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ORIGINAL ARTICLE

Hepatic Immune Response to Environmental Carcinogens

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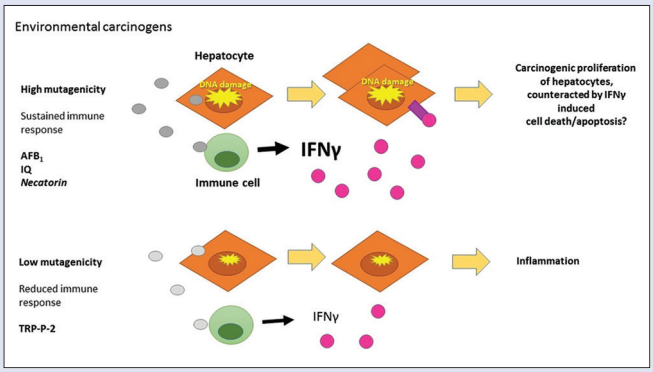
ABSTRACT

Aim: Environmental carcinogenic substances contribute to increasing incidence of hepatocellular carcinoma (HCC). We employed a sensitive method for the detection of DNA damage combined with analysis of the immune response to gain better knowledge how environmental carcinogens mediate pathology. **Materials and Methods:** Rat hepatocytes were isolated and stimulated with carcinogenic substances for the assessment of DNA damage. The mycotoxin aflatoxin B_1 (AFB_1), two heterocyclic amines from the cooking of meat amino-3-methylimidazo[4,5-f] quinoline (IQ) and 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole (TRP-P-2), and protein extract from the fungus *Lactarius necator* were assayed. Unscheduled DNA synthesis in hepatocytes was measured by the incorporation of radioactive thymidine during DNA repair. Stimulation of hepatocyte/immune cell preparation with the substances and measurement of IFNγ release at different time points determined their ability to induce an inflammatory response. **Results:** DNA repair in the hepatocytes was induced in response to 10^{-7} M AFB_1 and 10^{-9} M IQ. TRP-P-2 did not induce DNA repair; however, at 10^{-4} M, the fungus extract did this. Furthermore, liver-resident immune cells responded with differential production of IFNγ over time in response to stimulation by all the carcinogens, with AFB_1 being the most potent. TRP-P-2 showed the most significant reduction in IFNγ response over time. **Conclusion:** DNA damage in hepatocytes induced by environmental substances was detected at low molecular concentrations. The system did provide novel evidence for hepatic carcinogenicity by the fungus *L. necator*. Analysis of the response by liver-resident immune cells to the substances suggested that highly mutagenic substances induce prolonged inflammatory response.

Key words: DNA damage, hepatocytes, immune response, inflammatory response, mutagenic substances

SUMMARY

- Environmental carcinogens aflatoxin B_1 (AFB_1), amino-3-methylimidazo[4,5-f] quinoline (IQ), and extract of *Lactarius necator* did induce hepatocellular DNA damage
- The heterocyclic amine 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole (TRP-P-2) did not induce DNA damage but an inflammatory response in the liver
- Environmental substances also induced IFNγ release from hepatic immune cells
- The mutagenic substances AFB1, IQ, and *L. necator* extract did induce more prolonged immune response than TRP-P-2.



Abbreviations used: HCC: Hepatocellular carcinoma, AFB1: Aflatoxin B1, IQ: Amino-3-methylimidazo[4,5-f] quinoline, TRP-P-2: 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole, UDS: Unscheduled DNA synthesis, EGTA: Ethylene glycol-bis-(2-aminoethyl ether) N-N-N′-N′-tetraacetic acid, WME: Williams' medium E, 2-AAF: 2-Acetylaminofluorene, HBSS: Hanks' balanced salt solution, CYPs: Cytochrome p450 enzymes.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death.[1] HCC occurs worldwide but is more frequent in developing countries than in industrialized countries.[2] A number of risk factors are associated with HCC including hepatitis B and C virus infections, dietary intake of natural and artificial toxic substances, excessive alcohol consumption, and iron overload.[3,4] As the liver is detoxifying substances that have entered the bloodstream, it is exposed to their toxicity.[5] Carcinogenic substances include those that are present naturally and those that are by-products of food preparation. The fungus *Aspergillus* derived aflatoxin B_1 (AFB_1) is an example of such a substance. The urinary excretion of aflatoxin metabolites due to food contamination was in East Asia associated with a 4-fold increase in HCC.[6] Other examples of carcinogenic substances are heterocyclic amines developing during food preparation, such as 2-amino-3-methylimidazo[4,5-f] quinoline (IQ) and 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole (TRP-P-2).[7,8] Furthermore, edible mushrooms such *Lactarius necator* have been reported to contain carcinogenic substances.[9]

