Induction of Changes in Morphology, Reactive Nitrogen/Oxygen Intermediates and Apoptosis of Duck Macrophages by Aflatoxin B_1

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ABSTRACT : The purpose of this study was to investigate the effects of aflatoxin B_1 (AFB_1) on the ultracellular morphology alteration, apoptosis induction and reactive nitrogen and oxygen intermediates production of peritoneal macrophages (DPM) from mule ducks. The ducklings were purchased from a commercial hatchery, and were fed a corn-soybean based diet. As the ducklings were grown up to 3 wk of age, the Sephadex-elicited peritoneal exudative cells (PEC) were used as the source for duck peritoneal macrophages. The ultracellular morphology study showed that significant number of cells shifted from category I (normal cell with ruffled membrane) and II (cell membrane blebbing) to category III (cell membrane blebbing and even rupture) after DPM were incubated with AFB_1 (20 μg/ml) for 12 to 48 h. When DPM were exposed to AFB_1 *in vitro*, the production of NO, H_2O_2 and O_2^- in macrophages was reduced after 12-48 h incubation with previous LPS stimulation. There was a DNA laddering pattern observed in DPM incubated with AFB_1 5, 10, 20, 50 or 100 μg/ml for 12 h. Evidence also revealed that the percentage of apoptotic cells was increased along with the elevation of AFB_1 concentration. The results suggest that AFB_1 exposure causes duck macrophages going on apoptotic pathway through evidence of ultracellular morphology alteration and DNA laddering in agarose electrophoresis. The production of reactive nitrogen and oxygen intermediates of duck macrophages also depressed after AFB_1 exposure, and this implied that AFB_1 could cause deteriorated functions of bacteriocidal and tumoricidal activity in duck macrophages. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 11 : 1639-1645)***

Key Words : Aflatoxin B_1, Apoptosis, Reactive Nitrogen/Oxygen Intermediates, Macrophages

INTRODUCTION

Aflatoxin B_1 (AFB_1) is a secondary metabolite of fungus, *Aspergillus flavus* existing in feedstuff, and may induce hepatic tumor and some types of cancers (Robens and Richard, 1992). In addition, AFB_1 has been reported to impair immune responses (Corrier, 1991). For example, AFB_1 exposure decreases the clearance ability of colloidal carbon particle in the reticulo-endothelial system (Michael et al., 1973). Chemotaxic and phagocytic activity of blood monocytes are also impaired in chicken fed diets containing aflatoxin (Chang and Hamilton, 1979). Viable percentage and functions of chicken macrophages are decreased and alterations of cellular morphology including cell surface blebbing and nuclear disintegration are induced during aflatoxicosis (Neldon-Ortiz and Qureshi, 1991; 1992). Suppressed production of NO and H_2O_2 are obtained in murine peritoneal macrophages by AFB_1 pretreatment, resulting in reducing antitumor activities (Moon et al., 1999a).

Macrophages are responsible for presenting the antigen and for serving as supportive accessory cells to lymphocytes during the development of specific immunity. When stimulated by lipopolysaccharide, macrophages can be activated to tumoricidal or microbiocidal state and can release a variety of products (Nathan, 1987). Chicken macrophages also express some kinds of immune functions, such as phagocytosis of foreign particles and tumor cell destruction (Qureshi et al., 1986; Cheng et al., 2002). Moreover, macrophages could secrete prostaglandin and cytokines to modulate the activity of lymphocytes and other macrophages. (Kimball, 1990). Research has been reported that nitric oxide, superoxide anion and hydrogen peroxide are produced in activated macrophages, and involved in the killing of microorganisms as well as a function of tumoristatic mechanisms (Adams and Nathan, 1983; Hibbs et al., 1988).

