Comparative in vitro and ex-vivo myelotoxicity of aflatoxins B1 and M1 on haematopoietic progenitors (BFU-E, CFU-E, and CFU-GM): Species-related susceptibility

E. Roda a,*, T. Coccini b, D. Acerbi b, A.F. Castoldi b, L. Manzo a,b

a University of Pavia, Department of Internal Medicine and Therapeutics, Toxicology Division, I-27100 Pavia, Italy
b IRCCS Salvatore Maugeri Foundation, Toxicology Division, Institute of Pavia, Pavia, Italy

ARTICLE INFO

Article history:
Received 26 February 2009
Accepted 4 September 2009
Available online 9 September 2009

Keywords:
Aflatoxin
Mouse
Human
Bone marrow
Clonogenic assay

A B S T R A C T

Haematopoietic and myelotoxicity are adverse effects caused by mycotoxins. Due to the relevance of aflatoxins to human health, the present study, employing CFU-GM-, BFU-E- and CFU-E-clonogenic assays, aimed at (i) comparing, in vitro, the sensitivity of human vs. murine haematopoietic progenitors to AFB1 and AFM1 (0.001–50 μg/ml), (ii) assessing whether a single AFB1 in vivo treatment (0.3–3 mg/kg b.w.) alters the ability of murine bone marrow cells to form myeloid and erythroid colonies, and (iii) comparing the in vitro with the in vitro ex-vivo data.

We demonstrated (i) species-related sensitivity to AFB1, showing higher susceptibility of human myeloid and erythroid progenitors (IC50 values: about 4 times lower in human than in murine cells), (ii) higher sensitivity of CFU-GM and BFU-E colonies, both more markedly affected, particularly by AFB1 (IC50: 2.45 ± 1.08 and 1.82 ± 0.8 μM for humans, and 11.08 ± 2.92 and 1.61 ± 0.20 μM for mice, respectively), than the mature CFU-E (AFB1 IC50: 12.58 ± 5.4 and 40.27 ± 6.05 μM), irrespectively of animal species, (iii) regarding AFM1, a species- and lineage-related susceptibility similar to that observed for AFB1 and (iv) lack of effects after AFB1 in vivo treatment on the proliferation of haematopoietic colonies.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

In recent years there has been an increasing interest in both industry and regulatory bodies concerning the development and the validation of in vitro tests able to predict in vivo haematopoietic and myelotoxicity (Parchment, 1998).

Accumulating evidence suggest that the haematopoietic organs are readily affected by a wide variety of drugs and chemicals which may affect the haematopoietic cell renewal system at different stages interfering with cell proliferation and differentiation (Amess, 1993).

Haematopoiesis is a complex interplay between intrinsic genetic pathway of blood cells and their environment, regulated by cytokines. Under appropriate stimulations of these environmental regulatory proteins, erythroid and myeloid stem cells can give rise in vitro to phenotypically distinct colonies of differentiated cells. Colony development has been previously used as endpoint in vitro studies of several xenobiotic myelotoxicity, as described in recent pharmacology reviews on alternative in vitro test systems (Stephenson et al., 1971; Tepperman et al., 1974; Parchment, 1998; Parent-Massin, 2001; Pessina et al., 2001, 2005; Gribaldo 2002; Malerba et al., 2004). In vitro clonogenic assays sufficiently predictive for in vivo effects could play a pivotal role in bridging the gap between preclinical toxicology studies in animal models and clinical investigations, and help in the human risk assessment connected with food additives and environmental xenobiotics (Pessina et al., 2002).

Myelosuppression and immunosuppression are common adverse effects observed in toxicological syndromes caused by the ingestion of mycotoxins (Sharma, 1993; Tung et al., 1975; Dugyala et al., 1994).

Mycotoxins, secondary metabolites of fungi that grow on foodstuffs consumed by animals and man, have been found to induce signs of toxicity in different mammalian species. The occurrence of mycotoxins is considered to be a major risk factor affecting human and animal health (Fink-Gremmels, 1999).

