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Evaluation of Citrinin Toxicity on the Immune Functions of Mice[1]

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ABSTRACT

Citrinin, a nephrotoxic fungal metabolite produced by several species of *Penicillium* and *Aspergillus,* has been found to contaminate foods used by humans and animals. The present study investigated potential effects of this compound on the immune system. Male CD-1 mice received 0, 0.12, 0.6 or 3.0 mg of citrinin/kg i.p. every other day for 2-4 weeks. Food consumption and body or organ weights were not affected but kidneys were enlarged. Splenic cells from mice exposed to citrinin for 2 or 4 weeks were cultured with or without the mitogens, phytohemagglutinin (PHA), pokeweed mitogen (PWM) or lipopolysaccharide (LPS). Exposure to citrinin stimulated splenic lymphocyte proliferation. Antibody production by splenic cells in animals sensitized to sheep red blood cells (SRBC) increased in the two highest dose groups. Delayed hypersensitivity reaction, measured as a foot-pad swelling, in response to SRBC sensitization and subsequent challenge were not affected by citrinin treatment. In vitro addition of citrinin ($>1\times10^{-5}M$) to splenic lymphocytes was cytotoxic. These findings suggest that citrinin mildly stimulates the immune system but does not have consistent immunotoxic effects at the doses tested.

Citrinin, a secondary fungal metabolite produced by several species of *Penicillium* and *Aspergillus,* has been found to contaminate foods and feeds. Citrinin was considered as a potential antibiotic *(24),* but its toxic properties prevented its therapeutic use. Citrinin is nephrotoxic and implicated in disease outbreaks in both animals and humans. Acutely lethal doses administered to rabbits, guinea pigs, rats, swine, or mice caused swelling of the kidneys with eventual necrosis *(1,12,15,17,18).* In mice, citrinin is also embryocidal and fetotoxic *(13).* In rats, citrinin has similar effects and high doses are teratogenic *(25,26).*

Citrinin has been implicated in porcine and avian nephropathies and possibly in the fatal renal disease in humans, causing endemic Balkan nephropathy *(12,19,30).* The nephropathy of citrinin in rats was characterized by an enhanced excretion of dilute urine, glucosuria, proteinuria, and reduced glomerular filtration rate and renal blood flow *(3,22).* The mechanisms underlying the toxicity produced by citrinin are not clear.

The countries in which mycotoxin porcine nephropathy has been reported include Denmark *(16,21),* Norway and Sweden *(15)* and Ireland *(6).* Low levels of citrinin consumption may contribute to economic losses in the livestock and poultry industry by affecting weight gain and resistance to disease. The present investigation evaluated effects of low repeated exposures of citrinin on the immune functions of mice.

MATERIALS AND METHODS

Citrinin was prepared in Dr. Berndt's Laboratory (Dept. of Pharmacology, University of Mississippi Medical Center, Jackson, MS) from liquid cultures of *Penicillium citrinum* (NRRL 1842) grown in a 5% sucrose-2% yeast extract, liquid medium. Citrinin was isolated by methods reported by Davis et al. *(10).* The estimated purity of citrinin, measured by melting point, HPLC retention characteristics, and thin layer chromatography *(22),* exceeded 98%.

Citrinin was dissolved in 5% (w/v) sodium bicarbonate just before administration, and delivered in volumes of 0.1 ml/10 g of body weight. The final concentrations of citrinin in solutions were 0.012, 0.06 or 0.3 mg/ml, corresponding to the three treatment levels indicated below.

Experimental design

Male CD-1 mice weighing 23-25 g, from Charles River (Wilmington, MA), were acclimated for 10 d in a temperature-controlled (22 ± 2°C) and artificially illuminated room (12-h light cycle) free from known sources of toxic contaminants. Mycotoxin-free pelleted feed (Wayne Blox®, Allied Mills, Chicago, IL) and fresh water were available ad libitum; food consumption was measured at weekly intervals. Mice were divided into four groups and housed five per cage. After acclimation the mice received either 0, 0.12, 0.6 or 3.0 mg of citrinin/kg of body weight intraperitonially every other day for 2-4 weeks. Body weights were measured every other day throughout the experimental period. At the end of 2 or 4 weeks, the animals were decapitated, and selected organs collected and weighed. Blood was collected into heparinized tubes for red and white blood cell counts and smears were prepared for differential cell counts.