Modifications of the genotoxic substances by cytochrome p450 enzymes (CYPs) or other enzymes in the liver generate active

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intermediates that bind DNA, forming adducts. To assess the carcinogenicity of various substances, cultures of isolated hepatocyte have been employed. Techniques are available that detect the amount of DNA damage the substances inflict on the cells. Hepatocytes exposed to sublethal concentrations of genotoxic substances are undergoing unscheduled DNA synthesis (UDS) to repair damage caused by adducts. As hepatocyte cultures only proliferate at very low rate, measurement of incorporation radioactive nucleotides will correlate directly to amount damage inflicted to the DNA by the substance assayed.[10,11]

Hepatocytes constitute only about two-thirds of the total cell population in the liver. The remaining cells include endothelial cells, Kupffer cells, intrahepatic lymphocytes such as T cells, NKT cells, NK cells, B cells, and macrophages.[12] HCC does almost exclusively arise upon chronic hepatic inflammation. Hence, inflammation rather than a specific carcinogenic factor promote a tumor-prone hepatic microenvironment.[13] Paradoxically, the inflammatory environment is also associated with better survival in HCC patients.[14,15] Several studies have shown that naturally occurring carcinogenic substances in addition to inducing cancer by DNA damage also influence the immune system.[15-18]

In this study, we assessed UDS in hepatocyte preparations to assay naturally occurring mutagens. The substances were also analyzed for their ability to induce hepatic immune response *in vitro*.

MATERIALS AND METHODS

Reagents

Collagenase 1A, ethylene glycol-bis-(2-aminoethyl ether) N-N-N'-N'-tetraacetic acid (EGTA), trisodium citrate-2-hydrate, Williams' medium E (WME), Harris' hematoxylin and eosin (H and E), permount, 2-acetylaminofluorene (2-AAF), AFB_1, and TRP-P-2 were purchased from Sigma-Aldrich. Fetal bovine serum, gentamycin, Hanks' balanced salt solution (HBSS), and HEPES were purchased from Merck. (^{3}H) thymidine ([^{3}H] dT) was from PerkinElmer. IQ was from the Royal Society of Chemistry Worldwide Ltd. (Germany). *L. necator* was purchased from MycoBank, CBS-KNAW Fungal Biodiversity Center (The Netherlands) and IFNγ Elisa was purchased from Abcam. NTB-2 film emulsion, Kodafix, and D-19 developer were from Kodak.

Preparation of fungus extract

A fan was used to dry preparation of conserved fungus *L. necator.* Fungus extracts were homogenized and stored at room temperature until analysis. On analysis, the extracts were reconstituted using water. Protein concentration was determined using Bradford assay (Bio-Rad).

Animals

The study was performed in the College of Medical Veterinary Animal House under standard laboratory conditions, 12:12 light/dark photoperiod at 23°C ± 2°C. Twenty male Sprague–Dawley rats at 6–8 weeks of age, at a weight of 200 to 280 g were used in the study (Anticimex, Sweden). The rats were housed in polystyrene plastic cages. Approval of the study was received from the Ethics Board and the Office of the Vice President for Scientific Affairs and Postgraduate Studies at the University of Sulaimani.

In vivo treatment of rats with 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole, liver sections, and staining

In some experiments, rats were injected with 0.5 mg/0.1 ml saline, TRP-P-2 daily for a period of 6 days. Livers were subsequently removed and snap-frozen in liquid nitrogen. Frozen hepatic tissue was sectioned using a microtome, mounted on glass slides, fixed in 15% neutralized formalin, and subsequently stained with Harris's H and E as described below.

