Interactions between hepatitis B virus and aflatoxin B₁: effects on p53 induction in HepaRG cells

Myriam Lereau,¹,² Doriane Gouas,² Stéphanie Villar,² Ahmad Besaratinia,³ Agnès Hautefeuille,² Pascale Berthillon,¹ Ghislaine Martel-Planche,² André Nogueira da Costa,² Sandra Ortiz-Cuarian,² Olivier Hantz,¹ Gerd P. Pfeifer,³ Pierre Hainaut,²,⁴ and Isabelle Chemin¹

¹INSERM U1052, 151 cours Albert Thomas, 69424 Lyon Cedex 03, France
²International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France
³Department of Cancer Biology, Beckman Research Institute, City of Hope Comprehensive Cancer Center, 1500 East Duarte Road, Duarte, CA 91010-3000, USA
⁴International Center for Research and Training, Hospital AC Camargo, Rua Pirapitingui 204, São Paulo SP 01508 - 020, Brazil

Infection by hepatitis B virus (HBV) and dietary exposure to aflatoxin B₁ (AFB₁) are the main risk factors for the development of chronic liver disease and hepatocellular carcinoma (HCC). How these factors cooperate is still largely unknown. AFB₁ activation leads to DNA adduction and mutagenesis, with a specific mutation at codon 249 in TP53 (p.R249S). So far, only limited studies have addressed the effects of AFB₁ on HBV replication. We have analysed the effects of both risk factors on p53 induction during HBV infection in HepaRG, a cell line with hepatocyte-like morphology that metabolizes AFB₁ and supports HBV infection. Exposure to AFB₁ up to 5 μM induced a downregulation of HBV replication after 48 h, as measured by a decrease in viral antigens in the culture medium (HBsAg, HBeAg and large envelope protein) and in intracellular levels of HBV transcripts, DNA and HBsAg. Conversely, HBV infection did not significantly modify AFB₁-DNA adduct formation or repair as assessed by immunodot-blot assay, and the induction of p53 in response to AFB₁ was similar in infected and non-infected HepaRG cells. Overall, our results suggest that AFB₁ exposure decreases HBV replication, whereas DNA damage by AFB₁ and subsequent p53 induction is not affected by the presence of the virus. Thus, in HepaRG cell line, AFB₁ and HBV do not cooperate to increase DNA damage by AFB₁. Further studies on the effects of both factors in a context of chronicity are needed to better understand synergistic effects.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents 75–90% of primary liver cancers (McGlynn & London, 2005). With 694,000 deaths per year in 2008, HCC is the third leading cause of cancer-related deaths (Ferlay et al., 2010). Highest incidences are observed in sub-Saharan Africa and Southeast Asia (8–117 per 10⁵ in men and 5–75 per 10⁵ in women in 2008) (Ferlay et al., 2010). Worldwide, the main risk factors are chronic infections by hepatitis B (HBV) or C (HCV) viruses, accounting for 75–80% of global HCC cases, often in combination with consumption of foods contaminated by aflatoxin B₁ (AFB₁) or with alcohol abuse (Bosch et al., 1999). AFB₁ is classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) (WHO, 2002). In areas of highest incidence, HBV is endemic [prevalence of hepatitis B surface antigen (HBsAg) is higher than 8%] and exposure to AFB₁ is widespread. A synergistic effect of both factors has been observed in cohort studies, with a 5–10-fold higher risk of HCC in the presence of both factors as compared with HBV or AFB₁ alone (Kew, 2003; Lunn et al., 1997; Qian et al., 1994; Ross et al., 1992; Sylla et al., 1999; Wang et al., 1996).

HBV is a small hepatotropic virus, belonging to the family Hepadnaviridae, which can cause acute and chronic liver diseases (Ganem & Prince, 2004). HBV genomic DNA, identified as relaxed circular DNA (RC DNA), is partially double-stranded and converted into covalently closed circular DNA (cccDNA) after translocation into the nucleus. cccDNA is the template for viral transcription on Cancer (IARC) (WHO, 2002). In areas of highest incidence, HBV is endemic [prevalence of hepatitis B surface antigen (HBsAg) is higher than 8%] and exposure to AFB₁ is widespread. A synergistic effect of both factors has been observed in cohort studies, with a 5–10-fold higher risk of HCC in the presence of both factors as compared with HBV or AFB₁ alone (Kew, 2003; Lunn et al., 1997; Qian et al., 1994; Ross et al., 1992; Sylla et al., 1999; Wang et al., 1996).