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Lymphocyte cultures

Groups of mice, five animals per dose, were killed after 2 and 4 weeks of administration of citrinin. Spleens were collected into ice cold sterilized physiological saline solution (PSS), washed and mashed repeatedly with forceps to release their cellular components. Splenocytes were separated as described by Sharma and Gehring *(27)*. Lymphocyte blastogenesis was determined in culture by a method described previously *(27)*. The culture medium was RPMI 1640 (Gibco, Grand Island, N.Y.) containing 10% heat-inactivated fetal calf serum, 100 units of penicillin and 100 μg of streptomycin per ml. A 50-μl portion of splenocytes (8×10^6 cells/ml) was layered into each well of a micro tissue culture plate (Microtest II, Falcon 3040, Falcon plastics, Oxnard, CA). To each well 50 μl of additional medium and 50 μl of medium with or without an optimized concentration of mitogen (in triplicate) were added. The mitogens used were bacterial lipopolysaccharide (LPS) and phytohemagglutinin (PHA), (Sigma Chemical Co.), and pokeweed mitogen (PWM, Gibco) in concentrations of 33.3, 1.67, and 33.3 μg/ml, respectively. The plates were incubated for 52 h at 37°C in a humidified air containing 5% CO_2. After incubation, 50 μl of medium containing 0.5 μCi of ^{3}H-thymidine with a specific activity of 2 Ci/m mole, (New England Nuclear, Boston, MA) was added to each well and incubation continued for an additional 16 h. The cells were harvested and washed with PSS using a model M 12 cell harvester (Brandel, Rockville, MD) and collected on glass fiber disks (Whatman, grade 934AH, Clifton, NJ). The disks were placed directly into counting vials, dried, 5 ml of scintillation solution was added, and radioactivity was counted in a scintillation spectrometer (Tri-carb, Model 2660, Packard Instruments Co., Downers Grove, IL). The net dpm were calculated per 10^6 cells.

Splenic cell cultures after in vitro addition of citrinin

Splenic cells from untreated animals were collected and cultured as above, with or without addition of the same concentrations of mitogens described above. At the beginning of cultures, various concentrations of citrinin were added (see Results). Cells were harvested and the radioactivity was counted as mentioned above.

Antibody producing cells

Groups of mice, five at each dose, were sensitized intraperitonially with 0.25 ml of a 20% suspension of sheep red blood cells (SRBC) following a 2-week treatment with citrinin. The mice were killed 4 d later to determine the primary antibody response. Splenic cells were isolated, counted, and the number of plaque-forming cells (PFC) was determined according to the method of Cunningham and Szenberg using microchambers *(9,20)*. A mixture containing 45% splenic cells, 45% of 10% suspension of SRBC, and 10% pooled guinea pig complement was delivered to the chambers and incubated at 37°C for 45 min. Plaques were counted and expressed as plaque-forming cells per 10^6 splenic lymphocytes.

Cell-mediated immune response

After treatment with citrinin for 2 weeks, groups of mice, five at each dose, were sensitized intraperitonially with 0.25 ml of a 10% suspension of SRBC. Five days later, mice were rechallenged with 20 μl of 10% suspension of SRBC in the right hind foot-pad; the left pad received the same amount of PSS and served as a control. Seventeen hours after the challenge, ^{125}I-labeled bovine serum albumin, prepared as described by Boone et al. *(5)*, was injected intraperitonially. The mice were killed 2 h later; test and control feet were cut off at the junction of the lower and middle thirds of the tibia, and radioactivity was counted in gamma spectrometer (model 3320, Packard Instruments Co., Downers Grove, IL). Extravasation of ^{125}I-bovine serum albumin into the challenged and control foot-pads was used to evaluate the delayed-type hypersensitivity (DTH). The foot count radio was determined by dividing the counts of the test foot by the counts of the control foot.

Statistical methods

The results are expressed as the mean and standard error of mean. Data from these studies were analyzed by one way analysis of variance (ANOVA) followed by Fisher's Least Significant Difference test. All statistical calculations utilized the computer program Minitab (Minitab, Inc., University Park, PA) and were carried out on the Utah State University VAX/VMS-11 computer system.

