantly elevated compared to vehicle controls (AST: CCl4, median 288 U/l, CI: 151, 469; control, median 62 U/l, CI: 28, 149; ALT: CCl_4, median 541 U/l, CI: 355, 781; control, median 20 U/l, CI: 13, 25) (Fig. 2a). No increase in IgM RF was detected over the 4-week period (Fig. 2b), despite the liver damage.

We had identified an *E. coli* GLP that induces RF *in vivo* and examined its effect on CCl_4-induced liver injury as a

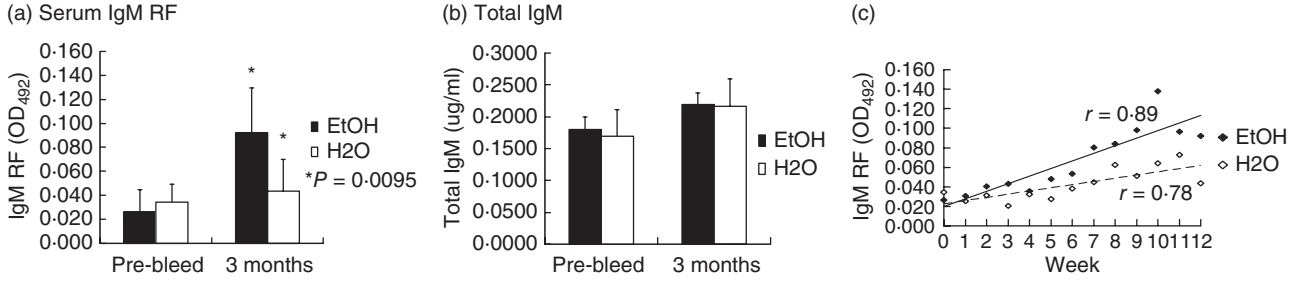

Fig. 1. Mean serum IgM rheumatoid factor (RF) (a) and total IgM (b) as measured by enzyme-linked immunosorbent assay (ELISA) in C57Bl/6 female mice consuming 8% ethanol (continuous exposure) or control mice consuming water for 3 months. Error bars represent standard deviation (s.d.). (c) Correlation between length of exposure to alcohol and serum IgM RF as measured by ELISA in C57Bl/6 female mice consuming 8% ethanol or water alone.

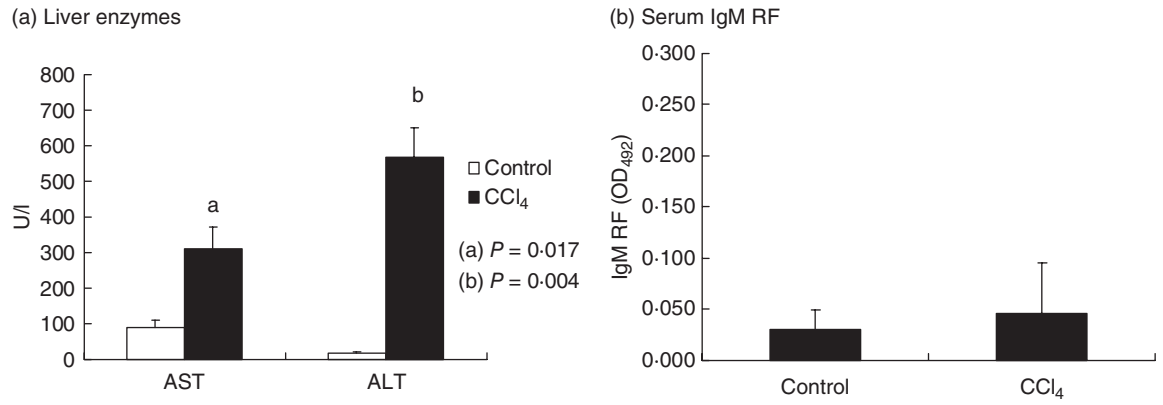

Fig. 2. (a) Mean serum and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in C57Bl/6 female mice treated with CCl_4 or mineral oil control for 4 weeks. IgM rheumatoid factor (RF) was measured by enzyme-linked immunosorbent assay (ELISA). Error bars represent standard error of the mean (s.e.m.). (b) Mean serum IgM RF in C57Bl/6 female mice treated with CCl_4 or mineral oil control for 4 weeks. Error bars represent s.d.