Preparation of hepatocyte cell culture

Hepatocytes were prepared using 200 g male rats, as described earlier.[19-21] The rats were anesthetized using pentobarbital at 50–75 mg/kg body weight intraperitoneal (i.p). A ventral midline incision was made from the xiphisternum to the pubic bone. The exposed liver lobes were carefully lifted, and a loose tie placed around the infra-hepatic inferior vena cava and another tie placed around the portal vein. A 21-gauge needle was inserted into the portal vein right which was ligated to ensure stability. Perfusion was commenced using a sterile solution (0.5 mM EGTA in Ca^{2+}, Mg^{2+} free HBSS, 10 mM HEPES, pH 7.35; Solution I) at 37°C. The perfusion rate was maintained for 1.5 min at 8 ml/min. Immediately following the start of perfusion, the subhepatic inferior vena cava was severed to permit the perfusate to escape. During the perfusion, the thoracic inferior vena cava was cannulated by puncturing the right atrium and the perfusate was collected. Ligation of the proximal segment of the subhepatic inferior vena cava was completed and the pump speed was increased to 40 ml/min for 2.5 min. After completion of perfusion, 250 ml of a second sterile solution (100 units/ml type 1A collagens in WME buffered with 10 HEPES, pH 7.35; Solution II) at 37°C was perfused through the liver at 20 ml/min. During this perfusion, the liver was covered with a sterile gauge (gauze) and a heat lamp (300 W) was positioned 55 cm above the rat to maintain 37°C. After completion of perfusion, the liver was placed in a sterile Petri dish containing WME and was trimmed of extraneous fat and connective tissue (37°C). The liver was then transferred to a new Petri dish containing Solution II. The porta hepatis was grasped with forceps and the capsule of the liver was opened at numerous points on both the inferior and superior surfaces using small scissors. Cells were detached by gentle combing with a round tooth stainless steel comb. The hepatic cells were resuspended and passed through a wide-bore pipette a couple of times to separate cell aggregates. The suspension was transferred to a 50-ml centrifuge tube and the volume was brought to 50 ml with WME supplemented with 10% calf serum and 50 g/ml gentamycin (WMES). The cells were sedimented at 50 ×*g* for 5 min and resuspended in WMES, up to 15–25 ml in the centrifuge tube. Viability and cell recovery were assessed using trypan blue staining and counting using a hemocytometer. This perfusion gave a yield of 15–20 × 10^6 hepatic cells/100 g body weight with a viability of 85%. Suspensions with 5 × 10^5 cells/ml in WMES were immediately seeded onto 25-mm round thermanox coverslips in 35-mm six well dishes (Merck) containing a final volume of 2.5 ml of WMES. The plates were either used directly for the assessment of hepatocytes in combination with immune cells or were placed in a 5% CO_2, 37°C incubator for 2 h. After the incubation, the medium was removed and the coverslips were washed with 2 ml of WME, leaving only the attached viable hepatocytes. These slides were subjected to DNA repair analysis in response to environmental toxins.

DNA repair assay of isolated hepatocytes

DNA repair analysis was performed as previously described.[21,22] The substances to analyze 2-AAF, AFB_1, IQ, TRP-P-2, and *L. necator* extract were dissolved in either EtOH or DMSO and 2 ml of serum-free WME, containing 10 Ci/ml (^{3}H) dT and were subjected to serial dilutions. Included were a positive control (2-AAF), a solvent control, and untreated cell. The hepatocytes were incubated with substances and (^{3}H) dT at 37°C for 18–20 h. Each coverslip was washed five times in each of three successive dishes, filled with 100 ml phosphate-buffered saline pH 7.35. The coverslips were then placed in a clean 6-well dish and covered with 2 ml of 1% sodium citrate. This treatment resulted in swollen nuclei,

permitting better quantification of the nuclear grains. The cells were then fixed using three changes of ethanol-glacial acetic acid (3:1), air-dried, and mounted cell-surface up, on glass slides with paramount.

