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DETECTION OF KOJIC ACID DURING ASPERGILLOSIS *

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INTRODUCTION

Aspergillosis in man and lower animals is caused by several species of the genus Aspergillus. The most common a etiologic agent of aspergillosis and systemic human disease is A. fumigatus. It also ranks as a major pathogen of lower animals, particularly birds (Austwick, 1965). Members of the A. flavus-oryzae group are less frequently involved in pulmonary aspergillosis (Emmons et al, 1970). other aspergilli that have been encountered as pathogens in man and lower animals are A. glaucus, A. restrictus, A. nidulans, A. terreus, A. niger (Emmons et al, (1970). Mice treated with cortisone alone or cortisone and antibiotic were shown to be highly susceptible to fatal pulmonary aspergillosis after inhalation of spores of A. flavus (Sidransky and Friedman, 1972). These animals were exposed to three concentrations of sprayed spores and retained approximately 3.6x10, 6.0x10 or 2.4x10 viable spores per left lungs. At the high level of spores retained mortality was 88%.

With current increased usage of antibiotics and adrenocortical steroids and immunosuppressants in transplants, the incidence of aspergillosis has appreciably increased (Rifkind et al, 1967). Moreover, aspergillosis has been reported in debilitated patients whose immune mechanisms were modified by severe alcohaolism, advanced pulmonary tuberculosis, hepatitis, diabetes or addiction to heroin (Visudhiphan et al, 1973).

It has been reported that some pathogenic aspergilli produce both exotoxins and endotoxins when growing in living tissues and that these substances may influence their pathogenic potential (Austwick, 1965). For example, kojic acid, produced in vitro by A. flavus and many other aspergilli including A. fumigatus (Prescott and Dunn, 1940), was found to cause epileptic type convulsions in animals (Werch et al, 1957). It has not been ascertained whether kojic acid is formed in vivo during aspergillosis.

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The definitive proof of production of this microbial toxin during aspergillosis in vivo would require a highly sensitive analytical method. We have reported (Abdalla and Grant, 1980) the development of an enzyme-linked immunosorbent assay (ELISA) with which small amounts of kojic acid can be detected in biological fluids. This detection method was used in the present study.

MATERIAL AND METHODS

Organism:

Aspergillus flavus-parasiticus, ATCC 15517, was used for infection of rats. it produced kojic acid when grown in a modified Czapek-Dox medium (Bently, 1957). Spores of this organism were produced on Moyer's sporulation medium (Raper and Fennel, 1965) and harvested with sterile sand. The sandspore suspension was dispensed into sterile 13x75 mm screwcap tubes to serve as inocula. The spores in one tube were suspended in 0.15 M sodium chloride solution containing 0.001% Tween 80(v/v). This suspension comprised the inoculum for sporulation medium in plates. The seeded plates were incubated at 28°C for four days. The spores were then harvested in sterile 0.15 M sodium chloride with Tween 80. The number of spores per ml was determined before inoculation of experimental animals.

Experimental infection of rats

In this experiment, three groups of ten day old male and female Wistar rats were given two subcutaneous injections of 10 mg prednisone (Sigma Chemical Company) suspended in 0.7 ml of 0.05 M sodium carbonate, pH 7.5. There were four rats in each group. The injections were given 3 days apart and each animal was weighed thereafter. Two groups showed slight weight gain. They were infected with the spores of A. flavus - parasiticus ATCC 15517. Each animal received 0.5 ml of the spore suspension, at a concentration of approximately 1 X 10 spores per ml, intraperitoneally and a third injection of the immunosuppressant. The control group received only prednisone. Twenty-four hours later, the three groups of rats were transferred to metabolic cages and put under observation. An animal in one infected group died 48 hours after infection. The remaining three became morbid and were exsanguinated 80 hours after infection.