HBV is a small hepatotropic virus, belonging to the family Hepadnaviridae, which can cause acute and chronic liver diseases (Ganem & Prince, 2004). HBV genomic DNA, identified as relaxed circular DNA (RC DNA), is partially double-stranded and converted into covalently closed circular DNA (cccDNA) after translocation into the nucleus. cccDNA is the template for viral transcription on Cancer (IARC) (WHO, 2002). In areas of highest incidence, HBV is endemic [prevalence of hepatitis B surface antigen (HBsAg) is higher than 8%] and exposure to AFB₁ is widespread. A synergistic effect of both factors has been observed in cohort studies, with a 5–10-fold higher risk of HCC in the presence of both factors as compared with HBV or AFB₁ alone (Kew, 2003; Lunn et al., 1997; Qian et al., 1994; Ross et al., 1992; Sylla et al., 1999; Wang et al., 1996).
and production of intermediate replicative (IR) and RC DNA in the newly formed intracellular capsids. AFB1 is a mycotoxin produced by \textit{Aspergillus} sp. that occurs as a food contaminant in hot and humid regions. In the liver, cytochromes P450 (CYP450), mainly CYP450 1A2 (CYP1A2) and 3A4 (CYP3A4), bio-activate AFB1 into exo-8,9-epoxide to form DNA adducts, preferentially on the N7-position of guanines. The primary adduct is rapidly processed into secondary lesions, the apurinic (AP) site and the ring-opened AFB1-formamidopyrimidine (AFB1-FAPY) adduct (SmA et al., 2002), which are repaired by nucleotide (NER) or base (BER) excision repair (Sarasin et al., 1977; Waters et al., 1992). Improper repair predominantly leads to G-to-T transversion mutations. In HCC cells of patients exposed to AFB1, a recurrent mutation occurs at codon 249 in the tumour suppressor gene TP53 (AGG to AGT, arginine to serine, R249S) (Aguilar et al., 1993; Bressac et al., 1991; Hsu et al., 1991). This mutation, detected in over 50% of HCC in high-incidence areas of China or Africa (Bressac et al., 1991; Hsu et al., 1991), is rare in HCC from non-aflatoxin contaminated areas and in cancers other than HCC (Ozturk, 1991).

\textit{TP53} encodes an all-round tumour suppressor protein, p53. Under normal conditions, p53 is maintained at low levels through binding to ubiquitin-ligases such as Hdm2 and degradation via the proteasome. However, in response to various stresses, p53 is stabilized by post-translational modifications including phosphorylation on serine 15 (Pser15-p53), binds specific DNA response elements and modulations including phosphorylation on serine 15 (Pser15-p53), binds specific DNA response elements and trans-activates genes involved in cell cycle arrest, apoptosis, DNA repair or senescence (Hollstein & Hainaut, 2010). These responses either facilitate repair of DNA lesions and maintenance of genetic integrity, or may induce apoptotic cell death and elimination of damaged cells.

Worldwide, the \textit{R249S} mutation is almost exclusively present in HCC of subjects co-exposed to chronic HBV and AFB1, suggesting that DNA damage by AFB1 and chronic infection by HBV may influence each other (Kew, 2003). Such an influence may occur at many levels. First, during infection, in a context of exposure to AFB1, HBV may decrease DNA repair and make cells more permissive to the formation of mutations (Jia et al., 1999). Second, during the development of chronic HBV infection, inflammatory stress may enhance AFB1 metabolism and DNA damage, increasing mutagenesis (Chemín et al., 1999). Third, in pre-cancer or cancer HCC cells, the mutant \textit{R429S} p53 protein may contribute to the survival of cells expressing viral antigens (Gouas et al., 2009).

In the absence of simple animal models for human HBV infection, the contribution of each of these mechanisms has been difficult to assess. In this study, we have taken advantage of a novel experimental system, HepaRG cells (Gripon et al., 2002), to address some aspects of these mechanisms, namely, the effects of HBV infection and replication on DNA damage by AFB1 and \textit{vice-versa}. HepaRG are derived from HCC. However, at confluence, they can differentiate into hepatocyte-like and biliary epithelial-like cells, the former being receptive to HBV infection (Gripon et al., 2002). Treatment with DMSO enhances differentiation and susceptibility to infection. Moreover, metabolic enzymes involved in detoxification (glutathione S-transferases, GST) and bioactivation (CYP450) of xenobiotics are expressed in HepaRG, allowing them to metabolize AFB1 (Aninat et al., 2006; Jenne et al., 2010).

\section*{RESULTS}

\subsection*{Cytotoxicity of AFB1}

We first investigated the cytotoxic effect of AFB1 on HepaRG cells using an MTS assay. Fig. 1(a) shows the morphology of HepaRG cells in proliferation and differentiation phases. In the latter, the presence of hepatocyte-like and epithelial-like cells is clearly recognizable. As shown in Fig. 1(b), AFB1 had no measurable cytotoxic effect after a pulse of 4 h of exposure at doses up to 10 \textmu M, followed by 48 h of culture in medium without AFB1 (denoted ‘4 h + 48 h’). For the purpose of the following experiments, two relatively low doses of AFB1 were used: 1 and 5 \textmu M. These doses were selected based on their ability to form detectable and dose-dependent AFB1-DNA adducts by immunodot-blot assay using an antibody that recognizes specifically FAPY-DNA adducts (Fig. 1c).