Among the groups of mycotoxins considered of major concern for human health, aflatoxins (AF) are both acutely and chronically toxic to mammals, being responsible for acute liver damage, liver cirrhosis, induction of tumours and teratogenic effects (Pitt, 2000). Among the four main naturally produced aflatoxins,
including aflatoxin B1, B2, G1 and G2, the AFB1 has been shown to cause immunosuppression (Corrier, 1991; Bondy and Pestska, 2000; Oswald et al., 2005).

In Europe, current strong regulation (EC, 2006, 2007) prevents the outbreaks of aflatoxicosis in humans and animals. However, a matter of concern remains the contamination of milk and dairy products by the hydroxylated metabolite of AFB1, the AFM1, excreted from hepatic metabolism (Neal et al., 1998; Fink-Gremmels, 1999).

The Joint FAO/WHO Expert Committee classifies AFB1 as a human carcinogen and proposes no safe dose. Although AFM1 is less carcinogenic (2–10% of potency) than AFB1, it is also a health danger, because this metabolite can survive pasteurization. It has comparable liver toxicity, can reduce the immunological functions in infants, and it is considered to be a human carcinogen (2B) by the International Agency for Research on Cancer (IARC, 1993, 2002) (Eaton and Gallhager, 1994; Chen et al., 2005). This is supported by a number of epidemiological studies demonstrating a positive association between dietary aflatoxins and liver cancer.

Immunosuppression caused by aflatoxin B1 has been demonstrated in various livestock species as well as in laboratory animals (Sharma, 1993). Ingestion of these mycotoxins has been associated with a wide range of adverse effects: e.g. acute mortality, stunted growth, acute hepatic failure and also impaired immunity, with a decreased host resistance to infectious disease (see Corrier, 1991 for a review). Furthermore, ingestion of aflatoxins has also been associated with several diseases such as leukopenia, anaemia, bone marrow aplasia and myelosuppression (Tung et al., 1975; Dugyala et al., 1994; Chen et al., 2005).

Bone marrow (BM), with its rapidly renewing cell populations, being one of the most sensitive tissue to cytotoxic agents (Bloom, 1993), plays a pivotal role in the immune functions and the toxins, that are able to damage BM cells, may profoundly alter immune response (Dugyala et al., 1994).

Previously, the myelotoxicity of aflatoxins has been demonstrated in animals by assessing bone marrow cells cellularity, microscopic observations, or chromosomal aberrations associated with crude extracts administered in acute and repeated doses (Barta et al., 1990; Ito et al., 1989).

Moreover, in vitro studies performed on myeloid progenitor cells, derived from mice, showed a concentration-dependent decrease in myeloid progenitors after AFB1 exposure (Cukrova et al., 1991; Dugyala et al., 1994). Due to the relevance of aflatoxins to human health, the present study aimed at (i) comparing, in vitro, the sensitivity of human vs. murine haematopoietic committed progenitors to AFB1 and its metabolite AFM1, by means of the CFU-GM (myeloid progenitors), BFU-E and CFU-E (erythroid progenitors) assays, and (ii) assessing whether a single in vivo treatment with different doses of AFB1 alters the ability of murine multipotent BM cells to form myeloid and erythroid colonies in vitro, with the ultimate goal to compare the in vitro results with the in vitro ex-vivo data, to fully assess different species-related sensitivities.

2. Materials and methods

The experimental design of the present study considered two different steps.

(1) An in vitro study: human BM cells (from Poietic Technologies) and murine BM cells (from control mice) were exposed in vitro to several concentrations of AFB1 and AFM1 (0.001–50 μg/ml) in clonogenic cultures for different hematopoietic progenitors, e.g. CFU-GM (Colony forming unit – Granulocyte-Macrophage), BFU-E (Burst forming unit – Erythroid) and CFU-E (Colony forming unit – Erythroid); the doses have been derived from previous study by Cukrova et al. (1991) and Dugyala et al. (1994).

(2) An in vitro ex-vivo study: BM cells, isolated from the femur of mice, given in vivo a single i.p. injection of AFB1 (or vehicle + control), were cultured for the appropriate time and finally CFU-GM, BFU-E and CFU-E colonies were scored.

