Effect of Aflatoxin B_1 upon Phytohemagglutinin-Transformed Human Lymphocytes[1] (34954)

HERBERT SAVEL,[2] BEN FORSYTH, WARREN SCHAEFFER, AND THOMAS CARDELLA

Departments of Internal Medicine and Medical Microbiology, University of Vermont College of Medicine, Burlington, Vermont 05401

The aflatoxins are a group of closely related toxic metabolites produced by certain strains of the mold *Aspergillus flavus*. Since the report that an outbreak of toxicity in poultry was due to contamination of peanut meal with aflatoxin (1), there has been a growing literature concerning the toxic (2), carcinogenic (3, 4) and teratogenic (5) effects of aflatoxin and its most abundant and potent component, aflatoxin B_1 (6).

Carcinogens have been observed to depress immune mechanisms (7, 8), especially the cellular immunity which is responsible for homograft rejection (9). Stutman (10) has reported a correlation between the sensitivity of various strains of mice to tumor development with 3-methylocholanthrene and immune depression by the carcinogen. Since lymphocyte transformation is considered to be a manifestation of lymphocyte or cellular immunity (11), it was decided to investigate the effects of aflatoxin B_1 on *in vitro* transformation of human peripheral blood lymphocytes by phytohemagglutinin and specific antigens.

Materials and Methods. Peripheral blood samples were obtained from patient volunteers at the Medical Center Hospital of Vermont who were not receiving corticosteroids or tranquilizers and who were not seriously ill. After the venipuncture, peripheral blood lymphocyte cultures were established by the method which has been described previously (12). In brief, the bulk of the polymorphonuclear leukocytes and monocytes was removed by adherence to soft glass and the lymphocyte-enriched buffy coat washed repeatedly to remove autologous plasma. The final leukocyte concentration was 40% to 70% small lymphocytes, and 10^6 lymphocytes/ml were suspended in 3 ml of medium (Minimal Essential Medium-Spinner) containing 20% fetal calf serum, 50 μg/ml of streptomycin, and 160 units/ml of penicillin. Penicillin was omitted from the cultures if the patients gave any history of definite or possible penicillin allergy.

Aflatoxin B_1, which was generously supplied by Dr. Gerald N. Wogan of the Massachusetts Institute of Technology, was dissolved in dimethyl sulfoxide (DMSO) (Fisher Scientific Company) at a concentration of 1.6 μg/ml filtered through a 0.45 sterile filter unit (Nalgene), and added to duplicate test cultures with a final DMSO concentration of 3%. Control cultures with and without DMSO were also established which indicated no toxicity of DMSO at this concentration. Phytohemagglutinin-M (Difco), 0.1 ml, was added to induce lymphocyte transformation. Purified PPD, which was supplied by the Parke-Davis Company, was added to PPD test cultures at a concentration of 10 μg/ml. Test cultures to live mumps antigen were established by adding 0.1 ml of mumps antigen prepared from the Enders strain of mumps, received from the Research Reference Reagents Branch, NIAID, NIH, and passed three additional times in rhesus monkey kidney.

The cultures were incubated with 50 μCi of tritiated thymidine (1.9 Ci/mmole) from days 5 to 6 of culture and harvested by the

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[2] Teaching and Research Scholar of the American College of Physicians.

TABLE I. Inhibition of Tritiated Thymidine Uptake (%) by Phytohemagglutinin-Stimulated Human Peripheral Blood Lymphocytes.[a]

Patient no.	Age	Sex	Diagnosis	Aflatoxin B_1 (μg/ml) 200 ($6.8 \times 10^{-4}M$)	50 ($1.7 \times 10^{-4}M$)	25 ($9 \times 10^{-5}M$)	8 ($3 \times 10^{-5}M$)	5 ($1.7 \times 10^{-5}M$)	0.5 ($1.7 \times 10^{-6}M$)	0.1 ($3 \times 10^{-7}M$)
1	25	F	Phlebitis	—	95	—	—	—	—	—
2	30	F	Brain tumor	—	—	—	93	66	—	0
3	32	F	Chronic bronchitis	—	90	—	—	63	0	—
4	57	F	Breast cancer	—	—	87	82	—	—	—
5	69	F	Breast cancer	80	—	—	—	—	—	—
6	18	M	Diabetes mellitus	—	93	—	—	—	25	—
7	28	M	Volunteer	—	—	—	—	63	7	—
8	43	M	Chronic bronchitis	—	95	—	—	85	—	—
9	45	M	Cholecystitis	—	55	—	—	28	26	—
10	51	M	Chronic bronchitis	—	45	—	—	0	0	—
11	52	M	Volunteer	—	79	—	—	21	—	—
12	53	M	Volunteer	—	91	—	—	—	—	—
13	62	M	Laryngeal cancer	—	—	—	—	48	—	12
14	70	M	Myocardial infarction	—	—	—	—	41	40	—
15	73	M	Myocardial infarction	—	—	—	—	76	34	—