\subsection*{Effects of AFB1 exposure on extracellular HBV}

Next, we assessed the effects of AFB1 treatment on HBV replication. Levels of viral antigens secreted in the culture medium were analysed by quantitative ELISA for HBsAg and semi-quantitative ELISA for HBeAg and the large (L) envelope protein. We observed a significant AFB1 dose-dependent decrease in the secretion of HBsAg, HBeAg and the L protein (Fig. 2a–c). With 5 \textmu M of AFB1, this decrease was of 41, 81 and 86%, respectively. These results indicate that AFB1 strongly downregulated the production of HBV proteins, including the L protein, which is considered as specifically associated with complete and mature virions.

\subsection*{Effects of AFB1 exposure on intracellular HBV}

To assess the effects of AFB1 on HBV replication, processing and intracellular accumulation, we quantified total intracellular viral DNA by PCR (Fig. 3a). A significant dose-dependent decrease in the amount of HBV DNA genome equivalents per micogram of total DNA was observed at 4 h + 48 h of exposure (\textit{P}<0.001, Student’s \textit{t}-test). Next, we used RT-quantitative (q)PCR to measure the levels of viral transcripts. In AFB1-treated cells, HBV transcript levels were decreased in a dose-dependent manner as compared with non-treated cells (\textit{P}<0.05 for 1 \textmu M and \textit{P}<0.01 for 5 \textmu M, Student’s \textit{t}-test) (Fig. 3b). In order to assess whether transcriptional inhibition may be specific for viral RNA, we analysed several cellular
transcripts by quantitative RT-PCR (see Supplementary Fig. S1, available in JGV Online). CYP1A2, CYP3A4 and GSTA1/2 transcripts were also found to decrease in a dose- and time-dependent manner, suggesting that AFB1 induced a more general inhibition of transcription in host cells. As decreased transcription leads to diminished synthesis of viral proteins, in particular HBsAg, the suppression of HBV transcription induced by AFB1 may account for the decrease in levels of secreted HBsAg. These observations were confirmed by measuring the relative amounts of intracellular HBsAg by semi-quantitative ELISA, which revealed a significant dose-dependent decrease after 4 h + 48 h of AFB1 (P<0.001, Student’s t-test) (Fig. 3c). Furthermore, in experiments exploring intracellular HBV levels at up to 4 h + 96 h of AFB1, a significant dose-dependent decrease in both RC DNA and cccDNA was observed after differential extraction of both forms (see protocol in Supplementary Methods and results in Supplementary Fig. S2, available in JGV Online).

Adduct formation and repair
To determine the influence of HBV on AFB1-induced DNA damage, we analysed adduct formation and repair by immunodot-blot assay. Adduct formation was rapid and short-lived, with a peak at 4 h after AFB1 exposure followed by return to basal levels after 4 h + 16 h (Fig. 4a). Quantification of dot signals at 5 μM of AFB1 revealed that the presence of HBV had no effect on adduct levels (Fig. 4b). Furthermore, the decrease in DNA adducts, indicative of DNA repair, was identical in both infected and uninfected cells. Adduction of AFB1 metabolites results in an unstable product that is rapidly processed into...
secondary FAPY-DNA adducts. It is conceivable that conversion may be affected by the presence of HBV. To address this point, we compared adduct levels in DNA treated or not with alkaline conditions that convert primary adducts into FAPY-DNA adducts. No significant difference was observed in dot signal intensity or decrease over time (Fig. 4c), as confirmed by quantification of dot signals (data not shown).

To investigate whether AFB1-DNA adducts could form within specific genes, chromatin-immunoprecipitation (ChIP) experiments were performed on DNA from HepaRG cells at 4 h + 4 h after 5 μM AFB1 treatment. Antibodies to AFB1-DNA adducts were used to precipitate sheared DNA (200–500 bp fragments) and the precipitated material was amplified by quantitative PCR using primers recognizing HBV DNA. As shown in Fig. 5, no or only background signals corresponding to HBV DNA copies were detected in non-HBV-infected cells. In contrast, in HBV-infected cells exposed to AFB1, a significantly increased number of HBV DNA copies was detected, suggesting that HBV DNA is a target for the formation of AFB1-DNA adducts.

**Effects of HBV and AFB1 on DNA synthesis at 4 h+48 h post-treatment**

As AFB1 is known to induce DNA damage, we wanted to determine whether HepaRG cells may undergo specific suppressive responses after AFB1 treatment. We first monitored DNA synthesis at 4 h + 48 h post-treatment by performing bromodeoxyuridine (BrdU) incorporation and FACS analysis. As shown in Fig. 6, without AFB1 treatment, HepaRG cells did not display any significant BrdU incorporation at 4 h + 48 h, consistent with the notion that these cells have undergone differentiation and are not proliferating anymore. Surprisingly, in cells treated with AFB1, BrdU incorporation became detectable and was distributed over the whole span of the S phase with a sizeable proportion of cells in late the S or G2/M phases (Fig. 6). This incorporation was dose-dependent but was essentially similar in the presence or in the absence of HBV. A similar pattern was observed after 4 h + 96 h of AFB1 exposure (data not shown). This observation suggests that AFB1 treatment leads to reinitiation of DNA synthesis in differentiated HepaRG cells, or to stimulation of proliferation in a subset of HepaRG cells that may have retained a progenitor status.