The in vivo acute test doses have been selected on the basis of previous studies showing a slight increase in chromosome aberrations in mouse BM after a single dose of 1 mg AFB1/kg body weight, with no increase in micronuclei (Anwar et al., 1994), and, on the contrary, a significant dose-dependent induction of micronuclei after i.p. administration of 2–8 mg AFB1/kg body weight in the rat (Raj et al., 2001).

2.1. Toxins

AFB1 and AFM1 were obtained from Sigma–Aldrich S.r.l., Milan, Italy.

For the in vitro experiments, stock solutions were prepared by dissolving AFB1 and AFM1 in acetonitrile (AFB1 = 10 mg/ml acetonitrile; AFM1 = 0.2 mg/ml acetonitrile). Stock solutions were then diluted with Iscove’s Modified Dulbecco’s Medium (IMDM, Gibco Life Technologies Italia S.r.l., San Giuliano Milanese, Italy) to reach the final concentrations.

For the in vivo treatment the AFB1 was dissolved in ethanol: corn oil (5%: 95%) just before the injection.

2.2. Murine bone marrow cells

2.2.1. Animals

All experimental procedures involving animals were performed in compliance with the European Council Directive 86/609/EEC on the care and use of laboratory animals.

Male CD-1 mice (3 weeks-old, weighing 14–16 g) were purchased from Charles River Italia (Calco, Italy) and allowed to acclimatize for at least two weeks before the exposure. Throughout the experimental time, animals were kept in an artificial 12 h light: 12 h dark cycle with humidity at 50 ± 10%. Animals were provided rat chow (4RF21 diet) and tap water ad libitum.

2.2.2. Isolation of murine bone marrow cells

This procedure was performed in rigorously sterile conditions on mice belonging to the different experimental groups specifically indicated in (i) Section 2.2.3 for the in vitro assay (treatment of cells obtained from control animals) and (ii) Section 2.2.4 for the in vivo exposure. Following animal sacrifice by cervical dislocation, intact femur were isolated by cutting muscle ligaments, cleaned, and placed in 100-mm Petri dishes containing 10 ml ice-cold IMDM supplemented with antibiotics (Penicillin 100 U/ml and streptomycin 100 μg/ml; Sigma–Aldrich S.r.l., Milano, Italy). The ends of each femur were cut just below the head and BM was flushed with 3 ml of IMDM without antibiotics. A single-cell suspension was produced by gently and repeatedly drawing the BM cells through a syringe fitted with a 23-gauge needle. BM cells were then filtered through a 100 μm cell strainer and washed by centrifugation at 2500 rpm for 10 min at 20 °C. Then, the pellet was resuspended in IMDM + 30% fetal calf serum (FCS), counted in a Bürker [10 μl of cell suspension was diluted with 90 μl of Hypotonic Liquid (Carlo Erba, Italia)]. Viability was usually 95% or greater, with no differences between experimental groups. The final cell suspension was adjusted to achieve the cell density required for the assay to be performed: (i) 3.0 × 10⁶ cells IMDM + 30% FCS for BFU-E and CFU-E, and (ii) 1.5 × 10⁶ cells IMDM + 30% FCS for CFU-GM.
2.2.3. Murine BFU-E/CFU-E and GM-CFU assay: in vitro experiment

Murine progenitor cells, collected as previously described, were washed, diluted in 30% FBS–IMDM, and then seeded in MethoCult-M3334 (StemCell Technologies, Vancouver, BC, Canada) for the BFU-E/CFU-E assay or in MethoCult-M3301 (StemCell Technologies, Vancouver, BC, Canada) for the GM-CFU assay. These media are specific for murine cells; the first one contains Iscove’s methylcellulose (1%), FBS (15%), BSA (1%), bovine pancreatic insulin (10 μg/ml), human transferrin iron-saturated (200 μg/ml), 2-mercaptoethanol (10^{-4} M), and glutamine (2 mM). Stimulation of the erythroid lineage was obtained by the addition of erythropoietin (3 U/ml). The latter MethoCult-M3301 contains all the components of the previous medium with the addition of CSF (10 ng/ml) to stimulate GM-CFU growth, and lacks 2-mercaptoethanol.

