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Aflatoxin B1 - induced inhibition of nitric oxide synthesis in macrophage and influence of its terminal furan ring in immunosuppressive activity

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Nitric Oxide (NO) synthesis and intracellular killing of *Candida albicans* by rat peritoneal macrophages exposed to AFB1 were studied. Aflatoxin B1 significantly inhibited Nitric Oxide (NO) production and microbiocidal activity by macrophages even in the presence of activating signals such as interferon - γ and lipopolysaccharide. Also, AFB1 with modification done at its terminal furan ring was evaluated for such activities. This toxin, with its terminal furan ring converted to ozonite by addition of ozone at the 2, 3-unsaturated carbon-carbon double bond, could not suppress the production of NO and killing of phagocytosed microbes by the macrophages indicating that immunosuppressive activity of this mycotoxin may be attributable to its terminal furan ring.

INTRODUCTION

Aflatoxin B1 (AFB1), a difurocoumarocyclopentanone, is a potent carcinogenic and immunotoxic secondary metabolite produced by *Aspergillus flavus* and *A.parasiticus* in the contaminated food (Moss 1991). One of the most serious threats that this toxin poses is the suppression of cell-mediated immunity (Hayes 1980). Recently increased incidence of immuno-suppression in AFB1 - exposed population has generated a flurry of research activity in the field related to mechanism of its immunotoxicity in order to develop new clinical medicine. Also, we have observed a significant impairment of phagocytic potential of macrophages exposed to AFB1 (Chatterjee & Mukherjee, 1993). Moreover, we found that some AFB1-treated macrophages that were still somehow able to retain their phagocytic potential, could not, however, kill the intracellular phagocytosed microbes. This finding has also been corroborated by Cusumano *et.al* (1990). Also, structure - activity relationship of AFB1 for immunosuppression has not hitherto been reproted. The lactone portion of coumarin ring system of this toxin, which has been implicated for carcinogenecity is, however, not the functional feature to express immunosuppressive property. Hence, drug development against AFB1 -induced immunosuppression remains elusive and a hectic research in this direction is certainly called for. It is well established that immunological stimuli such as interferon - γ (IFN - γ) and lipopolysaccharide (LPS) transmit signals to macrophage nucleus activating the latter to express cytokine inducible nitric oxide synthase (i NOS) which catalyses the synthesis of high concentration of NO from L-arginine and from molecular oxygen (Nathan and Xie, 1994). NO thus produced, kills tumor cells, bacteria and fungi (Liew and Cox 1991). Hence, impaired intracellular killing of microbes by macrophages exposed to AFB1 may plausibly be due to the inhibition of the expression of iNOS with the resultant suppression of NO production. However no work has yet been done in this direction. Also, it is of interest to investigate the role of its terminal furan ring having 2,3 - carbon-carbon double bond on the activity of toxin, as well as the impact of structural modification at this furan ring. In this communication, we report for the first time that AFB1 impairs NO synthesis and intracellular killing in macrophages, and the terminal furan ring of AFB1 may be one of the functional features to express such immunosuppressive activity.

MATERIALS AND METHODS

Serum

Serum was taken from blood samples of three-month-old rats (Norvegicus strain)

Yeast cell suspension

Cell suspension of *Candida albicans* was made in RPMI 1640 medium and was opsonized for 30 mins.

at 37°C with homologous serum. The final concentration of yeast cells was adjusted to 1.4×10^6 ml^{-1}.

Macrophage collection

Peritoneal cells were collected by flushing the peritoneal cavity of rats with 0.5 U ml^{-1} sterile heparin-0.85% saline solution. Paritoneal cells were repeatedly washed, centrifuged at 1500 rev min^{-1} at 10° C for 20 min and the cell pellets were resuspended in RPMI 1640 medium supplemented with 5% fetal calf serum at a concentration of 1×10^6 ml^{-1}.