Reinitiation of DNA synthesis and, possibly, of cell cycle progression, raises the possibility that HepaRG cells may acquire mutations as a result of DNA damage caused by AFB1. In previous publications, we and others have described and used a sensitive and quantitative method to detect mutant R249S DNA at low levels in a background of wild-type DNA (Lleonart et al., 2005). This method, short oligonucleotide mass analysis (SOMA), can routinely detect up to one copy of mutant DNA per 1000 copies of wild-type DNA. However, analysis by SOMA of DNA from AFB1-treated HepaRG cells at 4 h + 48 h and 4 h + 96 h failed to detect R249S mutation, suggesting that under these experimental conditions the mutant does not form at levels compatible with the SOMA detection method (data not shown).

**Induction of p53 and DNA damage**

HepaRG cells express detectable levels of p53 protein and sequencing of TP53 exons 2–11 and flanking splicing sites demonstrated the presence of wild-type sequence (see Supplementary Table S1, available in JGV Online). Western blot analysis identified a protein of expected size (53 kDa), which accumulated in a dose-dependent manner and underwent phosphorylation on serine 15 (Pser15-p53) in response to AFB1 treatment (Fig. 7a and Supplementary...
Fig. S3, available in JGV Online). This increase was not detected at the mRNA level (data not shown), suggesting that it corresponds to post-translational stabilization of wild-type p53 in response to DNA damage by AFB1. Next, we analysed the expression of Hdm2 and p21, the products of two p53 target genes. Western blots showed an increase in Hdm2 levels that parallels those of p53. In contrast, p21 levels did not vary in a p53-dependent manner, suggesting that p21 is not a significant target in differentiated HepaRG cells. HBV infection had no effect on these responses, suggesting that p53 induction is not modified by the presence of an actively replicating virus.

We next investigated DNA damage responses by analysing the levels of phospho-H2AX protein by Western blot (Fig. 7b): AFB1 induced a dose-dependent increase with the stronger signals being observed between 4 h and 4 h +16 h. These observations are consistent with the activation of a DNA-damage signalling pathway leading to p53 induction.

Fig. 4. Adduct formation and repair in HepaRG. (a) Adduct formation and repair was analysed by immunodot-blot assay after different times of AFB1 exposure in HBV-positive and -negative cells. (b) Dots were quantified in order to visualize repair kinetics by normalizing with HBV(-) or HBV(+) at 4 h for cells treated with AFB1 5 μM. Full line: HBV(-); dashed line: HBV(+). (c) Primary AFB1-DNA adducts were converted into FAPY-DNA adducts by alkaline DNA treatment (T) or were left untreated (NT) and were analysed by immunodot-blot assay. Blots are representative of three independent experiments.

Fig. 5. Detection of AFB1-DNA adducts in HBV genome. Adduct-IP experiments were performed on HepaRG cellular DNA. Quantitative PCR showed the presence of adducts at 4 h +4 h after 5 μM AFB1-treatment in HBX gene mainly in HBV-infected cells. *P<0.05, **P<0.01

Effects of p53 induction on HBV antigen secretion

Whereas results in Fig. 5 show that AFB1-DNA adducts may form in HBX (providing a mechanism to explain the inhibition of HBV replication by AFB1), we were interested in the possibility that activation of p53 by DNA-damage may also represent a mechanism that impairs HBV replication. To test this hypothesis, the HepG2/2.2.15 cell line was used, as a pilot study aimed at providing a proof of concept. This cell line is derived from a human hepatoblastoma cell line,
HepG2, by constitutive transfection of the complete HBV genome (Sells et al., 1987). Therefore, these cells constitutively produce and secrete HBV particles. HepG2/2.2.15 cells were treated with either doxorubicin (DNA-damaging agent) or nutlin-3 (inhibitor of Hdm2-dependent degradation of p53) for 24 h in order to activate p53, as shown by

**Fig. 6.** BrdU incorporation. FACS analysis showed an AFB1 dose-dependent BrdU incorporation in HepaRG cells at 4 h + 48 h post-treatment. Percentages are means of three independent experiments and represent BrdU-positive cells in the upper panel of each graph.