positive control. As expected, there was a significant increase in liver enzymes in response to CCl_4 injections compared to controls (Fig. 3a,b). Interestingly, there was a highly significant decrease in AST and ALT, together with an increase in IgM RF, when mice were injected with both CCl_4 and GLP compared with groups receiving CCl_4 alone (Fig. 3a–c). There was a trend toward a decrease in total IgM (Fig. 3d) when mice were treated with both GLP and CCl_4; however, it did not reach statistical significance.

BUN levels, a sensitive indicator of early kidney damage, were increased significantly in mice treated with CCl_4 compared to controls ($P = 0{\cdot}0002$) (Fig. 4). However, these levels were not pathological. Interestingly, injection of CCl_4 plus GLP significantly decreased BUN levels compared to mice treated with CCl_4 alone ($P = 0{\cdot}0044$). BUN levels were higher in mice treated with CCl_4 plus GLP compared to those treated with GLP alone ($P = 0{\cdot}0004$), indicating that GLP did not completely prevent kidney injury (Fig. 4).

Discussion

Although previous research has shown an association between liver disease and RF production in man [4,5,9], the mechanism(s) of induction and role of RF in liver disease in mice was unknown. We show that RF can be induced in response to chronic alcohol consumption. In addition, we demonstrate that low-dose CCl_4-induced liver damage does not lead to RF production over a 4-week period but that concurrent treatment with *E. coli* GLP, which induces RF, can protect against liver damage.

Alcohol consumption can cause increased intestinal permeability [21,22], allowing bacterial products from the normal gut flora to enter the portal circulation. Because TLR ligation along with BCR cross-linking of RF B cells can induce RF, it is likely that TLR ligands released by the alcohol contribute to the RF production in the gut and/or liver. Our model took advantage of the high preferential alcohol consumption by C57Bl/6 female mice [23]. A threshold level of alcohol consumption was required before RF were induced. The length of exposure to alcohol also impacted on RF induction. That prolonged exposure to alcohol can induce RF in self-administering mice is interesting, given that 90% of humans with alcoholic cirrhosis were IgA RF^+ [5].

From our studies, only mice with the highest alcohol consumption showed evidence of mild liver disease, consistent with previous findings [24,25]. This was not unexpected, as even in humans only 10–15% of alcoholics develop cirrhosis [26]. Mice with higher RF levels appeared to be slightly protected from the mild liver damage.

How alcohol turns on the RF response is unknown, but TLRs are probably involved. Murine B cells have been shown to express TLR2, TLR3, TLR4, TLR6 and TLR9 [27]. As the alcohol-induced RF response is not immediate, it appears that naive RF B cells are activated. Of all the major mouse strains, C3H/HeJ mice generate the highest spontaneous RF response *in vivo* [28], suggesting that TLR4 may not be required. Attempts to use TLR4-deficient C3H/HeJ mice were not successful because they consume very little alcohol voluntarily. Additional experiments using TLR-deficient mice would help to clarify the role of this pathway in RF B cell activation.