In the second group, two animals died 48 hours after infection; two became morbid and were exsanguinated 80 hours after the third injection of the immunosuppressant. Urine that accumulated in the metabolic cages were collected centrifuged at 10,000 X g for 10 minutes, and the supernatent adjusted to pH 7.0 for analysis.

Blood collected form the hearts of the Wistar rats was deproteinated with 4 M perchloric

acid. After centrifugation at 10,000 X g for 10 minutes, the supernatent fluid was collected and the pH adjusted 7.0 by dropwise addition of 4 M potassium hydroxide at 4°C. The perchlorate formed was separated either by centrifugation in the cold or by filtration with Whatman 2 filter paper and the fluid collected for anaysis.

Samples from clinical cases of aspergillosis:

Four serum samples from clinical cases of aspergillosis were received rom Drs. L. Ajello and L. Kaufman, Center for Disease Control, Atlanta, Georgia. Two of these samples a and b were from patients with aspergilloma and the other two c and d from patients with allergic bronchopulmonary aspergillosis. All for samples were positively diagnosed by immunodiffusion test.

A blood sample and part of the air sac from a Canadian goose which had reportedly died from pulmonary aspergillosis was received from the Department of Veterinary Pathology, Clorado State University, Ft. Collins, Colo. The serosal surface of the air sac was lined with greenish film of fungal mycelium. The causative agen was identified as A. fumigatus. The five samples were deproteinated with perchloric acid and analyzed for kojic acid by the ELISA technique.

Chromatographic detection of kojic acid:-

Chemical detection of kojic acid in the above samples was done on 0.25 mm precoated thin-layer chromatography plates of silica gel G (E. Merck, Darmstadt, Germany). Volumes of 100 ul or more of the undiluted, fractionated samples were spotted on the plates which were then developed to a distance of 10 cm in chloroformmethanol (4:1) or the organic phase from n-butanol-acetic acid-water (4:1:4) (Durackova et al, 1967). The plates were then dried and kojic acid was detected by spraying the plates with a solution of ferric chloride in n-butanol (10 g/L).

ELISA for kojic acid:-

The ELISA method used was that d eveloped by

Abdalla and Grant (1980) for analys is of kojic acid.

RESULTS

The presumptive presence of kojic acid in blood and urine samples from the experimentally infected groups of rats was indicated by weakly positive reactions with ferric chloride. Samples from the control groups gave negative reactions. Confirmation of the presence of kojic acid in the biological fluids of the infected animals was obtained with the use of ELISA (Table 1). The dilution end-point for detection was 10 for both blood and urine samples. This corresponds to kojic acid content of 0.01-1.0 ug/ml since the detection limit of the analysis was 100 pg/ml.

The kojic acid contents of four serum samples from cases fo human aspergillosis ranged from 0.01-10 ug/ml (Table II). Blood from a

Canadian goose with diagnosed pulmonary aspergillosis contained 0.1-1.0 ug/ml of kojic acid.

DISCUSSION

Many previous investigations on aspergellus toxins have focused on their clinical or pathological effects following ingestion. In this study, the question was asked whether A. flavus-parasiticus and A. fumigatus produce kojic acid in vivo and if its presence in biological fluids can be used as an indicator for aspergillosis. The finding of kojic acid in the blood and urine of rats with experimental aspergillosis but not in comparable samples from control animals, indicates that the mold produces kojic acid in an in vivo environment. Aspergillus fumigatus which is the most commonly encountered etiologic agent of aspergillosis in both humans and animals has been reported to produce kojic acid in vitro (Prescott and Dunn, 1940). The positive results of kojic acid detection by analysis of serum samples from four human cases of aspergillosis, presumably caused by A. flavus and in a blood sample from a Canadian ggose implies that this mycotoxin is also produced in vivo by this mold.