Two distinct mechanisms for cell death have been identified (Wyllie et al., 1980). One is necrosis, which is caused by catastrophic toxic or traumatic event and therefore the process is passive, unregulated, and aleatory. The other mechanism is apoptosis or programmed cell death, which is an active process of cellular self-destruction with unique defining morphological and molecular characteristics (Kerr et al., 1972; Wyllie et al., 1980). Morphologically, cells undergoing apoptosis will have nuclear and cytoplasmic condensation, followed by cell fragmentation. When nuclear DNA of apoptotic cells was examined, nucleotides of 180 to 200 bp were obtained (Gerschenson and Rotello, 1992). In another study, apoptosis was induced in HL-60 cells after exposure to AFB_1 (10 μg/ml) for 24 h (Ueno et al., 1995).

There was limited research on the induction of

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apoptosis in macrophages by AFB_1 exposure. Therefore, we examined the effects of AFB_1 on the alteration in ultracellular morphology, apoptosis induction and production of reactive intermediates in duck peritoneal macrophages.

MATERIALS AND METHODS

AFB_1 preparation

AFB_1 used in this study was obtained from pure crystal AFB_1 (Sigma, St. Louis, MO). In preparation of the reagent for assay, AFB_1 was first dissolved in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) to a concentration of 1 mg/ml as a stock solution. The AFB_1 stock solution was then covered with aluminum foil and stored in -20°C for later use. The final concentration of DMSO was 0.05% (w/v). Possible contamination with lipopolysaccharides (LPS) (Sigma, St. Louis, MO) was examined by *Limulus* assay (Sigma, St. Louis, MO). All AFB_1 preparations were found to be negative in LPS by *Limulus* assay.

Preparation of duck peritoneal macrophages (DPM)

Duck peritoneal macrophages were collected from 3-wk-old mule ducks. The ducklings were purchased from commercial hatchery and were fed a corn-soybean based diet containing 18.5% crude protein and 2,890 kcal ME/kg according to "Duck Nutrient Requirement Manual" compiled by National Taiwan University (Shen, 1988). During feeding period, feed and water were provided *ad libitum*. As the ducklings were grown up to 3 wk of age, the Sephadex-elicited peritoneal exudative cells (PEC) were used as the source of duck peritoneal macrophages. The procedure for the preparation of PEC was as previously described by Trembicki et al. (1984). Briefly, a 3% swollen Sephadex G-50 suspension in sterile saline (0.75%) was injected intraperitoneally into each duck at 1 ml per 100 g body weight. Approximately 42 h after injection, the ducks were sacrificed by cervical dislocation and PEC was collected by flushing out the peritoneal exudates with 30 ml per bird of cold saline containing 0.5 U/ml of heparin. The PEC suspension was kept on ice in siliconized tubes for about 15 min to allow any residual sephadex beads to settle. Supernatants containing cells were then decanted to new siliconized tubes. Cells were then centrifuged at 285×g for 15 min at 4°C. The resulting pellets of PEC were resuspended in 2 ml of complete medium consisting of RPMI-1640 (Gibco laboratories, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco laboratories, Grand Island, NY), and 2 mM L-glutamine, 100 U penicillin /ml, and 50 μg streptomycin /ml (all purchased from Sigma, St. Louis, MO). The number of viable PEC was determined by trypan blue exclusion on a hemocytometer and the cell concentration was adjusted to 1×10^6 viable DPM/ml for the assays. The other cells were stored in precooled medium containing 60% RPMI-1640, 20% FBS, and 20% DMSO in a concentration of 2×10^7 /ml and kept in liquid nitrogen until assay was performed.

Treatment of DPM with AFB_1

For the assay, DPM were quickly thawed in a water bath at 37°C and washed twice with RPMI-1640 medium. The cell viability and differential counts were re-evaluated and the cell concentration was adjusted to 1×10^6 cell/ml. DPM were then dispensed into 12-well culture plate (Costar Products, Cambridge, MA), and each well contained a sterile 15 mm round coverslip (Assistent, Bayerm, Germany). The cells were incubated at 41°C in an incubator containing 5% CO_2 for 2 h to form monolayers. The nonadherent cells were removed by washing twice with Dulbecco's phosphate buffer saline (DPBS) (Gibco Laboratories, Grand Island, NY). Thereafter, DPM were incubated with AFB_1 at concentrations of 0, 5, 10, 20, 50 or 100 μg /ml for 12 h. DNA was isolated from DPM and then separated by agarose gel electrophoresis for detecting of possible apoptosis. In another parallel experiment, DPM were treated with AFB_1 20 μg /ml for 2, 6, 12, 24 and 48 h, and quantitative assay of apoptotic cells and observation of ultracellular morphology by scanning electron microscope (SEM) were performed.