**Fig. 7.** p53 induction in HepaRG cells. Western blots were performed to analyse p53 and its target products p21WAF1 and HDM2 (a) and phospho-H2AX (b) upon AFB1-treatment in HBV-infected and non-infected cells. Positive (and negative) controls (a) were obtained from HepG2 cells treated or not with doxorubicin. Ku80: loading reference. Time-course corresponds to the following abbreviations: 0: '4 h', 4: '4 h + 4 h', 16: '4 h + 16 h', 48: '4 h + 48 h'.
Western blot (Fig. 8a). Hdm2 was clearly accumulated in nutlin-3-treated cells, whereas p21 was equally accumulated upon both types of treatment. Moreover no evidence of apoptosis was observed as shown by the absence of a sub-G1 cell population in flow cytometry analysis (Fig. 8d). Semi-quantitative ELISAs revealed a decrease in HBsAg and L protein secretion upon both treatments (Fig. 8b and c), suggesting that agents that activate p53, including a specific regulator of p53 stability that does not act through DNA-damage formation, may set in motion mechanisms that decrease the synthesis and secretion of HBV.

**DISCUSSION**

Epidemiological studies support the existence of a synergy between chronic HBV infection and exposure to AFB1 in hepatocarcinogenesis. Although HCC or chronic liver disease take place in a context of chronic HBV infection and long-term exposure to AFB1, it is conceivable that some synergistic effects may involve enhanced AFB1-induced DNA damage and mutagenesis in cells that replicate HBV, as well as enhanced HBV replication in cells that metabolize AFB1 (Jia et al., 1999). In this study, we have examined the short-term effects of exposure to AFB1 on the molecular parameters of infection by HBV, and the influence of HBV on AFB1-induced DNA damage. This study was made possible by the use of HepaRG cells, which are susceptible to HBV infection in culture (Gripon et al., 2002), express enzymes involved in AFB1 metabolism (Aninat et al., 2006; Jennen et al., 2010), and carry wild-type TP53 alleles (Supplementary Fig. S2). Although this system does not recapitulate the long-term effects of HBV chronicity, it represents to date the most sophisticated
Our results show that AFB1 decreased intracellular HBV DNA loads, mRNA levels and secretion of HBsAg, HBeAg and L proteins. These effects are consistent with an attenuation of viral replication and production of viral particles after a short exposure to AFB1. The mechanisms of this attenuation may involve many molecular processes and remain a matter of speculation. In the present study, we show that AFB1-DNA adduct may form within HBV DNA. To assess sequence-specific DNA adducts, we developed an original method based on the same principle as ChIP, taking advantage of the covalent binding of FAPY-adducts to DNA to immunoprecipitate and analyse short DNA fragments by PCR. We detected AFB1-DNA adducts in HBV DNA in HBV-infected cells treated with 5 μM AFB1. Adduct formation may result in hindrance of replication and in slowing down the process of HBV production. On the other hand, a pilot study aimed at providing a proof of concept and performed in another liver cancer cell model, HepG2/2.2.15, suggests that induction of p53 either by doxorubicin (a DNA damaging agent) or nutlin-3 (a specific, non-DNA damaging inhibitor of p53 degradation) contributes to inhibit the production of HBV antigens. Therefore, we suggest that treatment by AFB1 may trigger several parallel mechanisms that are detrimental for the replication and secretion of HBV. In this respect, AFB1 may act as a sort of natural antiviral agent.

Our results were obtained with doses of AFB1 (1–5 μM) well below the observed IC50 in HepaRG cells (>50 μM), but equivalent to the IC50 of 5 μM reported by another study (Aninat et al., 2006). The difference between the latter experiments and ours may be due to the fact that, instead of a continuous exposure for 24 h, we treated the cells with a 4 h ‘pulse’ of AFB1, followed by maintaining cells in fresh medium without AFB1 for 48 h. Thus, the effects we observed over 48 h were not due to the continuous perturbation of cell physiology by cumulative effects of AFB1, but were most likely a consequence of the initial, specific damage caused by the toxin. How the doses we used correlate with liver concentrations of AFB1 in humans exposed to the toxin is not clear. In the Gambia, a country with widespread AFB1 exposure, the ingested dose of AFB1 has been evaluated to vary between 4 and 115 ng per kg of body weight per day (Williams et al., 2004), but the concentration of the toxin in the liver is unknown.

The intracellular parameters of viral replication showed drastic changes upon AFB1 exposure. First, levels of viral DNA (total, RC and cccDNA) were significantly decreased as compared with non-treated cells. This suggests that exposure to AFB1 decreases the pool of cccDNA, thus reducing the production of viral mRNA as well as the downstream production of RC DNA. In turn, decreased transcription accounts for decreased levels of HBsAg, HBeAg and L protein in the culture medium. These results differ from those reported by Barraud et al. (1999) using the Pekin duck animal model. In this model, intraperitoneal injection of AFB1 over a 6-week period resulted in a significant increase in serum and liver Duck HBV (DHBV) DNA levels. These effects might be due to cumulative AFB1-induced oxidative and inflammatory stress rather than to DNA damage, since levels of AFB1-DNA adducts were similar in the liver of infected and non-infected animals (Barraud et al., 2001).