RESULTS

In mice, repeated exposure of citrinin for 2 or 4 weeks did not affect food consumption (data not shown) or body weight gain (Table 1) at the doses tested. There were no treatment-related effects on liver, thymus, or spleen weights; however, kidneys of mice receiving the highest dose were enlarged after the 2-week treatment (Table 1).

Citrinin exposure after 2 weeks caused a significant decrease in peripheral leucocytes in the group receiving the highest dose (Table 2). The differential counts indicated that this change was due to a decrease in lymphocytes. The leucocyte counts did not differ after 4 weeks of treatment nor were lymphocytes or neutrophils affected. Red cell counts were not affected by treatment at any time (data not shown).

Figure 1 illustrates how treatment with citrinin for 2 weeks affected the uptake of ^{3}H-thymidine by splenic cells. Exposure of mice to citrinin stimulated lymphocyte blastogenesis in splenic cultures with or without mitogens at all doses tested; a similar trend was observed with each of the three mitogens. The effect of 4 weeks of citrinin exposure on lymphocyte blastogenesis is shown in Fig. 2; responses were similar to those following exposure for 2 weeks. Uptake of ^{3}H-thymidine increased in splenic cells from citrinin-exposed animals. Antibody production by splenic cells from citrinin-treated animals is shown in Table 3. Spleens of animals challenged with SRBC were slightly larger in groups treated with citrinin. Primary antibody production was depressed in the group treated with the lowest dose but was stimulated at higher doses. There were no significant differences in delayed-type hypersensitivity response between treatments (data not shown).

Addition of citrinin directly to cultures from spleens of untreated animals did not increase blast formation as was observed following in vivo treatments. Representative results are indicated in Table 4. At higher concentrations of citrinin in culture, a cytotoxic effect was apparent in all cultures whether or not mitogens were present.

TABLE 1. *Selected organ and body weights of mice exosed to citrinin.*[a]

Dose (mg/kg body weight)	Body weight	Organ weights, g/100 g body weight			
		Liver	Kidney	Thymus	Spleen
2 Weeks					
0	34.4 ± 1.5	5.37 ± 0.13	1.54 ± 0.06	0.16 ± 0.01	0.29 ± 0.01
0.12	34.3 ± 0.7	5.12 ± 0.20	1.57 ± 0.07	0.20 ± 0.03	0.31 ± 0.01
0.6	34.5 ± 0.2	5.28 ± 0.17	1.54 ± 0.08	0.18 ± 0.01	0.31 ± 0.02
3	34.2 ± 1.1	5.70 ± 0.17	1.70 ± 0.06*	0.19 ± 0.02	0.31 ± 0.01
4 Weeks					
0	35.1 ± 0.3	4.40 ± 0.07	1.38 ± 0.04	0.14 ± 0.01	0.34 ± 0.02
0.12	38.0 ± 0.9	5.35 ± 0.08*	1.43 ± 0.07	0.14 ± 0.01	0.30 ± 0.02
0.6	37.3 ± 1.2	4.64 ± 0.20	1.48 ± 0.05	0.13 ± 0.01	0.31 ± 0.02
3	35.5 ± 0.8	5.09 ± 0.30	1.67 ± 0.13	0.14 ± 0.02	0.35 ± 0.01

[a]Values are given as mean ± S.E.M., n = 5.
*Significantly different from control value ($p<0.05$).

TABLE 2. *Effect of citrinin exposure of periphral leucocyte count.*[a]

Dose (mg/kg body weight)	WBC $\times 10^{-3}/\mu l$	Percent of WBC		
		Lymphocytes	Neutrophils	Monocytes
2 Weeks				
0	6.93 ± 1.17	75.2 ± 3.9	23.2 ± 2.8	1.0 ± 0.4
0.12	4.56 ± 0.42	74.0 ± 3.6	23.6 ± 3.0	2.2 ± 0.4
0.6	3.58 ± 0.39*	65.0 ± 7.2	33.0 ± 7.0	1.5 ± 0.5
3	3.65 ± 0.20*	54.6 ± 6.5*	44.0 ± 6.3	1.4 ± 0.5
4 Weeks				
0	5.30 ± 0.70	78.2 ± 3.9	20.5 ± 4.6	1.0 ± 0.6
0.12	3.46 ± 0.46	84.7 ± 4.1	13.0 ± 4.0	1.5 ± 0.5
0.6	6.06 ± 0.67	76.6 ± 5.2	23.2 ± 5.4	---
3	6.34 ± 0.91	72.2 ± 4.2	27.6 ± 4.1	---

[a]Values are given as Mean ± S.E.M., n = 5.
*Significantly different from control values ($p<0.05$).