The clonogenic assay was then performed by adding 100 μl of toxin solutions (in IMDM) and 300 μl of cells (3.0 × 10^5 cells/ml for the erythroid progenitors, or 1.5 × 10^6 cells/ml for the myeloid progenitors, respectively) directly to a 3 ml methylcellulose tube. The final concentrations range of AFB1 and AFM1 was 0.001–50 μg/ml. Finally, in order to obtain a triplicate for each dose, 1 ml methylcellulose-cell suspension was seeded in 35-mm dishes, and the cultures were incubated at 37 °C in 5% CO_2 for 3 and 10 days (BFU-E/CFU-E cultures) or 7 days (GM-CFU cultures).

2.2.4. Murine BFU-E/CFU-E and GM-CFU assay: acute in vivo treatment

Six mice per dose level received, respectively 0.3, 1, or 3 mg AFB1/kg body weight by single i.p. injection, while control animals were treated with the vehicle only (corn oil). Mice were euthanized by cervical dislocation 48 h after the treatment, and blood samples were analyzed from haematological indices. The time elapsed after injection (i.e. 48 h) was chosen based on previous data by Cukrova et al. (1992b) showing in vivo AFB1 effects on BM cell growth 2 days after treatment. Additionally, the mean body weight gains were calculated for each experimental group, along with the assessment of selected organ-to-body weight ratios. BM cells were isolated and cultured following the procedure described in the previous Sections 2.2.2 and 2.2.3, obviously with the unique exception that no toxins were added to the cell cultures.

2.3. Human bone marrow cells

2.3.1. Source of human progenitor cells

As the source of progenitors for the CFU-assays, human mononucleated BM cells were used. The cells were obtained frozen, from Poietic Technologies, Inc. (Gaithersburg, MD, USA) and thawed before using. Briefly, 1 ml of cells was rapidly thawed in a water bath at 37 °C and diluted in 1 ml of 2.5% human albumin (Sigma–Aldrich S.r.l., Milan, Italy), IMDM (Gibco Life Technologies Italia S.r.l., Milan, Italy), 10% fetal calf serum heat-inactivated (FCS, Sigma–Aldrich S.r.l., Milano, Italy), 10000U DNase/ml (Sigma–Aldrich S.r.l., Milan, Italy) IMDM solution. After 10 min, the solution was centrifuged at 1200 rpm for 15 min at 18–20 °C. The pellet was then diluted in 30% FCS—IMDM, counted in a Bürker (90 μl Hypotonic Liquid (Carlo Erba, Italia) + 10 μl cell suspension) (cell viability was 90 ± 5%), and used for the clonogenic test at a concentration of 1.5 × 10^5 cells/ml.

2.3.2. Human BFU-E/CFU-E and GM-CFU assay

BM cells, thawed as previously described, were seeded in MethoCult-H-4330 (StemCell Technologies, Vancouver, BC, Canada) for the BFU-E/CFU-E assay or in MethoCult-H4001 (StemCell Technologies, Vancouver, BC, Canada) for the CFU-GM assay. The MethoCult-H-4330 medium was minus colony-stimulating factor (CSF), but contained FBS (30%), bovine serum albumin (BSA, 1%), methylcellulose (1%), 2-mercaptoethanol (10^{-4} M), glutamine (2 mM) and 30/μl erythropoietin.

The MethoCult-H4001 medium, without mercaptoethanol, contains CSF, methylcellulose (1%), FBS (30%), BSA (1%), 2-mercaptoethanol (10^{-4} M), glutamine (2 mM), and GM-CSF (10 ng/ml).

The procedure for the clonogenic assay was similar to that used for murine assays, performed by adding 100 μl of toxins solutions (AFB1 and AFM1 in IMDM) and 300 μl of cells (1.5 × 10^6 cells/ml) directly to a 3 ml methylcellulose tube. Similar were also the AFB1 and AFM1 final concentration tested. In order to obtain a triplicate for each dose of aflatoxins, 1 ml methylcellulose-cell suspension was seeded in 35-mm dishes, and the cultures were incubated at 37 °C in 5% CO_2 for 7 and 15 days.