Our results also provide some insights on whether HBV replication may modulate adduct formation by AFB1. We did not detect a significant difference in the amount of FAPY-DNA adducts or in the type of adduct formed (AFB1-N7-guanine vs FAPY) between infected and non-infected cells. Moreover, these adducts were not detectable anymore after 4 h of AFB1 exposure levels returned to basal, with no difference between infected and non-infected cells. This observation suggests that despite being arrested in cell cycle, HepaRG cells maintain a capacity to eliminate DNA adducts. In addition, exposure to AFB1 induced a dose-dependent increase in DNA damage (as detected by phospho H2AX) and in p53, but not in p21WAF1 levels, again with no difference between cells infected by HBV and non-infected cells. We conclude that HBV infection and replication do not significantly modulate the sensitivity of HepaRG cells to genome-wide DNA-damage induced by AFB1.

Surprisingly, our results show that, following exposure to AFB1, induction of p53 was not followed by an increase in p21WAF1, a classical p53 target gene. The reason for this absence of p21WAF1 activation is unknown. It may be due to the specific status of differentiated HepaRG cells, which are post-mitotic, differentiated and therefore non-dividing cells. In such a context, the transcription of p21WAF1 might be constitutively repressed. Of note, different types of p53 activating treatment resulted in a clear increase in p21WAF1 in dividing liver cancer cells (HepG2/2.2.15) as well as in non-differentiated HepaRG cells (data not shown). Perhaps in relation with the absence of p21WAF1 induction, HepaRG cells appeared to activate BrdU incorporation over the whole spectrum of the S phase, suggesting that they can reinitiate de novo synthesis of the entire DNA under exposure to AFB1. It is not known whether this phenomenon is due to the reactivation of cell cycle in differentiated HepaRG cells or to stimulation of proliferation in a small number of cells with liver progenitor status that persist in the culture after differentiation. This phenomenon did not differ between infected and non-infected cells. DNA synthesis and replication under damage induced by AFB1 may facilitate the formation of mutations at adducted positions as a consequence of inappropriate DNA repair or DNA synthesis. Only a small number of studies have analysed DNA repair in HepaRG cells. Andrieux et al. (2007) have shown that stimulation with epidermal growth factor increased NER activity and expression of ERCC1 (excision repair cross-complementary 1) in proliferating HepaRG, but they did not analyse these effects in differentiated cells.

http://vir.sgmjournals.org
Overall, our results indicate that, when given as a time-limited pulse, AFB$_1$ operates as a modulator of HBV replication, assembly and secretion in HepaRG cells. It decreases all intracellular parameters of HBV replication, transcription and antigen synthesis, as well as the secretion of HBsAg, HBeAg and L protein. On the other hand, presence of replicating HBV does not significantly affect DNA damage by AFB$_1$ or HepaRG cell responses to this damage. It should be noted, however, that different events might take place in the context of chronic infection accompanied by sustained AFB$_1$ exposure, where cumulative damage by both agents is compounded by inflammatory, cytotoxic and liver regeneration responses. Further studies are needed to more precisely compare the levels of adduct formation and repair at base positions of relevance for HCC, in particular in codon 249 of TP53.

### METHODS

#### HepaRG cell culture, HBV infection and AFB$_1$ treatment.

HepaRG cells have been described previously (Gripon et al., 2002). Cells were grown for 2 weeks at 37 °C, 5 % CO$_2$ in proliferation medium [Williams’ E medium (Gibco-Invitrogen), 10% non-decomposed FCS (HyClone Fetalclone II; Thermo Scientific), 2 mM glutamine (Gibco-Invitrogen), 50 U penicillin (Gibco-Invitrogen) ml$^{-1}$, 50 U streptomycin (Gibco-Invitrogen) ml$^{-1}$, 5 µg human recombinant insulin (Sigma-Aldrich) ml$^{-1}$, 0.5 µM dexamethasone (Sigma-Aldrich), 20 µg gentamicin (Gibco-Invitrogen) ml$^{-1}$]. For differentiation, cells were grown for an additional 2 weeks at 37 °C, 5 % CO$_2$ in proliferation medium supplemented with 1.8 % DMSO (Sigma-Aldrich) and 5 % epidermal growth factor (AbCys) ml$^{-1}$. HepaRG were infected with HBV genotype D isolated from HepG2/2.2.15 cells as described previously (Hantz et al., 2009). Infection was performed at 100–200 viral particles per cell in the presence of 4 % polyethylene glycol (PEG 8000; Sigma-Aldrich) in differentiation medium overnight at 37 °C, 5 % CO$_2$. After extensive washing with PBS, the medium was replaced and at day 12 post-infection, cells were treated for 4 h in serum-free differentiation medium with AFB$_1$ (Sigma-Aldrich). Cells were harvested using a scraper immediately or 4 h ('4 h + 4 h'), 16 h ('4 h + 16 h') and 48 h ('4 h + 48 h') after switching medium to differentiation medium. Pellets were washed with PBS and frozen at −80 °C until use.