Examination by scanning electron microscopy

At each designated time of treatments, DPM were fixed in 2.5% glutaraldehyde (Electron Microscope Sciences, Washington, PA) in 0.15 M DPBS for 30 min. They were then post-fixed in 1% (w/v) osmium tetroxide (Electron Microscope Sciences, Washington, PA) in 0.15 M DPBS for another 30 min, dehydrated in graded concentration of ethanol (35% v/v, 50%, 70%, 85%, 90%, 100%, 100%, and 100%) and dried in a critical-point drying apparatus (Tousimis Research Corp., Rockville, MD). The cells were coated with gold-palladium in a sputter coater (SPI Supplies Inc., West Chester, PA) and examined under a scanning electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 5 or 10 kv. A total of 300 randomly selected DPM were examined on each coverslip preparation and the percentages of cells of various categories were calculated. In order to quantify the differences between AFB_1-exposed and control group, DPM were divided into three categories based on morphological characteristics. In category I, cells have ruffled membrane surface with many long, radiating, finger-like pseudopodia (Figure 1A); in category II, cells are rounding with scattered short and thin pseudopodia and small blebs (Figure 1B); in category III, cells are rounding and surface blebbing with occasional rupture of the cell membrane (Figure 1C).

1A. Category I: cells with ruffled membrane surface with many long, radiating, finger-like pseudopodia.

1B. Category II: cells rounding with scattered short and thin pseudopodia and small blebs.

1C. Category III: cells rounding and surface blebbing with occasional rupture of the cell membrane.

Figure 1. Morphological categories of duck peritoneal macrophages after exposure to AFB_1 20 μg/ml for 2-48 h.

Reactive nitrogen/oxygen intermediates determination

For reactive nitrogen/oxygen intermediates (nitric oxide, superoxide anion, and hydrogen peroxide) determination, DPM were seeded at a concentration of 1×10^6cell/ml on 96-well tissue culture plates (Costar Products, Cambridge, MA). Non-adherent cells were removed after 2 h at 41°C by washing twice with DPBS. After treated with AFB_1 0 or 20 μg/ml in phenol red-free RPMI 1640 medium with LPS (1 μg/ml) for 48 h. After macrophages were stimulated with LPS, the accumulation of NO in culture supernatant was measured. In the measurement, 100 μl of supernatant was removed from each well into an empty 96-well plate, and mixed with an equal volume of Griess reagent (one part 0.1% naphthylethylenediamine dihydrochloride to one part 1% sulfanilamide in 5% phosphoric acid) as described by Green et al. (1982). After 10 min incubation at room temperature, the presence of NO was quantified by reading the plates at A540 on an ELISA reader (Bio-Tek, Winooski, VT). NO concentration was calculated from a $NaNO_2$ standard curve.

Production of superoxide anion was measured by the reduction of ferricytochrome-c by superoxide (Pick and Mizel, 1981). Briefly, macrophage monolayers were covered by 100 μl per well of 160 μM solution of ferricytochrome-c in phenol red-free RPMI 1640 medium and treated with LPS (1 μg/ml). The plate was placed in a 41°C, 5%CO_2 humidified incubator for the desired length of time. The plate was then transferred to ELISA reader and the absorbance was measured at 550 nm. The amount of superoxide anion produced per well was calculated from the extinction coefficient and expressed by the formula:

$$\text{nmoles superoxide anion per well} = (\text{absorbance at 550 nm} \times 100)/6.3.$$

Hydrogen peroxide production was measured by the procedures described by Pick and Keisari (1980). At each time point, medium was removed from 96-well plates and 100 μl phenol red-free RPMI 1640 medium, 50 μl LPS (1 μg/ml), 25 μl phenol red (1.2 mg/ml) and 25 μl horseradish peroxidase (100 unit/ml) were then added (all except RPMI 1640 purchased from Sigma, St. Louis, MO). After incubation for 3 h at 41°C in 5% CO_2 incubator for the desired length of time, the absorbance of each well was measured at 610 nm in an ELISA reader.

