APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1983, p. 1420–1422 Vol. 46, No. 6
0099-2240/83/121420-03$02.00/0

Toxicity of the *Alternaria* Metabolites Alternariol, Alternariol Methyl Ether, Altenuene, and Tenuazonic Acid in the Chicken Embryo Assay

GREGORY F. GRIFFIN AND FUN S. CHU*

Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

Received 13 June 1983/Accepted 12 September 1983

The effects in the chicken embryo assay of four *Alternaria* metabolites (alternariol [AOH], alternariol methyl ether [AME], altenuene [ALT], and tenuazonic acid [TA]) were investigated. Administered to 7-day-old chicken embryos by yolk sac injection, AOH, AME, and ALT caused no mortality or teratogenic effect at doses up to 1,000, 500, and 1,000 μg per egg, respectively. TA exhibited a calculated 50% lethal dose of 548 μg per egg, with no teratogenic effect observed at either lethal or sublethal doses.

Molds belonging to the genus *Alternaria* are ubiquitous in nature and are common contaminants of foods and feeds (12). Since the molds can grow at low temperatures, they are often involved in the spoilage of refrigerated produce (11, 18). When grown in culture, many *Alternaria* strains produce secondary metabolites that have been shown to be toxic in a variety of test systems (10, 14, 19). The major metabolites that have been most extensively studied are alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), and tenuazonic acid (TA) (Fig. 1).

Alternaria spp. have been implicated in a poultry disease outbreak called poultry hemorrhagic syndrome (7, 8). Extracts of *Alternaria* cultures, or rations on which *Alternaria* spp. have been cultured, have been shown to be toxic to chicken embryos and day-old chicks (4–6, 16). In one study (16), grains molded by a number of different *Alternaria* isolates were analyzed for toxin content. Although all four toxins were found, only those diets containing TA showed toxicity. Purified TA also produced mortality in chicken embryos (3) and day-old chicks (9).

However, studies on the effects in poultry systems of the other major *Alternaria* metabolites are necessary to disclose their potential roles in the observed toxicity of the molds. This note reports a study on the effects of these metabolites, administered singly and in purified form, in the chicken embryo assay.

Alternaria alternata RL 671-2 and RL 8442-3, obtained from L. M. Seitz, Grain Marketing Research Laboratory, U.S. Department of Agriculture, Manhattan, Kans., were used for toxin production. AOH, AME, and ALT were produced with strain RL 671-2, according to the procedure of Chu and Bennett (2). AOH, AME, and ALT were quantified by high-performance liquid chromatography, using a reversed-phase column and a water-acetonitrile-glacial acetic acid eluting system. (Details of this high-performance liquid chromatography method will be described elsewhere.) For TA production, strain RL 8442-3 was cultured and extracted in a similar fashion, except that ethyl acetate was used as the extracting solvent in place of methanol. TA was purified from the crude extract by the 5% $NaHCO_3$ extraction technique of Davis et al. (3). For increased storage stability, TA was converted to its copper salt by the procedure of Rosett et al. (15); the free acid was recovered by shaking the salt with 0.2 N HCl–chloroform by the procedure of Stinson et al. (17). TA was quantified spectrophotometrically (3).

Eggs from white Leghorn-Rhode Island Red crosses were incubated at the Poultry Research Laboratory, Department of Poultry Science, University of Wisconsin, in incubators equipped with automatic turning trays (James Manufacturing Co., Ft. Atkinson, Wis.). They were candled on day 6 of incubation, and nonviable eggs were discarded.

Toxins were dissolved in dimethyl sulfoxide (Aldrich Chemical Co., Milwaukee, Wis.) and administered on day 7 of incubation. A modification of the yolk sac injection method of Archer (1) was used, in which the shell over the air sac was punctured and the toxins were injected vertically into the yolk. One hundred microliters of toxin was injected per egg. Eggs were candled on day 8 of incubation and every other day thereafter through day 19, when the eggs were removed from the turning racks and placed in

compartmented baskets for hatching. Hatched chicks were weighed and examined for gross external abnormalities. All eggs which remained unhatched by day 23 were opened, and the embryos were examined for gross external abnormalities. Dose-response data were treated by the graphical probits method of Miller and Tainter (13).

The maximal doses of AOH, AME, and ALT administered were 1,000, 500, and 1,000 μg per egg, respectively, the limit determined by the solubility of the metabolites in dimethyl sulfoxide. No mortality, or difference in weight or appearance of hatched chicks from that of controls, was observed at any of the doses tested.