#### HepG2/2.2.15 cell culture and treatments.

HepG2/2.2.15 cells (Sells et al., 1987) were cultured at 37 °C, 5 % CO$_2$ in minimum essential medium (Gibco-Invitrogen) supplemented with 10% FCS (Gibco-Invitrogen), 100 U penicillin ml$^{-1}$, 100 U streptomycin ml$^{-1}$, 2 mM glutamine, 1 mM non-essential amino acids (Gibco-Invitrogen), 1 mM sodium pyruvate (Gibco-Invitrogen) and 0.4 mg G418 (Gibco-Invitrogen) ml$^{-1}$ for HBV genome selection. HepG2/2.2.15 cells were treated with either 1 µM doxorubicin (Sigma-Aldrich) or 10 µM nutlin-3 (Cambay Chemical) for 24 h and harvested.

#### Evaluation of AFB$_1$ cytotoxicity on HepaRG cells.

After 1 month of growth in 96-well plates as described in the previous section, HepaRG cells were treated with AFB$_1$ (0–50 µM) in serum-free differentiation medium. After 4 h, AFB$_1$-containing medium was replaced by fresh serum-containing differentiation medium and cells were further cultured for 48 h. Cytotoxicity was evaluated by an MTS assay in 150 µl medium and using 20 µl per well of CellTiter 96 AQuant One Solution reagent (Promega).

#### Cell extractions.

DNA was extracted from HepaRG cells using a proteinase K/saturated NaCl method as described previously (Besaratinia et al., 2009). Briefly, cell pellets were treated with 0.5 µg proteinase K (Qiagen) ml$^{-1}$ overnight at 37 °C in Tris/HCl pH 8.0 (0.5 M), EDTA pH 8.0 (20 mM), NaCl (10 mM), SDS (1 %), followed by addition of NaCl (6 M). After vortexing, samples were incubated at 56 °C for 10 min and centrifuged for 30 min at 17 600 g. The supernatant was transferred into a new tube and DNA was precipitated by adding two volumes of pre-chilled absolute ethanol, kept overnight at −20 °C, pelleted by centrifugation for 10 min at 17 600 g at 4 °C, washed with 70 % ethanol and dissolved in Tris (10 mM) EDTA (1 mM) pH 7.5 buffer. RNA was extracted from HepaRG cells using the Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer’s instructions. Proteins were extracted from HepaRG and HepG2/2.2.15 cells by resuspending cell pellets in RIPA-like buffer containing Tris pH 7.4 (50 mM), NaCl (250 mM), SDS (0.1 %), DTT (2 mM), NP-40 (0.5 %), protease inhibitors (500 mM PMSF, 0.5 mg leupeptin ml$^{-1}$, 2 mg aprotinin ml$^{-1}$, 1.4 mg pepstatin A ml$^{-1}$), and phosphatase inhibitors 1:100 (Halt Phosphatase Inhibitor Cocktail; Thermo Scientific) and were kept on ice for 30 min. After centrifugation at 15 600 g for 15 min at 4 °C, supernatants were collected, protein concentrations were measured using the Bradford method and extracts were stored at −80 °C until Western blot analysis.

#### Analysis of HBV antigens by ELISA.

To measure extracellular HBV antigens (Ag), HepaRG cell culture media were collected at 4 h + 48 h. A quantitative ELISA was performed to analyse HBsAg according to the manufacturer’s instructions (Architect HbsAg; Abbott Diagnostics). HBsAg was analysed by semi-quantitative ELISA (Monolisa HBsAg/Ab Plus; Bio-Rad) according to the manufacturer’s instructions. HBV L envelope protein was monitored by semi-quantitative ELISA by detecting the pre-S1 domain as described previously (Le Guillou et al., 2000). To measure intracellular HBsAg, 10 µg proteins were diluted in PBS and analysed for HBsAg by semi-quantitative ELISA (Monolisa HBsAg Ultra; Bio-Rad) according to the manufacturer’s instructions. HepG2/2.2.15 cell culture media were collected after the 24 h treatment with nutlin-3 or doxorubicin. HBsAg and L protein were monitored by semi-quantitative ELISA as described for HepaRG.

#### Conversion of AFB$_1$-N7-Gua to FAPY-DNA adducts.

Six micrograms DNA were incubated with 15 mM Na$_2$CO$_3$, 30 mM NaHCO$_3$, pH 9.6 for 2 h at 37 °C (Besaratinia et al., 2009). DNA precipitation was performed with 3 M sodium acetate pH 5.2 and pre-chilled absolute ethanol. Pellets were dissolved in 15 µl Tris (10 mM) EDTA (1 mM) pH 7.5 buffer.