DNA isolation and gel electrophoresis

DPM in a 12-well plate were lysed with 400 μl digestion buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA in 0.5% SDS), and incubated overnight at 37°C with proteinase K (20 μg/ml). Samples were extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) solution and total DNA contained in the aqueous phase was precipitated with 1/10 volume 3 M sodium acetate, pH 5.2, and 2 volumes cold 100% ethanol at 4°C for 30 min. DNA pellets were obtained by centrifugation (13,000×g, 10 min), washed twice each with 70% and 100% ethanol, air dried, and then resuspended in 50 μl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and nucleic acid concentration was measured at 260 nm. Electrophoresis was performed on a 2.5%agarose gel, containing 0.5 μg/ml ethidium bromide. A 100 bp DNA ladder (GIBCO-BRL, Gaithersburg, MD) was included as a size marker.

Quantitation of apoptotic cells

Cells undergoing apoptosis would lose phospholipid asymmetry on its membrane and expose phosphotidylserine on the outer leaflet of the plasma membrane. The detection of exposed phosphotidylserine by Annexin V has been shown to be a general and early marker of apoptosis (Vermes et al., 1995). Cells were then stained with propidium iodide which can be used to distinguish necrosis from apoptosis by fluorescence microscopy, in which apoptosis shows green color and necrotic cells show yellowish-green color. In this assay, an Annexin-V-FLUOS staining kit (Boehringer Mannheim GmbH, Berlin) was used to detect the apoptotic cells. After DPM monolayer

was exposed to AFB_1 in 12-well plates and washed twice with DPBS, 100 μl stain solution (20 μl Annexin-V labeled solution mixed with 1,000 μl Herpes buffer and 20 μl propidium iodide) was added to the plate, covered with coverslips and allowed to react for 10-15 min at room temperature. The coverslips were observed under fluorescence microscope with 200 cells counted per coverslip. The cells stained with green color were judged as apoptotic, and the results were given as means of three experiments.

Statistical analysis

The tau-b test and analysis of variance (ANOVA) (SAS Institute, 1988) were used for the analysis of the differences in morphological data between AFB_1-exposed and control groups. Reactive intermediates data were from three independent experiments and presented as means±SD. Results were analyzed using Student's t-test. AFB_1-exposed groups were compared with the control group to obtain the significance of difference (* $p<0.05$; ** $p<0.01$).

RESULTS

Ultracellular morphology alterations

SEM revealed an apparent morphological variation in the surface structure of DPM. For quantitation of the changes of morphology, three categories were used to characterize the features which were shown in Figure 1A, 1B and 1C. Although minor changes in percentages in the three morphological categories occurred in the control group during the whole incubation time (Table 1), there was a significant decrease in the percentage of cells in category II, and a significant increase in the percentage of cells in category III as the incubation time prolonged. In the AFB_1-exposured groups, categories II and III changed the most. The differences started to appear at 6 h of incubation and became significant by 12 h. When the incubation period was extended to 48 h, almost all of the DPM were shifted to category III in the AFB_1-exposed group. Although there were 40-60% of the DPM in control group with surface blebs through the study, the severity was much less apparent than that in AFB_1-exposed group.

Reactive nitrogen/oxygen intermediates

The effects of AFB_1 on the production of reactive nitrogen and oxygen intermediates were examined. The production of NO in DPM was reduced to about 89%, 80%, 56% and 46% of the control group for those groups exposed to AFB_1 for 6, 12, 24 and 48 h, respectively (Figure 2A). The significant differences were noticed in 12 to 48 h incubation. As for O_2^-production in DPM, it was suppressed to about 82%, 81%, 64% and 56% of the control group for those groups exposed to AFB_1 for 6, 12, 24 and 48 h, respectively (Figure 2B). The significant differences were observed in 12 to 48 h of incubation. H_2O_2 production in DPM was reduced to about 97%, 91%, 79% and 81% of the control group for those groups exposed to AFB_1 for 6, 12, 24 and 48 h, respectively (Figure 2C). There were also significant differences in 12 to 48 h incubation.