As alcohol consumption did not cause severe marked liver injury we investigated CCl_4-induced liver damage, known to cause elevated liver enzymes. We found that whereas CCl_4 induced substantial liver damage as measured by AST and ALT level increases, there was no increase in RF over the 4-week study when compared to the vehicle controls. Treating mice with an *E. coli* GLP at the same time as CCl_4 led to a striking decrease in AST and ALT, accompanied by an increase in RF. Whether the decrease in liver enzymes caused by injection of GLP was due to circulating RF B cells, soluble RF or a completely unrelated effect is unknown, although the weak negative correlation between RF and liver enzymes in

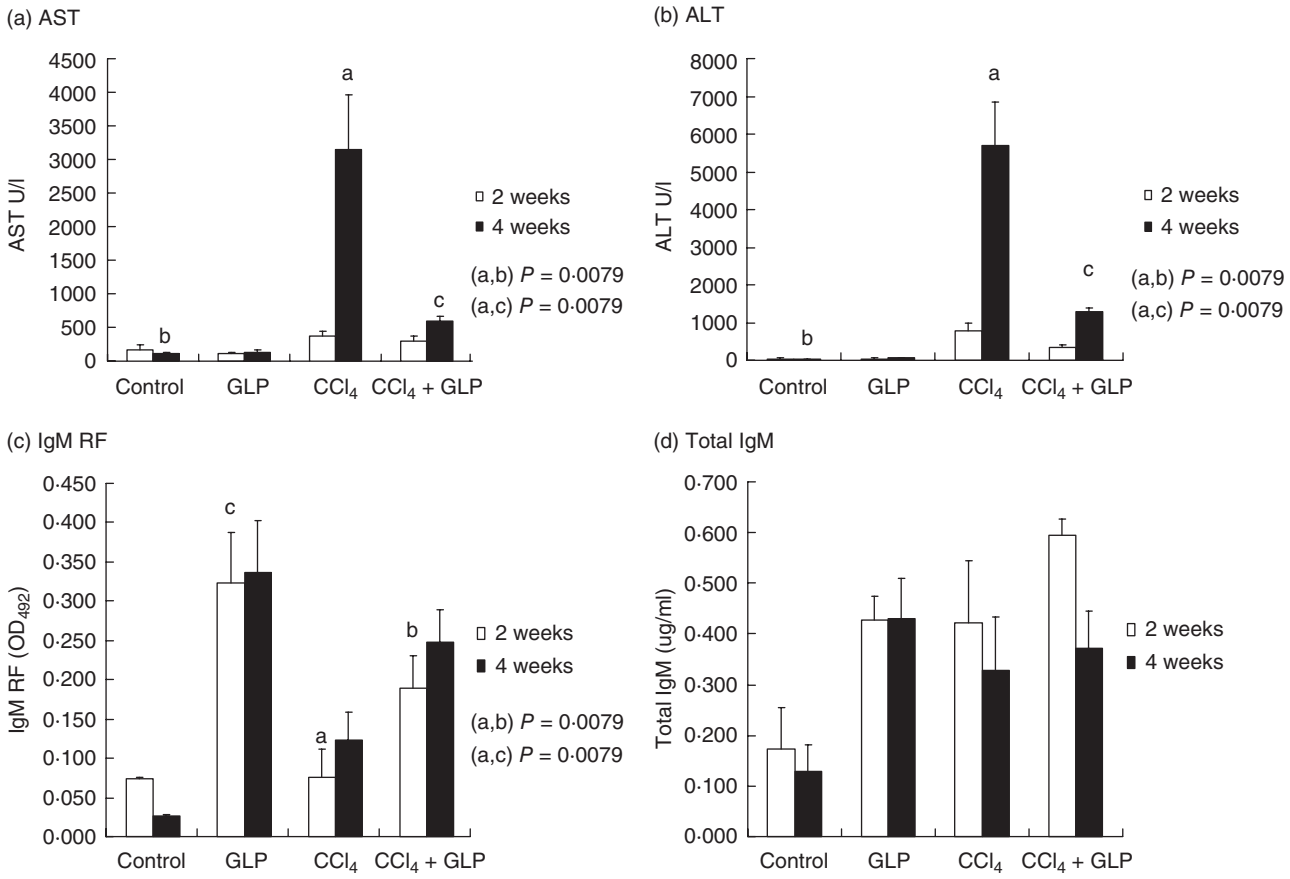

Fig. 3. (a) Mean serum aspartate aminotransferase (AST) and (b) alanine aminotransferase (ALT) in mice treated with CCl_4, glycolipoprotein (GL), both or mineral oil control for 2 or 4 weeks. Error bars represent s.e.m. (c) Mean serum IgM rheumatoid factor (RF) and (d) total IgM in C57Bl/6 female mice treated with CCl_4, GLP, both or mineral oil control for 2 or 4 weeks. Error bars represent s.d.