#### Immunodot-blot assay.

To assess AFB$_1$-DNA adduct formation, an immunodot-blot assay was performed as described previously (Besaratinia et al., 2009). DNA (500 ng) was denatured at 95 °C for 5 min and put immediately on ice. Samples were loaded onto an Amersham Hybond-N$^+$ membrane (GE Healthcare) that was then soaked in 0.4 M NaOH for 20 min at room temperature and saturated in PBS/Tween 0.2 %/non-fat milk (NFM) 5 % (Régia) overnight at 4 °C. Immunodetection was performed using a primary antibody against FAPY-DNA adduct (Zhang et al., 1991) (6A10 antibody, Immunodot-blot assay.)
monoclonal, dilution 1:10000; Novus Biologicals) and a secondary antibody (goat anti-mouse, dilution 1:5000; DAKO) for 1 h. Antibodies were diluted in PBS/Tween 0.2 %/NFM 5 %. Signal detection was done by chemo-luminescence using the Amersham ECL plus Western blotting System (GE Healthcare) and Amersham Hyperfilm ECL (GE Healthcare). Relative intensities were determined using Bio-Rad Imaging Equipment applying Image Lab analyser (Bio-Rad).

DNA adduct, immunoprecipitation and HBV DNA quantitative PCR. Presence of FAPY-adducts within specific sequences was analysed using a method derived from the principle of ChIP, based on the notion that adducts are covalently linked to DNA at specific positions. Experiments were performed using ChIP-Adem-kit Protein G (Ademtech) according the manufacturer’s instructions. Briefly, 10 μg DNA was sonicated for four pulses (5 s On/5 s Off) with 20 % amplitude. 6A10 antibody (2 μg) was used to capture DNA fragments containing FAPY-DNA adducts. The isolated DNA was finally resuspended in 30 μl water and HBV DNA was quantified by real-time PCR relative to an external plasmid DNA standard on a LightCycler instrument using LightCycler FastStart DNA Masterplus SYBR Green I (Roche) and the primers HBV1745fw (5'-GGAGGGA-GTTGCC-) and HBV1844rev (5’-GGAGGGA-TACATAAGGGTCTTGCAG-3') (Lucifora et al., 2011; Unterpasser et al., 2006). Results were expressed in HBV-DNA copy numbers ml⁻¹ of resuspended DNA after ChIP.

BrdU incorporation and FACS analysis. After infection and AFB1 treatment as described above, HepaRG cells were incubated with 1 μg BrdU (BD Biosciences) ml⁻¹ for 90 min, harvested by trypsinization, washed with PBS, resuspended in ethanol 70 % and kept at 4 °C for at least 30 min. Cells were then treated with 3 M HCl for 20 min, washed twice with PBS, treated with 0.1 M borax for 3 min, washed twice with PBS/FCS 2.5 %, incubated with 10 μl antibody against BrdU (BD Pharmingen FITC Mouse Anti-BrdU Set; BD Biosciences) for 20 min in the dark, and washed with PBS/FCS 2.5 %. Thirty microlitres of 0.1 mg propidium iodide (Sigma-Aldrich) ml⁻¹ was added to each sample. FACS analysis was performed on a FACScalibur using Cell Quest analyser software (BD Biosciences).

Western blot analysis. Proteins (20–30 μg) were diluted into Laemmli buffer, denatured for 5 min at 95 °C and separated on SDS-PAGE. After transfer onto a PVDF Western blotting membrane (Roche Applied Science), non-specific sites were blocked by incubation in PBS/ NP-40 0.1 %/NFM 5 % for 1 h. Immunodetection was performed overnight at 4 °C using primary antibodies against p53 (DO7, monoclonal, 1:3000; Dako), Pser15-p53 (polyclonal, 1:1000; Cell Signaling Technology), p21 (Ab1 antibody EA10, monoclonal, 1:500; Calbiochem), Hdm2 (Ab2 antibody 2A10, monoclonal, 1:200; Calbiochem), phospho-H2AX (Ser139, JBW301, monoclonal, 1:1000; Upstate) and Ku80 (monoclonal, 1:20000; GeneTex) or β-tubulin (TUJ1, monoclonal, 1:1000; Covance) as loading reference, followed by peroxidase-conjugated secondary antibodies (goat anti-mouse: 1:10000, goat anti-rabbit: 1:8000; Dako) for 1 h. Antibodies were diluted into PBS/NP-40 0.1 %/NFM 1 % except for Pser15-p53 and phospho-H2AX antibodies which were diluted into TBS/Tween 0.1 %/NFM 1 %. Signal detection was done by chemo-luminescence using the Amersham ECL Western blotting System and quantified as described for immunodot blots.

Statistical analysis. Each experiment was repeated at least three times. Measures were averaged for triplicate experiments and SE were calculated. Statistical analyses were conducted using a paired Student’s t-test assuming that experimental conditions correspond to population of equal variance. P<0.05 was considered statistically significant.

ACKNOWLEDGEMENTS

The authors thank Luc Barraud, Philippe Chevallier, Isabelle Borde and Françoise Berby for technical assistance. This work was supported by grants from ‘Ligue Nationale Contre le Cancer Savoie’, ‘Association pour la Recherche sur le Cancer’, ICRETT award from ‘International Union Against Cancer’ and EXPLO’RA DOC fellowship from Région Rhône-Alpes.

REFERENCES