Development of silver grains incorporated into the nuclei

In darkness, slides were submerged in a prewarmed NTB-2 emulsion for 30 min at 45°C and removed and left to dry overnight. The slides were stored in the dark at 4°C. After 7 days of exposure, the slides were developed using D-19 developer for 4 min, placed in a stop bath of acidified tap water for 30 s, immersed in Kodafix for 10 min, washed in tap water for 5 min, and left to dry.

Analysis of DNA repair (unscheduled DNA synthesis) in hepatocytes

The level of DNA repair in the hepatocyte cultures induced by a mutagenic substance was quantified by determining the net increase in nuclear grains. Grains were counted using a microscope at ×1200. Only cells, which were viable at the time of fixation, indicated by swollen nuclei and evenly coated with emulsion, were scored. About 20 cells, randomly selected from the coverslip, were counted. Counts were obtained for each nucleus while the background was determined by a nuclear-sized area of cytoplasm. Net nuclear grain counts were calculated by subtracting the highest cytoplasmic count from the nuclear count. This scoring method was designed to avoid false-positive results. Results of individual experiments were reported as the mean ± standard deviation of net gain counts for triplicate coverslips. A test substance was reported as positive when it produced a statistically significant increase in nuclear grains when using the one-tailed Mann–Whitney *U*-test. Cytotoxicity of the test substance was identified by the complete absence of S-phase cells in the autoradiograph and by general morphology. The substance was reported negative in this assay if the net nuclear count was not significantly increased at the highest nontoxic dose or at all concentrations up to the limit of solubility.

Harris' hematoxylin and eosin staining

Slides were stained with Harris' hematoxylin for 3–10 min and rinsed with tap water for 5 min. The slide was then submerged in ammonia water (2–3 ml concentrated ammonium hydroxide per 1000 ml H_2O), rinsed in tap water, treated with 70% ethanol, and stained with eosin S (1%) for 60 s. The slides were then washed by immersion in 95% EtOH (3 × 2 min) followed immersion in 99.5% EtOH (3 × 2 min). Subsequently the slides were left to dry. The coverslips were mounted onto glass slides with mounting medium and assessed using a light microscope (Leica). Images were captured with a digital imaging system (Kodak).

Immunoassay analysis for detection of IFNγ

Liver cell preparations, placed on coverslips in six-well plates, were treated with 1.5×10^{-3} M each of 2-AAF, AFB_1, IQ, TRP-P-2, or *L. necator* protein extract. Culture supernatants were removed at 12, 24, and 48 h after start of treatment and centrifuged to remove residual cells. The level of IFNγ released was determined using ELISA according to the manufacturers' instructions (Abcam).

Statistical analysis

Data were analyzed by one- or two-tailed Mann–Whitney U-test used as and where appropriate. Statistical calculations were performed using Stat view SE + Graphics software (GraphPad Software, Fay Avenue, Suite, La Jolla, CA 92037 USA).

RESULTS

Hepatic DNA repair in response to environmental toxins

We exposed rat hepatocytes to naturally occurring toxic substances with the aim to study their DNA repair inducing ability as a marker of hepatocarcinogenic activity. Initially, to verify that our system was able to detect damage to DNA, the hepatocytes were exposed to 2-AAF at concentrations ranging from 10^{-6} to 10^{-9} M. 2-AAF is a carcinogenic and a mutagenic derivative of fluorine which is commonly used in the study of carcinogenesis.[23] Our system relies on the measurement of the increase in the rate of alkaline elution of DNA which is a marker of UDS.[10,11] Intranuclear grains counted correlate to radioactive thymidine incorporation during UDS. This is part of the excision repair process during removal of DNA adducts. The number of grains was compared to the number of grains present in cytoplasm, the latter reflecting background. 2-AAF was found to potently inducing DNA repair at a concentration as low as 10^{-9} M [Table 1]. The substance further showed a dose-dependent increase in DNA repair up to the 10^{-6} M level. The controls and the dose groups were compared using a one-tailed Mann–Whitney *U*-test. In all cases, a statistical difference at the 5% confidence interval was found, confirming statistically significant differences.