DNA agarose gel electrophoresis

In order to identify the possible cell apoptosis, the DNA fragmentation phenomenon may serve as an evidence. DNA from the control DPM did not show laddering (Figure 3, CTRL). After DPM were exposed to different concentrations of AFB_1 (5, 10, 20, 50 or 100 μg/ml) for 12 h, DNA fragmentation identified as ladder phenomenon was present (Figure 3, lane 1-5), indicating the appearance of apoptosis.

Quantitation of apoptotic cells

The percentages of apoptosis of DPM after exposure with different concentrations of AFB_1 (0, 5, 10, 20, 50 or 100 μg/ml) for 12 h, were shown in Figure 4. The results indicated that the percentage of apoptosis of DPM was increased linearly with the elevation of AFB_1 dosage. There were 4.0, 9.6, 34.5, 52.8, 61.2 and 86.7% increases,

Table 1. The alteration of ultracellular morphology in duck peritoneal macrophages after exposure to AFB_1[a]

Time after AFB_1 exposure (hr)	Category (%)					
	Control DPM			DPM exposed to AFB_1 20 μg/ml		
	I	II	III	I	II	III
2	3.2±0.5	45.5±1.0	51.3±1.3	3.4±0.5	45.0±1.5	51.6±1.2
6	3.2±0.3	43.8±1.6	53.0±1.7	4.1±0.7	40.1±6.4	56.8±6.8
12[b]	4.0±0.2	41.7±3.1	54.3±3.0	2.1±0.5	25.5±4.9	72.4±5.0
24[b]	4.4±0.5	37.1±3.2	58.5±3.0	1.8±0.5	6.2±1.8	92.0±1.7
48[b]	4.8±0.7	35.0±4.3	60.2±4.7	0.4±0.3	0.8±0.3	98.8±0.6

[a] A total of 200 randomly selected DPM were examined on each coverslip preparation for each time hour after AFB_1 (20 μg/ml) exposure and the mean percentages of cell of various categories were calculated. Data expressed are means of three independent experiments and standard deviation, n=9 per time interval.

[b] Highly significant difference between control group and AFB_1 exposed group ($p<0.01$).

Category I: cells with ruffled membrane surface and with many long, radiating, finger-like pseudopodia; category II: cell rounding with scattered short and thin pseudopodia and small blebs; category III: cell rounding and surface blebbing with occasional rupture of the cell membrane.