Conventional serological tests are currently the method of choice for diagnosis of aspergillosis. However, variations in individual antibody responses may necessitate the inculsion of a battery of aspergillus extracts to obtain the greatest number of positive reactions (Pepys and Longbottom, 1973). Antigenic components are shared between some aspergilli and other organisms. These are capable of reacting with shared precipitins found in sera of certain patients to produce falsc positive diagnosis (Longbottom and Pepys, 1964). Moreover, cases of false negatives could also be encountered. No antibody to A. fumigatus was detected in sera from fifteen patients with widespread invasive aspergillosis using techniques of double diffusion in agar, complement fixation, immunoelectrophoresis and indirect fluorescent antibody (Young and Bennett, 1971). Out of eighty patients with acute leukaemia, ten were proved at autopsy or lung biopsy to have invasive aspergillosis.
Seven out of the latter converted form a negative to a positive A. fumigatus immunodiffusion test, whereas three patients with documented aspergillosis did not develop a positive immunodiffusion test (Shaefer et al, 1967).

Since kojic acid is a secondary metabolite produced by most members of the genus Aspergillus commonly implicated in aspergillosis and since its detection in body fluids is now possible with ELISA, it is suggested that kojic acid analysis in cases of suspected aspergillosis may aid rapid diagnosis and possibly permit earlier treatment.

SUMMARY

Kojic acid was dtected by an enzyme-linked immunosorbent assay (ELISA) in biological fluids of

immunosuppressed Wistar rats that had been infected with A. flavus-parasiticus ATCC 15517. Positive tests were obtained at dilutions of 10 in urine and deproteinated blood. In deproteinated serum samples from four clinical cases of human aspergillosis, kojic acid was dtected at dilutions of 10, 10, 10, and 10. Kojic acid was also detected in a 10 dilution of a blood sample from a Canadian goose with mycotic air sacinfection.

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Table I

Detection of Kojic Acid in Urine and Blood from Rats Infected with Aspergillus flavus-parasiticus

Reciprocal serial dilution	Absorbance: Mean of values within 10% of median with S.D. *			
	Infected Rats	Control Rats	Infected Rats	Control Rats
	Urine		Deproteinated whole blood	
10	0.175 ± 0.007	0.158 ± 0.003	0.175 ± 0.007	0.157 ± 0.008
10	0.284 ± 0.006 **	0.159 ± 0.005	0.280 ± 0.012 **	0.157 ± 0.003
10	0.342 ± 0.005	0.160 ± 0.006	0.357 ± 0.010	0.157 ± 0.004
10	0.338 ± 0.009	0.160 ± 0.004	0.342 ± 0.005	0.157 ± 0.003

* Standard deviation.

** Limit of detection of kojic acid in infected rats by ELISA.

Table 2

Absorbance Values for Detection of Kojic Acid by ELISA in Sera of Human Patients With Diagnosed Aspergillosis

Reciprocal serial dilution	Patients				
	(a)	(b)	(c)	(d)	control sera
	Urine				Deproteinated whole blood
10	0.242 ± 0.01 *	0.234 ± 0.004	0.226 ± 0.004	0.272 ± 0.012	0.183 ± 0.001
10	0.268 ± 0.008 ***	0.272 ± 0.012	0.233 ± 0.004	0.383 ± 0.005 ***	0.181 ± 0.005
10	0.383 ± 0.916	0.287 ± 0.004	0.272 ± 0.012	0.438 ± 0.006	0.179 ± 0.003
10	0.350 ± 0.005	0.319 ± 0.001	0.308 ± 0.005 ***	0.414 ± 0.006	0.182 ± 0.008
10	ND ****	0.352 ± 0.015 ***	0.476 ± 0.016	ND	ND
10	ND	0.432 ± 0.012	0.450 ± 0.006	ND	ND
10	ND	0.403 ± 0.005	ND	ND	ND

* Mean of values withen 10% of median and standard deviation.

** Samples (b) and (a) gave positive ferric chloride reaction with deproteinated undiluted serum samples; kojic acid was de ected by ELISA in dilutions of 10, 10, 10, 10, respectively.

*** Detection limit of kojic acid by ELISA in sample.

**** Not done.