Next, we exposed hepatocytes to the mycotoxin AFB_1 which has a known association with HCC.[6] The substance was used at concentrations ranging from 10^{-7} to 10^{-9} M. AFB_1 was found to inducing UDS with activity detected at a concentration of 10^{-7} M [Table 1]. Interestingly, some chromosomes were very sensitive

Table 1: Assessment of DNA repair in hepatocyte cultures treated with 2-acetylaminofluoene, mycotoxin aflatoxin B_1, 2-Aminl-3-methyl-Imidazo (4, 5-F) quinoline (amino-3-methylimidazo[4,5-f] quinoline), 3-amin0-1- amino-1methyl- 5H- pyrido- (4, 3-B) - Indole (3-amino-1-methyl-5H-pyrido- (4,3-b)-indole), and *Lactarius necator*

Chemical	Dose	Mean±SD		
		Cytoplasmic count	Nuclear silver grain counts (corrected)	
			Treated	Control
2-AAF	10^{-6} M	17.2±12.9	54.7±25.3	−5.2±9.3
	10^{-7} M		30±12.2	
	10^{-8} M		13±9.7	
	10^{-9} M		2.3±8.0	
AFB1	10^{-7} M	18.3±9.8	48.0±21.0	21±12.3
	10^{-8} M		30±14.5	
	10^{-9} M		15±13.0	
IQ	10^{-6} M	23.7±9.5	>100 *	4.0±11.7
	10^{-7} M		30±18.0	
	10^{-8} M		21.0±18.0	
	10^{-9} M		12.0±11.0	
TRP-P-2	10^{-5} M	23.7±9.5	32.3±14.0	16.0±16.2
	10^{-6} M		21.4±13.0	
	10^{-7} M		12.0±16.8	
	10^{-8} M		−0.9±17.8	
	10^{-9} M		16.8±13.0	
Lactarius necator	10^{-4} M	26.9±14.3	22.9±19.8	5.2±19.0
	10^{-5} M		21.4±13.0	
	10^{-6} M		14.0±10.2	
	10^{-7} M		11.3±17.3	
	10^{-8} M		9.2±12.3	

At least three separate experiments were performed for each substance to evaluate the mutagenic effect. SD: Standard deviation; 2-AAF: 2-acetylaminofluoene; AFB1: Aflatoxin B1; IQ: Amino-3-methylimidazo[4,5-f] quinolone; TRP-P-2: 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole

to the mutagenic effect of AFB_1, while other chromosomes were resistant (data not shown).

Heterocyclic amine derived during the preparation of red meat has also been reported to induce HCC.[24-26] One of the metabolites IQ was evaluated and was shown to be highly mutagenic. Furthermore, for this substance, the DNA repair showed a dose–response profile (concentrations: 10^{-9}–10^{-6}M), with mutagenic ability detected at concentrations of 10^{-9} M [Table 1]. We then assayed the heterocyclic aromatic amine TRP-P-2 also generated during preparation of meat. Contrasting to previous reports,[25] we could detect only low levels of DNA repair in response to TRP-P-2 as grains in the nuclei could be seen only at the highest dose tested [10^{-5} M, Table 1]. When the hepatocytes in our culture system were exposed to the amine, the substance induced vesicle formation in cells (data not shown). To verify the lack of DNA repair induction, we treated rats with i.p. injections of 0.5 mg TRP-P-2/day for 6 days and subsequently removed, sectioned, and stained livers with H and E. The morphological appearance showed several histological signs of liver injury, necrosis, vesicle containing cells, and invasion of neutrophils [Figure 1]. These changes were considered to be a drug-induced liver injury.

The fungus *L. necator* is commonly consumed in the northern hemisphere. However, it has been found to contain a carcinogenic substance, necatorin.[9] We prepared protein extracts from conserved fungi and assayed the mutagenicity of these in our system. A mutagenic effect was seen at a concentration of 10^{-4} M total protein used in the assay.