2.4. Colony scoring

2.4.1. Erythroid lineage

BFU-E and CFU-E were scored after 7 and 15 days of incubation, for human cells, and after 3–5 and 8–10 days, for murine cells, respectively, using an inverted microscope (Zeiss, Axiosvert 25) with magnification × 25.

2.4.2. Myeloid lineage

The human CFU-GM colonies were scored after 14 days of incubation, whereas murine CFU-GM were scored after 7 days of incubation, using an inverted microscope (Zeiss, Axiosvert 25) with magnification × 25.

The criteria adopted for the identification of the different colony types (immature/mature erythroid or myeloid) have been previously reported by Pessina et al. (2001) and Malerba et al. (2002).

2.5. Data analysis

Cell proliferation is expressed as a percentage of growth, with 100% corresponding to the number of colonies in the vehicle dishes. Colony formation linearity was used as acceptance criteria for colony growth in control dishes (data not shown). The IC_{50} values (concentration of toxin necessary to reduce by 50% the cells viability) were calculated from concentration–response curves in which percent inhibition was plotted against the natural logarithm of the molar concentration. Data will be expressed as mean ± SD of at least three experiments, each carried out in triplicate. Statistical analyses were performed by ANOVA followed by Dunnett’s post hoc test. Values of p < 0.05 were considered statistically significant.

3. Results

The toxic effects of AFB1 and its metabolite AFM1 on the formation and on the proliferation of erythroid colony forming unit (CFU-E), erythroid burst forming unit (BFU-E) and granulocyte/macrophages colony forming unit (CFU-GM), from human and murine BM progenitors, were evaluated after in vitro and in vitro-ex-vivo (murine) exposure to the two toxins.

3.1. Marine data: In vitro treatment

Regarding to the in vitro study, AFB1 and AFM1 concentration–response curves obtained in three different assays on murine erythroid and myeloid progenitor cells are shown in Fig. 1a, b and c. As clearly detectable observing the dose–response curves, both toxins reduced the colony formation in a concentration-dependent fashion, with an overt toxicity particularly evident in BFU-E and CFU-GM at the highest dose (Fig. 1a and c; Table 1).
The IC\(_{50}\) values calculated for CFU-E, BFU-E and CFU-GM (Table 1) clearly demonstrated that AFB1 seemed to affect more markedly both the myeloid lineage (CFU-GM) and the immature erythroid progenitors (BFU-E) (IC\(_{50}\) values of 11.08 ± 2.92 and 1.81 ± 0.20, respectively), than the more mature CFU-E (IC\(_{50}\) = 40.27 ± 6.05). In particular, the BFU-E are the most affected colony types (Fig. 1a) in that the dose of 50 µg AFB1/ml was strongly toxic, those of 2, 5 and 10 µg AFB1/ml were markedly suppressive, (toxicity values of 50–80%), and even the lower doses (from 0.001 to 0.1 µg AFB1/ml) significantly inhibited the BFU-E proliferation processes (about 20%). A similar trend of colony proliferation inhibition was induced by AFM1 in CFU-E, BFU-E and CFU-GM, although in a lesser extent manner (see IC\(_{50}\) values in Table 1).

3.2. Human data: in vitro treatment

Fig. 2 shows the proliferation inhibition of human erythroid (Fig. 2a and b) and myeloid (Fig. 2c) colonies caused by both AFB1 and AFM1 toxins in a dose-dependent manner, demonstrating a similar trend of toxicity observed in mice. In particular the IC\(_{50}\) values indicated that the myeloid progenitors (CFU-GM) and the immature erythroid progenitors (BFU-E) were about 5 and 10 times respectively more sensitive than the respective more mature CFU-E to AFB1 (Table 1).

With respect to the myeloid lineage (Fig. 2c), the most affected colony type, the doses of 10 and 50 µg AFB1/ml were totally toxic, the dose of 5 µg AFB1/ml was strongly suppressive, and the 2 µg AFB1/ml dose still caused 50% inhibition. Exposure to AFM1 caused similar results to those obtained by AFB1 for all treated colony types.