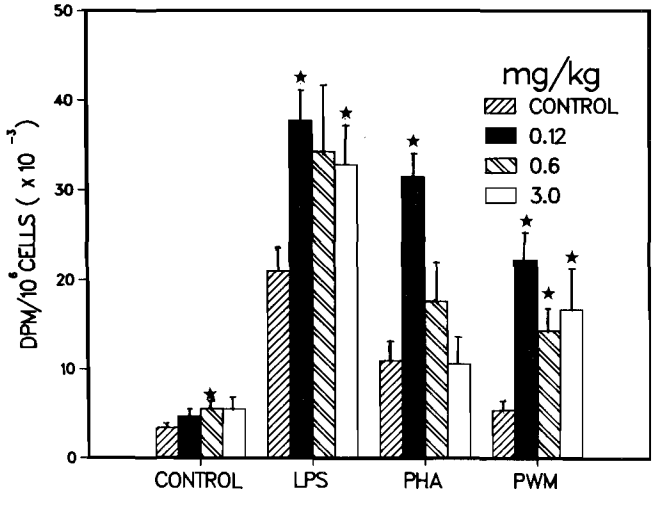

Figure 1. *Effect of 2-week citrinin exposure on the uptake of ^{3}H-TdR by splenic lymphocyte alone (control) or in the presence of LPS PHA, or PWM. Mitogen concentrations have been indicated in the text. Error bars denote S.E.M., n=5. *Significantly different from control $p<0.05$.*

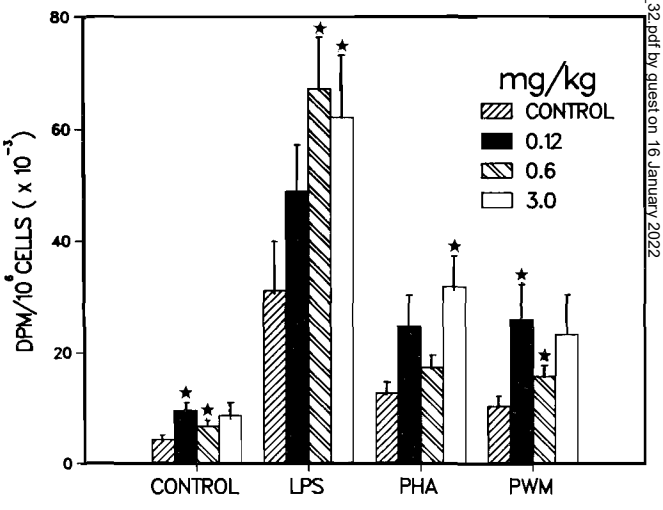

Figure 2. *Effect of 4-week citrinin exposure on the uptake of ^{3}H-TdR by splenic lymphocyte alone (control) or in the presence of LPS, PHA or PWM. Optimized mitogen concentrations were 33.3, 33.3 and 1.67 μg/ml for LPS, PWM and PHA, respectively. Error bars denote S.E.M., n=5. *Significantly different from control $p<0.05$.*

TABLE 3. *Effect of citrinin on the antibody response[a] to sheep red-blood cells.*

Dose (mg/kg/body weight)	Spleen weight (g/100 g b.w.)	Plaque forming cells[b]
0	0.44 ± 0.04	898 ± 38
0.12	0.53 ± 0.03	458 ± 64*
0.6	0.56 ± 0.03	1280 ± 70*
3	0.56 ± 0.03	1317 ± 188

[a]Values are given as mean ± S.E.M., n = 5.
[b]Plaques/10^6 splenic lymphocytes.
*Significantly different from control value ($p<0.05$).