Hepatic immune-response to environmental toxins *in vitro*

Immune cells are present in the liver. These cells have been shown to be important in the induction of HCC, but persistent inflammation is also associated with better prognosis.[14] We assessed the immune response to the genotoxic substances previously assessed for UDS, by exposing total liver cell preparations to these. For this, we used cell preparations containing nonadherent immune cells in addition to hepatocytes. The immune response was determined by measurement of IFNγ production by the cells at 12, 24, and 48 h of carcinogen exposure. After 12 h of incubation of the liver cells with 1.5×10^{-3} M of each substance, significantly increased production of IFNγ was measured compared to controls. AFB_1 exposure resulted in the most pronounced immune response and release of IFNγ (223.9 ± 74.8 pg/mL) three-fold higher than control cells (66.3 ± 41.1 pg/mL, $P < 0.0001$). IQ, TRP-P-2, and *L. necator* protein extract exposure resulted in 127.4 ± 42.2 pg/mL ($P = 0.0004$, compared to controls), 146.7 ± 34.1 pg/mL ($P < 0.0001$), and 109.1 ± 29.6 pg/mL ($P = 0.0032$) release of IFNγ, respectively. The change in level of IFNγ release was assessed by comparing levels at 12 and 48 h [Figure 2]. Treatment with all substances resulted in a significant reduction of IFNγ production after 48 h stimulation, TRP-P-2 treatment showing the most significant reduction in cytokine release (3.4-fold, 146.7 ± 34.1 to 42.75 ± 15.64 pg/mL).

In conclusion, we have been able to assess DNA repair induction/HCC inducing ability by several naturally occurring genotoxic substances. *L. necator* hepatocyte genotoxicity has not been reported before. In addition, we were able to detect substance-specific responses from the liver residing immune cells over time. The study suggests that persistent inflammation induction over time correlates with the substances mutagenic ability.

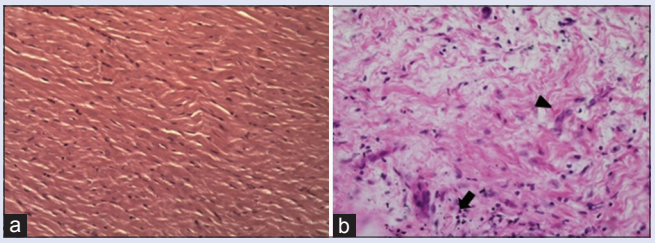

Figure 1: Morphological appearance of liver from rats (a) control without treatment and (b) treated with TRP-P-2. Rats were treated for 6 days with 0.5 mg TRP-P-2/day intraperitoneally. Livers were subsequently removed, sectioned, and stained as described in the Materials and Methods section. Visualized are interlobular connective tissue, blood vessels, and a multitude of a vesicle containing cells (arrow head) and signs of neutrophilic infiltration (arrow) (×100, H and E). Reproduction size at column width. TRP-P-2: 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole

DISCUSSION

In this study, we have used a sensitive system to assess naturally occurring substances ability to induce UDS in hepatocytes as a surrogate marker for initiation of HCC. Naturally occurring mutagens that are found in food could induce DNA damage in the hepatocytes at nanomolar concentrations. We were able to measure UDS induced by an extract from the fungus *L. necator*, which has not been reported before. By studying liver preparations containing resident immune cells, we were, in addition to DNA repair, able to assess the environmental toxins ability to induce substance-specific immune responses.

The liver is a primary target for ingested naturally occurring toxins. These are there metabolized by CYPs. AFB_1 is metabolized by the cytochrome enzymes CYP1A and CYP3A to exo-AFB1-8,9-epoxide.[5] Unfortunately, this epoxide is highly reactive and genotoxic, covalently binding to nucleic acids in the nuclei of the hepatocytes forming adducts such as 8,9-dihydro-8 (N7 guanyl)-9-hydroxy-AFB1.[2] Excision repair to remove adducts can introduce mutations in the DNA sequence. Such a mutation occurs for example often at 249ser in the p53 tumour-suppressor gene. This mutation has been observed in 30%–60% of HCC tumors in aflatoxin-endemic areas.[13] Furthermore, intake of heterocyclic aromatic amines formed during meat preparation, IQ and TRP-P-2, has been shown to induce dose-dependent increase of DNA adducts and carcinogenesis in the liver.[24-26] In our study, IQ induced DNA repair, but we were unable to replicate an induction of UDS by TRP-P-2. To verify the lack of mutagenicity by TRP-P-2, we assessed the compound *in vivo* and found liver damage but no signs of DNA repair. The intracellular vesicles found in the hepatocytes in response to treatment could be an early stage of apoptosis as has been described before.[7] Necatorin from *L. necator* has previously been shown to be carcinogenic but not before to induce DNA repair in the liver.[9] In this study, total protein extract of *L. necator* was used without exact knowledge of the concentration of necatorin in the preparation.