[a] Human peripheral blood lymphocytes (3×10^6) in 3 ml MEM-spinner containing 20% fetal calf serum, penicillin 160 units/ml, and streptomycin 50 μg/ml and 0.1 ml PHA M (Difco), with 0.1 ml of tritiated thymidine (1 mCi/ml, 1.9 Ci/mmole) added day 5, cultures harvested day 6.

previously described Millipore membrane technique (12). The Millipore membranes were placed in scintillation fluid, counted in a liquid-scintillation counter, and the results expressed as the total counts per minute of tritiated thymidine incorporated in each culture of 3×10^6 lymphocytes. The uptake of tritiated thymidine was utilized as a measure of the degree of lymphocyte stimulation because of the greater objectivity and sensitivity of this technique as compared with the simple determination of blast percentage (13).

Results. The addition of aflatoxin B_1 to phytohemagglutinin-stimulated cultures of human peripheral blood lymphocytes resulted in an inhibition of tritiated thymidine uptake as compared to control cultures without aflatoxin B_1 (see Table I). A significant degree of inhibition was observed with aflatoxin concentrations of 5 μg/ml (1.7×10^{-5} *M*) and a lesser degree of inhibition was still observed with 0.5 μg/ml (1.7×10^{-6} *M*). The addition of 3% DMSO vehicle to similar phytohemagglutinin-stimulated lymphocyte cultures had no effect upon tritiated thymidine uptake. In a patient who was tuberculin positive, it was observed that incubation with 50 μg/ml of aflatoxin B_1 produced 62% inhibition of the increased uptake of tritiated thymidine in the PPD-exposed lymphocyte cultures. Aflatoxin B_1 at a concentration of 50 μg/ml was similarly observed to completely suppress the increased uptake of tritiated thymidine produced by mumps antigen in a patient who was mumps-sensitive. Slides prepared from duplicate cultures of phytohemagglutinin-stimulated human peripheral blood lymphocytes, which were stained with Wright–Giemsa stain, revealed that the phytohemagglutinin-exposed lymphocytes which were incubated with aflatoxin B_1 at a concentration of 50 μg/ml appear to have transformed normally to immunoblasts.

Cultures were incubated with phytohemagglutinin and aflatoxin B_1 (50 μg/ml), and the cells were then washed and placed in new media containing only phytohemagglutitin after 1, 2, 4, and 20 hr of incubation. In two such experiments, it was observed that washing the cells after 1, 2, or 4 hr incubation with aflatoxin B_1 and placing the cells in new media containing only phytohemagglutinin prevented the inhibition of tritiated thymidine uptake which was observed in the unwashed cultures. However, washing of the cells and removal of the aflatoxin B_1 after 20 hr of incubation produced a degree of inhibition identical to that observed in the unwashed cultures containing aflatoxin B_1.

Discussion. The present study indicates that the extremely potent carcinogen aflatoxin B_1 is able to inhibit the stimulated uptake of tritiated thymidine by phytohemagglutinin in human peripheral blood lymphocytes, and to decrease the stimulation induced by potent antigens—in this case, tuberculin and mumps. The molar concentrations of aflatoxin B_1 that produced a significant degree of inhibition appear to be smaller than was reported for other immunosuppressant drugs (14). Many of the commonly used carcinogenic agents have been observed to produce inhibitory effects on numerous components of the immune system (7, 8), especially an inhibition of delayed or cellular type immunity (9), and it appears that aflatoxin B_1 may have a similar spectrum of action. Aflatoxin B_1 is believed to act by inhibition of DNA synthesis (15, 16), an effect detectable within the first few hours after exposure of human embryonic lung fibroblasts to aflatoxin (17). Aflatoxin B_1 is believed to act by combining with the DNA and thus preventing DNA, RNA, and protein synthesis (18). The present study indicates that the inhibitory effect of aflatoxin B_1 on tritiated thymidine uptake of human lymphocytes can be reversed if the cells are washed and replaced in aflatoxin B_1-free media after up to 4 hr incubation with the drug. However, after 20 hr incubation, the aflatoxin B_1 appears to be bound to the cell, and repeated washing does not reverse the aflatoxin B_1 inhibitory effect upon tritated thymidine uptake between days 5 and 6. Another possible explanation is that at 4 hr the aflatoxin B_1 has not yet entered the cells.

Aflatoxin B_1 has been observed to produce effects on human Chang liver cells in tissue

culture (19) and on human embryonic lung fibroblasts (17). The present data suggest that the human peripheral blood lymphocytes are as sensitive to the effects of aflatoxin B_1 as are the human embryonic liver cells, and that the aflatoxin B_1 appears to block a pathway common to both antigenic and phytohemagglutinin stimulation. Studies are in progress to determine whether there are any specific morphologic alterations in the aflatoxin B_1-exposed lymphocytes. Studies on the possible systemic immunosuppressant effects of aflatoxin B_1 in animals appear to be warranted by the present observations.

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