TA elicited dose-related mortality responses over the range of 150 to 1,500 μg per egg (Fig. 2). The calculated 50% lethal dose in this system for TA is 548 μg per egg. Weight and appearance of hatched chicks also did not differ from controls. Examination of embryos which died or failed to hatch did not disclose any gross external abnormalities. The embryos themselves were not opened for examination of internal morphology.

The chicken embryo assay is often used as a convenient assay for toxicity of mycotoxins (1, 3, 21) as well as other compounds (20). The results of this study show that AOH, AME, and ALT exhibited no toxic effects in this system at the maximal doses used. In addition to the lack of mortality, these metabolites exerted no gross teratogenicity in the developing embryo, an effect of significance in this assay when both fungal extracts (1) and other chemicals (20) are tested. TA, although producing mortality within the dose range tested, elicited no gross teratogenic effect at either lethal or sublethal dose levels.

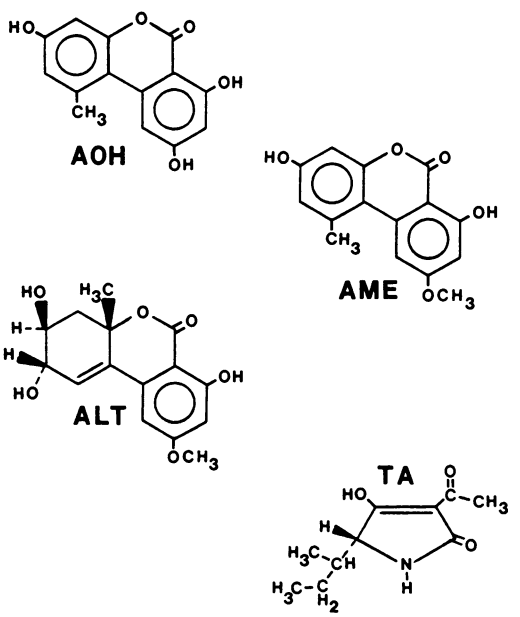

FIG. 1. The major *Alternaria* metabolites examined in this study: alternariol (AOH), alternariol methyl ether (AME), altenuene (ALT), and tenuazonic acid (TA).

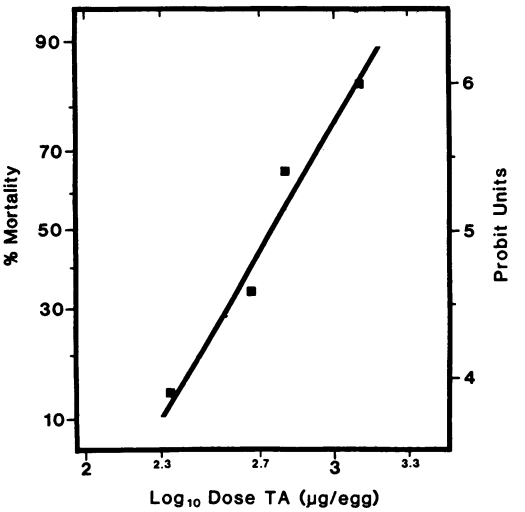

FIG. 2. Sensitivity of chicken embryos to TA. Assay conditions are described in the text. Twenty-five to thirty eggs were used per dose group. Calculated 50% lethal dose ± standard error from this curve is 548 ± 66 μg per egg.

In a recent study in our lab, day-old chicks fed a standard diet supplemented with purified AME at levels of up to 100 mg/kg of feed for 4 weeks exhibited no mortality or significant loss in performance (G. F. Griffin, V. K. Tsiagbe, M. L. Sunde, and F. S. Chu, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, O45, p. 247). These data with the present results indicate a general lack of toxicity of AME in poultry systems. If studies with the related metabolites AOH and ALT show similar results, a role for these metabolites in the toxicity of *Alternaria*-infested rations may be ruled out. Further study of these, and of TA and other *Alternaria* secondary metabolites, is in order to elucidate fully their contributions to the observed toxic effect of these rations in poultry.

This work was supported by the College of Agricultural and Life Sciences, the University of Wisconsin—Madison, and by U.S. Department of Agriculture grant no. 59-2551-0-2-084-0.

The authors wish to express appreciation to Mary M. Jens and S. Christopher Bennett for valuable technical assistance. The advice and assistance of Milton L. Sunde of the Department of Poultry Science, and of the staff of the Poultry Research Laboratory, University of Wisconsin—Madison, are also gratefully acknowledged.