### Table 1

<table>
<thead>
<tr>
<th>IC(_{50}) (µM)</th>
<th>Human</th>
<th>Murine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haematopoietic colonies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB1 BFU-E</td>
<td>1.82 ± 0.8</td>
<td>1.81 ± 0.20</td>
</tr>
<tr>
<td>AFB1 CFU-E</td>
<td>12.58 ± 5.4</td>
<td>40.27 ± 6.05</td>
</tr>
<tr>
<td>AFB1 CFU-GM</td>
<td>2.45 ± 1.08</td>
<td>11.08 ± 2.92</td>
</tr>
<tr>
<td>AFM1 BFU-E</td>
<td>2.01 ± 0.9</td>
<td>3.9 ± 2.18</td>
</tr>
<tr>
<td>AFM1 CFU-E</td>
<td>15.7 ± 3.81</td>
<td>60.5 ± 10.05</td>
</tr>
<tr>
<td>AFM1 CFU-GM</td>
<td>2.40 ± 1.02</td>
<td>11.5 ± 2.08</td>
</tr>
</tbody>
</table>

* p < 0.05 (comparing the same cell type in human vs. murine).
3.3. Human vs. murine in vitro data

First, our investigation clearly demonstrated that human BM progenitors were more sensitive (about 4 times) than murine BM cells to both toxins, especially for CFU-E and CFU-GM.

Second, irrespectively of the animal species (human or murine), both AFB1 and AFM1 affected more markedly both the myeloid lineage (CFU-GM) and the immature erythroid progenitors (BFU-E), than the more mature CFU-E colonies (Figs. 1 and 2, Table 1).

3.4. Murine data: in vitro ex-vivo experiment

Regarding the in vitro formation of CFU-E, BFU-E and CFU-GM colonies after acute in vivo exposure to AFB1 (single i.p. administration 0.3, 1, 3 mg/kg b.w.), the toxin neither causes overt toxicity on erythroid lineage nor induces significant myelotoxic effects at any dose (data not shown).

Table 2 reports the results of the haematological parameters measured 48 h after treatment. There were no significant alterations of erythrocyte and leucocyte counts in that the values were within the physiological range at any utilized dose. Similarly, at sacrifice, no significant body weight gain was registered (Fig. 3); furthermore no observable effects were seen in weight of liver, spleen and thymus (Fig. 3). There were no changes in the liver-to-body weight ratio for any of the treatment as also deductible from Fig. 3 (data not shown).

4. Discussion

The present study clearly evidences the in vitro toxic effects of aflatoxins, namely AFB1 and AFM1, on myeloid and erythroid human and murine committed progenitors. In particular, comparing in vitro human and mice data, both toxins (i) displayed similar dose-dependent toxic effects on both species stem cells, with a stronger sensitivity of the human with respect to the murine BM progenitors, and (ii) affected more markedly the myeloid and the immature erythroid lineage irrespectively of the animal species (human or murine).

Regarding our in vitro ex-vivo findings, they seemed to demonstrate that a single i.p. administration of AFB1 (0.3, 1 and 3 mg/kg b.w.) did not cause overt toxicity on erythroid lineage nor induces significant myelotoxic effects at any utilized dose, and furthermore no significant alterations of haematological parameters were observed 48 h after the treatment.

Previous in vivo data clearly demonstrated the granulopoietic toxicity of AFB1 in 5–6 week-old male Fischer rats given single i.p. injections of 1 mg/kg (=1/5 of LD50) or 0.1 mg/kg (=1/50 of LD50), followed by a successive rescue reaction (Cukrova et al., Table 2

Blood parameters and leucocyte formula of the CD1 mice at the day of sacrifice, 48 h after acute in vivo treatment with AFB1 (0.3, 1 and 3 mg/kg b.w.). Data are expressed as the mean ± SD of a single experiment (n = 6 per experimental group) performed in triplicate.