NO and intracellular killing

Peritoneal cells suspension was allowed to adh ..o tissue culture-type petridishes for 1 h at 37° C in a 5% CO_2 atmosphere. Cells were then washed with medium to remove non-adherent cells. The adherent mono-layer cells were resuspended in fresh medium activated with INF-γ-(40 units ml^{-1}) and LPS (10 ng ml^{-1}) (Sigma Co.) and incubated in a 5% CO_2 atmosphere at 37° C. In parallel experiments, N^G - monomethyl L-arginine (L-NMMA), an inhibitor of iNOS, was added to the culture at the concentration of 500 μ M. After 24 h, macrophages were suspended in yeast cell suspension of *C. albicans* in serum and incubated in a humidified 5% CO_2 atmosphere for 1 h at 37° C. Then the supernatant, in triplicate, was removed for the assay of NO_2 (an indicator of NO production) by the Griess reaction (Stauehr and Nathan, 1989). In brief, 50 ml culture samples were combined in plate with a 1/1 mixture of 1% sulfanilamide in 2.5% H_3PO_4 and 0.1% naphthylethlenediamide in 2.5% H_3PO_4. Plates were incubated at room temperature for 10 min and absorbance was determined at 550 nm. NO_2 concentrations were measured in triplicate using a standard curve of sodium nitrite from 125 to 1 μM prepared in culture medium.

Macrophages on the plate were stained with 15 ppm acridine orange solution and phagocytizing macrophages and intracellular killing were observed under UV light following Cusumanao *et.al* (1990). The killed *C. albicans* cells stained reddish yellow whereas viable *C. albicans* and the cell nucleus stained green. A differential count of macrophages containing *C. albicans* was performed, and the intracellular killing activity was expressed as the percentage of positive cells containing dead *C. albicans*. Experiments were conducted in triplicate by randomly observing 400 macrophages in each slide.

Aflatoxin B1 exposure

Macrophages were plated out as described above and after washing, the adherent cells were resuspended in the fresh RPMI 1640 medium. To test the effects of AFB1 on NO synthesis and intracellular killing by peritoneal macrophages, AFB1 (Sigma Co.) in the concentrations of 0.0001, 0.001, 0.01 μg ml^{-1} (which are much lower than the cytotoxic dose) (Chatterjee and Mukherjee, 1993) was added to the macrophages in medium 24 h before, and 24 h after the addition of IFN-γ (40 units ml^{-1}) and LPS (10 ng ml^{-1}) as well as the same time as IFN-γ and LPS. The culture was incubated in a 5% CO_2 atmosphere at 37°C. Then macrophages were suspended in yeast-cell suspension, incubated and the intracellular killing and NO production were determined as described above.

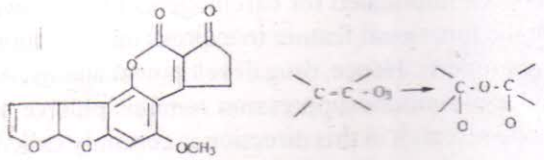
Cytotoxicity test

Viable cell counts were made using Trypan blue exclusion.

Modification at furan ring

Ozone (1.2 mg/l), at the flow rate of 40 mL/min at room temperature was made to react with 5 ml of 50 ppm AFB1 (Sigma Co.). Then the terminal furan ring becomes converted to ozonite by the addition of ozone at 2,3-unsaturated carbon-carbon double bond (Scheme 1). After such modification, AFB1 was evaluated for its immunosuppressive activity by asseying NO production by Griess reaction (Stauehr and Nathan, 1989) and testing intracellular killing following Cusumano *et al.* (1990).

Scheme 1



Aflatoxin B1

RESULTS AND DISCUSSION

Peritoneal macrophages were able to synthesize NO and kill intracellular *C. albicans* when activated with IFN - γ and LPS. Nevertheless, NO systhesis and microbiocidal activity were both inhibited in the presence of L-NMMA, an inhibitor of iNOS, which indicates that the intracellular killing of *C. albicans* by macrophages is mediated by NO (Table 1).