LITERATURE CITED

1. **Archer, M.** 1974. Detection of mycotoxins in foodstuffs by use of chick embryos. Mycopathol. Mycol. Appl. **54**:453–467.
2. **Chu, F. S., and S. C. Bennett.** 1981. High-performance liquid chromatographic preparation of alternariol, alternariol methyl ether, and altenuene. J. Assoc. Off. Anal. Chem. **64**:950–954.
3. **Davis, N. D., U. L. Diener, and G. Morgan-Jones.** 1977. Tenuazonic acid production by *Alternaria alternata* and *Alternaria tenuissima* isolated from cotton. Appl. Environ. Microbiol. **34**:155–157.
4. **Diener, U. L., G. Morgan-Jones, R. E. Wagener, and N. D. Davis.** 1981. Toxigenicity of fungi from grain sorghum. Mycopathologia **75**:23–26.
5. **Diener, U. L., R. E. Wagener, G. Morgan-Jones, and N. D. Davis.** 1976. Toxigenic fungi from cotton. Phytopathology **66**:514–516.
6. **Doupnik, B., Jr., and E. K. Sobers.** 1968. Mycotoxicosis: toxicity to chicks of *Alternaria longipes* isolated from tobacco. Appl. Microbiol. **16**:1596–1597.
7. **Forgacs, J., and W. T. Carll.** 1955. Preliminary mycotoxic studies on hemorrhagic disease in poultry. Vet. Med. (Kansas City, Mo.) **50**:172.
8. **Forgacs, J., H. Koch, W. T. Carll, and R. H. White-Stevens.** 1958. Additional studies on the relationship of mycotoxicoses to the poultry hemorrhagic syndrome. Am. J. Vet. Res. **19**:744–753.
9. **Giambrone, J. J., N. D. Davis, and U. L. Diener.** 1978. Effect of tenuazonic acid on young chickens. Poultry Sci. **57**:1554–1558.
10. **Harvan, D. J., and R. J. Pero.** 1976. The structure and toxicity of the *Alternaria* metabolites. Adv. Chem. Ser. **149**:344–355.
11. **Harwig, J., P. M. Scott, D. R. Stoltz, and B. J. Blanchfield.** 1979. Toxins of molds from decaying tomato fruit. Appl. Environ. Microbiol. **38**:267–274.
12. **Hyde, M. B., and H. B. Galleymore.** 1951. The subepidermal fungi of cereal grains. II. The nature, identity, and origin of the mycelium in wheat. Ann. Appl. Biol. **38**:348–357.
13. **Miller, L. C., and M. L. Tainter.** 1944. Estimation of the ED_{50} and its error by means of logarithmic-probit graph paper. Proc. Soc. Exp. Biol. Med. **57**:261–264.
14. **Pero, R. W., H. Posner, M. Blois, D. Harvan, and J. W. Spalding.** 1973. Toxicity of metabolites produced by the "Alternaria," p. 87–94. Environmental health perspectives, Expt. issue no. 4, June 1973. DHEW Publication no. NIH 73-218. Dept. of Health, Education, and Welfare, NIEHS, NIH Research Triangle Park, N.C.
15. **Rosett, T., R. H. Sankhala, C. E. Stickings, M. E. U. Taylor, and R. Thomas.** Metabolites of *Alternaria tenuis* auct.: culture filtrate products. Biochem. J. **67**:390–400.
16. **Sauer, D. B., L. M. Seitz, R. Burroughs, H. E. Mohr, J. L. West, R. J. Milleret, and H. D. Anthony.** 1978. Toxicity of *Alternaria* metabolites found in weathered sorghum grain at harvest. J. Agric. Food Chem. **26**:1380–1383.
17. **Stinson, E. E., D. D. Bills, S. F. Osman, J. Siciliano, M. J. Ceponis, and E. G. Heisler.** 1980. Mycotoxin production by *Alternaria* species grown on apples, tomatoes, and blueberries. J. Agric. Food Chem. **28**:960–963.
18. **Stinson, E. E., S. F. Osman, E. G. Heisler, J. Siciliano, and D. D. Bills.** 1981. Mycotoxin production in whole apples, oranges, and lemons. J. Agric. Food Chem. **29**:790–792.
19. **Templeton, G. E.** 1972. *Alternaria* toxins related to pathogenesis in plants, p. 169–192. *In* S. Kadis, A. Ciegler, and S. J. Ajl, (ed.), Microbial toxins, vol. VII. Academic Press, Inc., New York.
20. **Verrett, M. J., W. F. Scott, E. G. Reynaldo, E. K. Alterman, and C. A. Thomas.** 1980. Toxicity and teratogenicity of food additive chemicals in the developing chicken embryo. Toxicol. Appl. Pharmacol. **56**:265–273.
21. **Verrett, M. J., J. Winbush, E. F. Reynaldo, and W. F. Scott.** 1973. Collaborative study of the chick-embryo bioassay for aflatoxin B_1. J. Assoc. Off. Anal. Chem. **56**:901–904.