<table>
<thead>
<tr>
<th>AFB1 dose (mg/kg b.w.)</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes (10⁶ µl)</td>
<td>5.17 ± 1.77</td>
<td>6.77 ± 0.53</td>
<td>6.11 ± 2.24</td>
<td>6.58 ± 2.30</td>
</tr>
<tr>
<td>Leukocytes (10⁹ µl)</td>
<td>7.08 ± 4.07</td>
<td>5.68 ± 1.75</td>
<td>4.93 ± 2.43</td>
<td>6.13 ± 1.88</td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td>9.28 ± 2.02</td>
<td>11.50 ± 2.99</td>
<td>10.47 ± 3.58</td>
<td>11.37 ± 3.07</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>29.40 ± 7.99</td>
<td>36.00 ± 10.58</td>
<td>31.17 ± 11.84</td>
<td>34.50 ± 11.55</td>
</tr>
<tr>
<td>Leucocyte formula:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>17.00 ± 10.39</td>
<td>24.40 ± 10.53</td>
<td>31.00 ± 11.83</td>
<td>36.00 ± 8.85</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>58.40 ± 7.99</td>
<td>66.60 ± 8.17</td>
<td>61.33 ± 13.13</td>
<td>53.67 ± 2.94</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.67 ± 0.52</td>
<td>0.80 ± 0.84</td>
<td>2.33 ± 2.16</td>
<td>1.67 ± 1.37</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.17 ± 0.41</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td><strong>Physiological ranges</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes (10⁶ µl)</td>
<td>5.17 ± 1.77</td>
<td>6.77 ± 0.53</td>
<td>6.11 ± 2.24</td>
<td>6.58 ± 2.30</td>
</tr>
<tr>
<td>Leukocytes (10⁹ µl)</td>
<td>7.08 ± 4.07</td>
<td>5.68 ± 1.75</td>
<td>4.93 ± 2.43</td>
<td>6.13 ± 1.88</td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td>9.28 ± 2.02</td>
<td>11.50 ± 2.99</td>
<td>10.47 ± 3.58</td>
<td>11.37 ± 3.07</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>29.40 ± 7.99</td>
<td>36.00 ± 10.58</td>
<td>31.17 ± 11.84</td>
<td>34.50 ± 11.55</td>
</tr>
<tr>
<td>Neutrophils (%)</td>
<td>17.00 ± 10.39</td>
<td>24.40 ± 10.53</td>
<td>31.00 ± 11.83</td>
<td>36.00 ± 8.85</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>58.40 ± 7.99</td>
<td>66.60 ± 8.17</td>
<td>61.33 ± 13.13</td>
<td>53.67 ± 2.94</td>
</tr>
<tr>
<td>Eosinophils (%)</td>
<td>0.67 ± 0.52</td>
<td>0.80 ± 0.84</td>
<td>2.33 ± 2.16</td>
<td>1.67 ± 1.37</td>
</tr>
<tr>
<td>Basophils (%)</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.17 ± 0.41</td>
<td>0.00 ± 0.00</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>AFB1 dose (mg/kg)</th>
<th>0</th>
<th>0.3</th>
<th>1</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Histograms showing body weight gain and different organ weight of the CD1 mice exposed to different doses of AFB1 (0.3, 1 and 3 mg/kg b.w.) at the day of sacrifice (48 h after the single i.p. injection). Data are expressed as the mean ± SD of a single experiment (n = 6 per experimental group) performed in triplicate.
ity for the AFB1-8,9 epoxide, giving them a pronounced detoxifica-

constitutive levels of a hepatic alpha class of GST, with a high affin-

ments is in agreement with several data available in literature.

dent (0.1, 0.5, 1, 5, 10

The lack of myelotoxicity found in our investigation may be as-
scribed at first to the short time of exposure to the toxin in vivo (48 h, until the sacrifice); as previously stated, alterations in gran-
ulopoiesis have been reported in mice after a two-week-repeated
AFB1 treatment (Dugyala et al., 1994). Furthermore, the different
species (rats vs. mice), the route of administration (i.p. vs. orally/
gavage), the duration of the treatment (single vs. repeated doses),
and the evaluation of different endpoints (myelotoxicity vs. geno-
toxicity/mutagenicity) may explain the different rate of suscepti-
bility reported in the above mentioned literature and our data.