DISCUSSION

Low and repeated exposure to citrinin for 2 or 4 weeks had no effect on the weights of primary or secondary immune organs of mice. It also did not affect liver, body weight gain or food consumption but did cause an enlargement of the kidneys, a target organ for citrinin toxicity *(3,4,7,23)*. Exposure to citrinin for 2 weeks resulted in a dose-related decrease in peripheral leucocyte counts, with a decrease in lymphocytes; however, the peripheral leucocyte counts were normal after 4 weeks of citrinin exposure. The initial effects on parameters observed after a 2-week exposure may be due to inflammatory responses to citrinin *(1,12)*.

Lymphocyte blastogenesis indicated that citrinin did not suppress DNA synthesis; rather, all dose levels tested stimulated the lymphocyte proliferation. Uptake of ^{3}H-thymidine was greater in splenic cells from citrinin-exposed animals that had been cultured with a lipopolysaccharide from *Escherichia coli,* which exerts a non-specific stimulatory effect on murine B cells *(2)*. A similar type of stimulation was seen with cultures treated with the plant lectins, PHA and PWM, which stimulate thymus-dependent cells and also B lymphocytes, respectively *(28)*. Results were similar after 2 or 4 weeks of citrinin treatments.

Plaque formation study indicated antibody-producing activity was stimulated by citrinin. Results of the plaque-forming cells agree with those confirming stimulatory effect of citrinin on lymphocytes, as indicated by proliferation of lymphocytes. The reason for a decreased number of PFC at the lowest dose level of citrinin is not apparent. The delayed-type hypersensitivity response indicated that citrinin had no effect on this cell-mediated immune function within the dose levels tested.

These results indicated that citrinin stimulated the humoral immunity of mice within the dose ranges evaluated, but since citrinin toxicity varies with the species *(14)*, these results may not be applicable to other species. The failure to stimulate delayed hypersensitivity may be due to the insensitivity of the assay system employed, since PHA-induced lymphocyte proliferation suggested a stimulatory effect on T-cell function. Although the effects on the immune system were non-specific, it is not clear if these were related to the nephrotoxic effect of citrinin.

There is considerable information on drugs that are used clinically to alter immune function, but few reports concern environmental contaminants, especially mycotoxins. Some mycotoxins like aflatoxins, *Fusarium* toxins and ochratoxin A *(8,11,29)* are immunosuppressive, but the effects of the other commonly occurring mycotoxins on immune system have not been evaluated. Results of this study indicate that low repeated exposures to citrinin do not depress the immune response in mice. Stimulation of immune responses by citrinin suggests the need for further studies. The immunologic stimulation after in vivo administration of citrinin may not be a direct effect of this chemical since it caused cytotoxity in vitro; however, a metabolite or alterations of immune functions subsequent to organ damage may have a role. In any event, citrinin does not appear to possess considerable immunotoxic properties.

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TABLE 4. *Effect of in vitro citrinin exposure on lymphocyte blastogenesis.*[a]

	DPM per 10^6 cells			
Conc. (*M*)	No mitogen (DPM × 10^{-3})	LPS (DPM × 10^{-3})	PHA (DPM × 10^{-3})	PWM (DPM × 10^{-3})
Control	5.3 ± 0.6	47.0 ± 10.0	20.6 ± 1.0	15.8 ± 1.1
1×10^{-5}	4.8 ± 1.0	49.7 ± 9.2	22.9 ± 2.2	15.6 ± 2.3
2.5×10^{-5}	3.4 ± 0.1*	38.7 ± 8.2	22.9 ± 1.8	12.1 ± 1.6
5×10^{-5}	3.3 ± 0.3*	5.0 ± 1.3*	6.7 ± 0.7*	3.4 ± 0.4*
7.5×10^{-5}	3.5 ± 1.0	4.4 ± 0.4*	2.2 ± 0.3*	2.5 ± 0.4*
10^{-4}	2.6 ± 0.6*	2.7 ± 0.3*	1.8 ± 0.2*	2.4 ± 0.6*

[a]Values are given as mean ± S.E.M. (n = 5, cells from each animal cultured in triplicate). Values at citrinin concentrations of lower than 10^{-5} *M* showed no effect at any condition and have not been shown here.
*Significantly different from control values ($p<0.05$).

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