Table 1 : NO production and intracellular killing by rat peritioneal macrophages

Activating Signals	NO production* (μM)	Intracellular killing* (% Phagocytizing macrophages containing dead *C. albicans*)
IFN - γ + LPS	36.5[a]	62[a]
IFN - γ + LPS + L - NMMA	3.0[b]	4[b]

*Values with different superscripts in each vertical column are significantly different. (P>0.01).

However, AFB1, when added to the culture 24 h before IFN - γ and LPS, significantly inhibited the synthesis of NO and intracellular killing of *C. albicans* by macrophages even in the presence of the activating signals such as IFN-γ and LPS (Table 2). It appears that AFB1 affected the macrophages in such a way that the latter either did not receive or could not act upon the activating signals. Also, it is possible that AFB1 may affect the signalling pathways. The inhibition was dose-dependent and was not due to any cytotoxic effects since the viability of the cells treated with or without AFB1 were almost similar (Data not shown).

In contrast, AFB1 could not inhibit the macrophages from getting activated and producing NO as well as from NO-mediated intracellular killing of *C. albicans* when it was applied to the culture 24 h after IFN- γ and LPS (Table 2). This may be due to the possibility that the macrophages had already received or acted upon the signals (IFN-γ and LPS) much before AFB1 could exert its action on the macrophages.

However, the toxin with its terminal furan ring converted to ozonite and 2, 3-C=C double bond eliminated, could not suppress the production of NO and killing of phagocytosed *C.albicans* (Table 3). The observed depressive effects of AFB1 on the microbiocidal activity through NO production impairment and this finding that immunosuppressive activity of AFB1 may be attributable to the terminal furan ring of the compound have not hitherto been reported. Thus the present work gives important new insight into the structure-activity relationship of this toxin for inhibiting nitric oxide production as well as intracellular killing by macrophages, which would help

Table 2 : Efficacy of AFB1 in suppressing NO production and intracellular killing

AFB1 Conc (μg/mL)	Application Time	% Viable macrophage*	Nitric Oxide (μM)*	Intracellular Killing *, +
0.0001		78[a]	21.5[a]	29[a]
0.001	24 h before	80[a]	12.7[b]	22[b]
0.01	IFN - γ + LPS	82[a]	3.0[c]	12[c]
0.0001	At the same	80[a]	18.0[a]	28[a]
0.001	time as	77[a]	10.1[b]	20[b]
0.01	IFN - γ + LPS	79[a]	4.4[c]	12[c]
0.0001		81[a]	34.6[d]	59[d]
0.001	24 h after	80[a]	32.8[d]	66[d]
0.01	IFN - γ + LPS	78[a]	34.0[d]	64[d]

* Values are means of 5 replicates. Values with different superscripts in each vertical column are significantly different (P>0.01). +. % phagocytizing macrophages containing dead *C.albicans*.

Table 3 : Influence of modification of terminal furan ring of AFB1 on NO production and intracellular killing

AFB1with furan ring modified Conc (μg/mL)	Application Time	Nitric Oxide (μM)*	Intracellular Killing *, +
0.0001	24 h before	37.0[a]	65[a]
0.001	IFN - γ + LPS	35.2[b]	63[b]
0.01		38.0	64[a]
0.0001	At the same	36.0[a]	66[a]
0.001	time as	34.2[a]	62[a]
0.01	IFN - γ + LPS	34.0[a]	64[a]
0.0001	24 h after	35.0[a]	65[a]
0.001	IFN - γ + LPS	37.4[a]	65[a]
0.01		37.0[a]	62[a]

* Values are means of 5 replicates. Values with different superscripts in each vertical column are significantly different (P>0.01). +, % phagocytizing macrophages containing dead *C.albicans*.

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