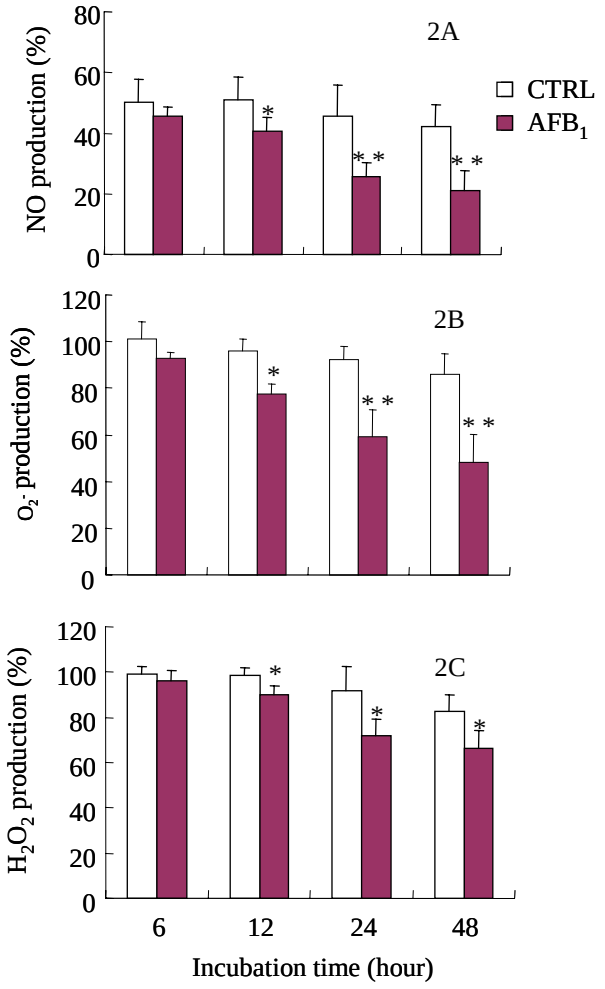

Figure 2. Effect of AFB$_1$ exposure for different time on NO (2A), O_2^- (2B) and H_2O_2 (2C) production by macrophages *in vitro*. Peritoneal macrophages were exposed to AFB$_1$ (20 μg/ml) and stimulated by LPS (1 μg/ml) for 48 h. At different time of AFB$_1$ exposure, NO, O_2^- and H_2O_2 production were measured at OD 540 nm, 550 nm and 610 nm, respectivey. The percentage of reactive nitrogen and oxygen intermediate production were obtained by comparing with that of control group (CTRL). Data presented as means±SD of 3 different experiments, * $p<0.05$; ** $p<0.01$.

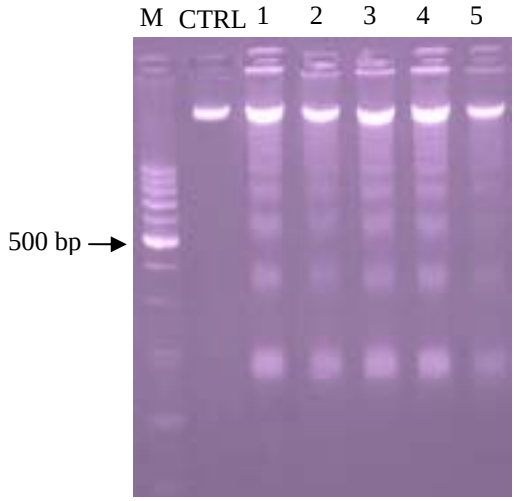

Figure 3. The DNA ladder phenomenon of duck peritoneal macrophages was produced after exposure with different concentrations of AFB$_1$.
M: 100 bp ladder marker, CTRL: control, Lane 1-5 presented AFB$_1$ 5, 10, 20, 50 and 100 μg/ml treatment for 12 h, respectively.

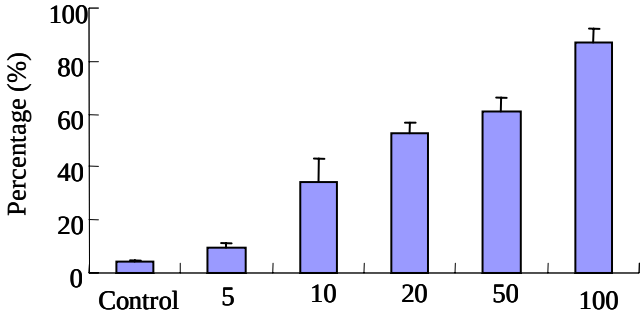

Figure 4. The percentages of apoptosis of duck peritoneal macrophages after exposure to different concentrations of AFB$_1$ at 0 (control), 5, 10, 20, 50 and 100 μg/ml for 12 h. Duck peritoneal macrophages were stained with Annexin-V-FLUOS staining kit, and cells with green color under fluorescence microscope were recognized as apoptotic cells. The percentages of apoptosis were means of triple experiments. Data presented as means±SD.

respectively, in the order of dosage. The highest interval increase was 25.5% when dosage of AFB$_1$ was elevated from 50 μg /ml to 100 μg/ml.