the GLP-treated mice suggests a minor role. It is likely that GLP could activate liver Küppfer cells to produce the hepatocyte protective cytokine interleukin (IL)-10, but additional studies would be required to investigate this.

TLR ligation probably contributes to the *E. coli* GLP-induced RF response, as in other studies in the laboratory using macrophage cell lines deficient in MyD88 no nitric oxide (NO) was produced in response to GLP when compared to wild-type MyD88$^{+/+}$ macrophage cell lines (data not shown), which produced elevated levels of NO. The precise TLR(s) activated by GLP are unidentified, but it is unlikely to be TLR9 as DNAse treatment of GLP did not affect its ability to induce RF either *in vitro* or *in vivo* (data not shown).

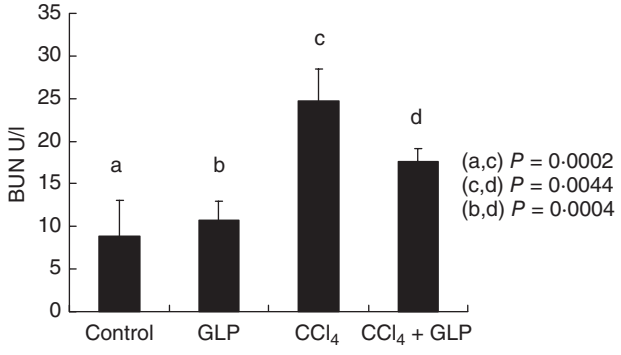

Fig. 4. BUN levels in mice treated with CCl_4, glycolipoprotein (GLP), both or mineral oil control for 4 weeks. Error bars represent s.d.

Signalling through TLR2 is possible, as in other studies we have found that Pam3Cys, a lipoprotein, which signals through TLR2, can induce RF *in vivo*. We have found that RF, induced by Pam3Cys in combination with CCl_4, were also weakly inversely correlated with liver enzyme levels ($r = -0{\cdot}385$ and $-0{\cdot}332$ for AST and ALT, respectively), indicating that such hepatoprotection is not induced by only GLP.

GLP induced RF in CCl_4-treated mice at lower levels than for mice treated with GLP alone, reflecting the toxic nature of CCl_4. Novobrantseva *et al.* [29] have reported that ~50% of hepatic lymphocytes are B cells and that CCl_4 caused a 10-fold loss in hepatic B cells 1 day post-CCl_4 administration; however, by day 5 the hepatic B cells returned to normal levels. Less B cell death would be anticipated in our studies with the use of 4·4-fold less CCl_4.

Although CCl_4 mainly targets the liver, it does have systemic effects as demonstrated by a significant but not pathological increase in BUN, an early marker of kidney damage.

Severe CCl_4-induced kidney damage has been reported in both mice and rats [30,31]. Interestingly, in our study GLP also appeared to provide some protection at that site.

We demonstrate that RF can be induced in response to chronic alcohol consumption, probably secondarily to TLR ligation. In contrast, CCl_4-induced acute liver damage was not associated with a RF response, which indicates that inflammation alone is not sufficient. Importantly, it is clear that RF do not necessarily contribute to liver damage. In both models, there was an inverse correlation between RF and liver enzyme levels. TLRs may be involved in the hepatoprotection observed, but further studies are needed to determine which cells and/or cytokines contribute to the hepatocyte protection. The RF response appears to reflect host defence.

Acknowledgements

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