Furthermore, it should be also mentioned that, notwithstanding the broad similarities in aflatoxin biotransformation across species, there are some key species differences in rate of metabolism, partic-

ularly regarding the affinity for and the catalytic activity of the main
enzymes involved, particularly glutathione S-transferase (GSTs).
GSTs are the main biosynthetic enzymes involved in AFB1-exo-8,9
epoxide conjugation representing the main detoxification pathway.
The order of GSH conjugation to AFB1 among species is mouse >
rat > human, with humans exhibiting comparatively low conjuga-
tion, and, consequently a higher susceptibility (Raney et al., 1992;
Kirbi et al., 1993, 1994). Additionally, several in vivo studies, using different rodent species, further showed the lowest susceptibility of
mice, evaluated by the determination of AFB1-DNA levels and
AFB1-albumin adducts (Wild et al., 1996; The Efsa Journal, 2007).

Indeed, our in vivo findings seem to be in line with the above ex-
plain marked resistance of mice to AFB1, probably due to their high
costituitive levels of a hepatic alpha class of GST, with a high affini-

ity for the AFB1-8,9 epoxide, giving them a pronounced detoxifica-
tion power.

Conversely to the in vitro data, our in vitro investigations in mice assessed that both AFB1 and AFM1 caused a dose-dependent inhi-

bition of the growth of erythroid and myeloid committed prog-
nitors, affecting more markedly the myeloid (CFU-GM) and the
immature erythroid lineage (BFU-E) than the more mature CFU-E
colonies.

The trend of toxicity observed in our murine in vitro experi-
mens is in agreement with several data available in literature. Cukrova et al. (1991) demonstrated in vitro a concentration-depend-
ent (0.1, 0.5, 1, 5, 10 μg AFB1/ml) suppression of granulopoiesis according to the results of the CFU-GM assay, with the concentra-
tions of 5 μg/ml and 0.5 μg/ml being these two doses highly and
slightly suppressive, respectively. BM cells from control CD-1 mice
cultured in vitro with 1–50 μM AFB1 also displayed a suppression of
all the three types of colonies (Dugyala et al., 1994).

Evaluating our murine data for AFB1 toxicity, obtained with the
two different experimental approaches (in vitro vs. in vivo), the
in vitro IC50 data (Table 1) underscore the moderate sensitivity of
murine cells, with special reference to CFU-GM and CFU-E, in line
with the poor susceptibility of mice to AFB1 myelotoxic effects in
vivo. As above hypothesized, the differences among in vivo and
in vitro data may be ascribed to the short time of in vivo exposure
to the toxin; noteworthy, in in vivo experiments the myeloid and
erythroid committed progenitors were continuously exposed to

AFB1 throughout all the culturing period until the day of scoring
(from minimum 3 to maximum 10 days).

In human cultures, the inhibition of proliferation of erythroid and
myeloid colonies displayed a dose-dependent toxicity similar but,
markedly, more pronounced to that observed in murine cells.

In particular, the specific IC50 values for each type of stem cells
were about 4 times lower in humans than in mice.

Moreover, AFB1 IC50 values clearly demonstrated that the mye-
loid progenitors (CFU-GM) and the immature erythroid progeni-
tors (BFU-E) were about 5 and 10 times respectively more
sensitive than the respective more mature CFU-E to AFB1; a similar
trend of toxicity was induced by AFM1 in all human colony types,
as clearly deductible from IC50 values.

Notably, our in vitro findings regarding AFM1, in both human and
murine cells, seem to be in line with previous literature data,
showing that, similarly to AFB1, the conjugation of AFM1-epoxide
with reduced GSH, representing the main detoxification pathway,
is catalyzed by mouse but not human liver cytosol (Neal et al., 1998).

Taken together, our in vitro findings could be probably ex-
plained by (i) the lack of a detoxification pathway in both murine
and human myeloid and erythroid progenitors cultured in vitro; (ii)
the poor resistance of human cells to AFB1 and AFM1, probably due
to the scarce detoxication pathway typical of human biotransfor-
mation metabolism of these toxins.

An overall evaluation of our findings clearly indicate (i) the need
of an integrated study strategy to rightly evaluate the toxic poten-
tial of a toxin, taking into careful consideration the species-related differencies and (ii) the need of caution in designating the meta-

bolic conversion of AFB1 to AFM1 as an essentially detoxification
process.
due to contamination risks of these products by aflatoxins (2006/504/EC).