However, DNA damage and mutagenesis alone is not sufficient for induction of hepatic carcinogenic. HCC does almost exclusively arise upon chronic hepatic inflammation. This, rather than a specific carcinogenic factor promotes a tumour-prone hepatic microenvironment.[13] Paradoxically, an inflammatory environment is also associated with improved survival in hepatocellular carcinoma patients. NK and T cells have been identified in tumors of patients and their presence associated with longer survival.[14] Furthermore, the time of establishment of a protective immune microenvironment is of importance. Early establishment during tumorigenesis has shown to impact disease progression significantly. IFNγ together with Toll-like receptor ligands stimulates cancer cells or macrophages to produce chemokines CXCL10, CCL5, and CCL2. These chemokines induce tumor infiltration by Th1, $CD8^+$ T, and NK cells, leading to cancer cell killing and tumor control.[27] IFNγ can also induce cell cycle arrest by

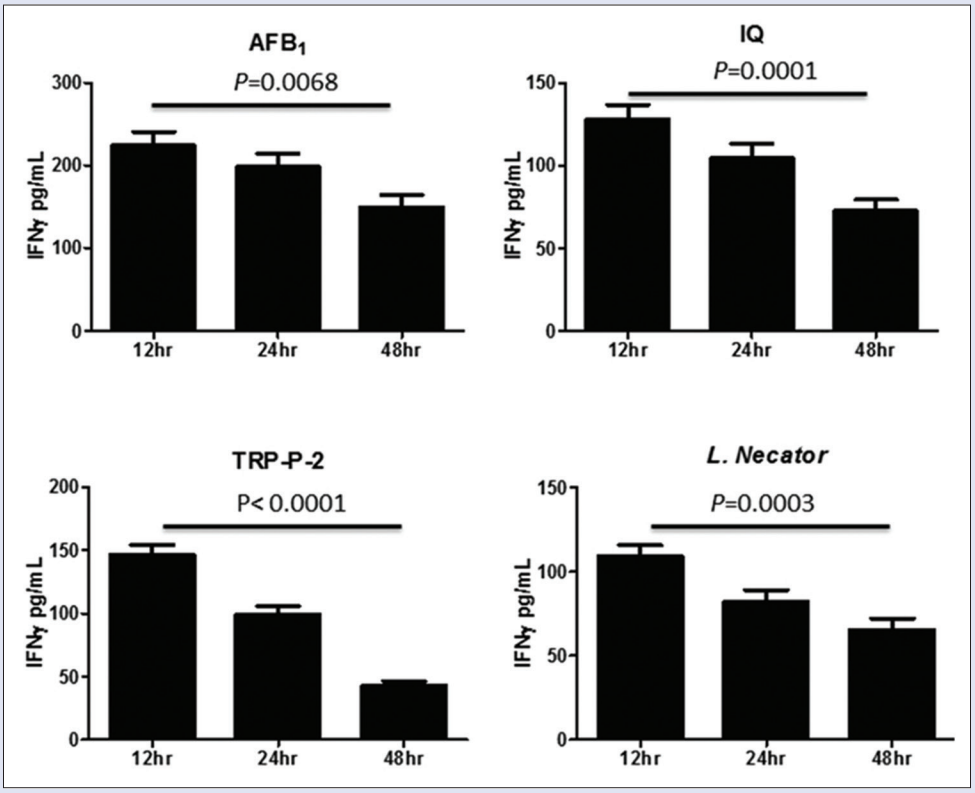

Figure 2: Hepatic immune response to 2-AAF, AFB_1, IQ, TRP-P-2, and *Lactarius necator* protein extract. Rat hepatocyte/immune cell preparations were isolated as described in the methods section. The cell preparations were stimulated with 2-AAF, AFB_1, IQ, TRP-P-2, or *Lactarius necator* at a concentration of 1.5×10^{-3} M of each substance or left untreated for 12, 24, and 48 h (n = 20 for each stimulation). Cell supernatants were subsequently removed for measurement of IFNγ as described in the methods section. Results are presented ± standard deviation. Significant differences compared to the control were determined using Mann–Whitney U-test. $P < 0.0001$ was considered statistically significant different comparing levels of IFNγ release at 12 and 48 h. Reproduction size at column width. 2-AAF: 2-acetylaminofluoene; AFB_1: Aflatoxin B_1; AFB1: Aflatoxin B1; IQ: Amino-3-methylimidazo[4,5-f] quinoline; TRP-P-2: 3-amino-1-methyl-5H-pyrido-(4,3-b)-indole

direct binding to tumor cells.[28] The importance of IFNγ to resolve HCC has been shown in a clinical trial where the cytokine with IL-2 in a combination of chemotherapy was shown to induce tumor regression in 70% of HCC patients.[29]

In the present study, we found that stimulation of hepatocyte preparations with all the environmental toxins induced IFNγ production by the resident lymphocytes, with AFB_1 being the most potent. The different levels of IFNγ from the different substances at different time points suggest that they influence immune cells differently. Until now, studies of AFB_1's influence on the immune system have shown variable results. Studies have often been performed in livestock to determine the role of AFB_1 contamination in feed. Several studies have, unlike our findings, reported immune suppressive activity by the mycotoxin.