DISCUSSION

Macrophages play an important role in defending the invaded pathogen by chemotaxis, phagocytosis and antigen presentation mechanism; any factors, therefore, affecting the functional integrity of macrophages might increase the disease susceptibility of host. AFB$_1$ has been known as a hepatocarcinogen and is also reported to impair the immune system. When lymphocytes were directly treated with AFB$_1$, cell proliferation and antibody formation were decreased (Corrier, 1991). When AFB$_1$ was administered to animals, its active metabolites can damage liver by forming adduct with DNA (Robens and Richard, 1992), and cause impairment of the efficacy of mononuclear phagocytic system to inhibit cytokine production and to decrease macrophage adherence ability (Neldon-Ortiz and Qureshi, 1992). However, the mechanism by which AFB$_1$ exerts these effects on macrophages is not fully understood. Our data showed that AFB$_1$ could inhibit the production of reactive nitrogen/oxygen intermediates, these effects were correlated with macrophage dysfunctions reported by other researchers (Moon et al., 1999a). We also suggest that

apoptosis of macrophages induced by AFB_1 exposure may play a vital mechanism in immune suppression on macrophages.

In studies on macrophages, Nathan (1987) indicated that some active products released from macrophages served as the nonspecific primary defense against foreign infectious agents. Neldon-Ortiz and Qureshi (1991) also showed that AFB_1 might reduce the function of macrophages.

When duck peritoneal macrophages were exposed to AFB_1 *in vitro*, the production of NO, H_2O_2 and O_2^- in macrophages was reduced during 12-48 h incubation after LPS stimulation. Our results showed that the suppressive effect of AFB_1 on the production of reactive intermediates was time-dependent and an accumulative manner was also revealed. In mammals, mice was exposed *in vivo* on AFB_1, the production of NO, H_2O_2 and O_2^- in macrophages was reduced (Moon et al., 1999a).

Macrophages produce NO by oxidizing the guanidino nitrogen of L-arginine by nitric oxide synthetase (Marletta et al., 1988). Being a very short-lived radical, NO is converted to more stable products such as nitrite and nitrate (Stuehr and Marletta, 1985). Evidence from several fields of research has suggested the involvement of this pathway in the tumoristatic and microstatic effector mechanisms of macrophages (Adams et al., 1991; Suk et al., 1993). The report that AFB_1 decreases transcription and translation of nitric oxide synthetase in macrophages after *in vitro* pretreatment with AFB_1 suggests that AFB_1 may suppress the production of NO, H_2O_2 and O_2^- in an irreversible manner (Moon et al., 1999b). Furthermore, a critical role of H_2O_2 and O_2^- in tumor lysis has been reported (Adams and Nathan, 1983). O_2^- is subsequently converted to H_2O_2 by either spontaneous or enzyme-mediated dismutation (Root and Metcalf, 1977). Our results suggest that the tumoricidal and bacteriocidal activity of peritoneal macrophages may be impaired after exposure to AFB_1.

The DNA laddering phenomenon observed in this study and collaborating the result of morphological alteration reveals that apoptosis is occurred more actively in macrophages after AFB_1 exposure. Based on these results, it led to speculate that AFB_1 may impair the defense ability of macrophages. AFB_1 may turn on apoptosis pathway by signal transduction or through its mediated metabolite, such as 2,3 epoxide. The reports showed that AFB_1 might induce mutation of Ki-ras gene in hepatocarcinogenesis and at codon 249 of the p53 tumor suppressor gene (Soman and Wogan, 1993; Nose et al., 1993). It was also reported that AFB_1 might increase phosphatidyl metabolism resulting in an increase in intracellular signal transduction (Mistry et al., 1995). However, the results by Liu et al. (2002) showed that aflatoxin did not induce the apoptosis in swine alveolar macrophages. It is possible that different cell types or animals species may have different response to the toxic effects of AFB_1. Our results obtained with the Annexin-V-FLUOS staining method suggested that the number of cells exhibiting DNA fragmentation was much greater in the presence of AFB_1 as compared with controls. We also observed a close relationship between assessment of apoptosis by SEM and Annexin -V-FLUOS staining.

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