[16,30] However, it has also been reported that AFB_1 influence blood cells are dependent of dosage.[17] In agreement with our study AFB_1 fed to swine increased the number of both monocytes and immune globulins in the blood.[31] No reports have previously described the ability of IQ, TRP-P-2, and necatorin to induce an immune response in the liver. It has however been shown in mice that in an inflammatory environment (govern by the high level of IFNγ), IQ gained the ability to mutate DNA. The substance was nitrosylated by macrophages forming N-NO-IQ.[15,18] Mediators of inflammation (i.e., myeloperoxidase, HOCl, and acid pH) can activate N-NO-IQ to forming DNA adducts.[32] The environmental carcinogens induced the most IFNγ release from the hepatic immune cells after 12 h stimulation. IFNγ levels remained high after 48 h of stimulation with AFB_1 and *L Necator* extract [Figure 2]. However TRP-P-2 that was in our system unable to induce mutagenesis showed the most reduction in IFNγ production after 48 h. Taken together, these findings support the notion that persistent inflammation is required for development of HCC.[13]

In the liver, IFNγ is produced by NK cells, NKT cells, and $CD4^+$ and $CD8^+$ T cells, so any these cells could have contributed to the cytokine release we detected.[12] We speculate that persistent IFNγ release, induced by the environmental toxins assessed. serves as break preventing tumorigenesis in the liver. In support of this, IFNγ has previously been shown to prevent carcinogenesis induced by AFB_1.[33] Further studies are warranted to determining the specific effects of the carcinogenic toxins on individual liver resident lymphocyte subsets and possible dual role in both inducing and limiting carcinogenesis development.

CONCLUSION

We have, in this manuscript, assessed mutagenic abilities of AFB_1, IQ, and *L. necator* in hepatocytes. Such activity conferred by *L. necator* extract is described here for the first time. Such mutations are believed to be one of the events toward the development of HCC. Furthermore, stimulation of immune cells present in hepatocellular preparations with the substances in this study induced specific response in terms of IFNγ production. Prolonged IFNγ production was most evident for environmental substances able to induce mutagenesis. Given the role of IFNγ in limiting tumor development, we propose the induction of an immune cell-driven negative feedback response to the mutagens in the liver.

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Conflicts of interest

There are no conflicts of interest.

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