Biological and Chemical Detection of Trichothecene Mycotoxins of Fusarium Species

Y. UENO, N. SATO, K. ISHII, K. SAKAI, H. TSUNODA, and M. ENOMOTO

Microbial Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, Tokyo, Japan; The Food Research Institute, the Ministry of Agriculture and Forestry, Tokyo, Japan; and The Institute of Medical Science, the University of Tokyo, Tokyo, Japan

Received for publication 28 August 1972

The procedure for biological and chemical detection of trichothecene-type mycotoxins and its application to the screening of Fusarium for toxic strains were described.

The potent carcinogenicity of the hepatotoxic metabolites of Penicillium islandicum Sopp (13, 15) and Aspergillus flavus (16) and the neurotoxicity of the metabolites of P. citreo-viride Bouarge (10, 14) led to the theories suggesting possible relationships between consumption of mycotoxins and development of etiologically unknown diseases in the world. It is therefore desirable to develop sensitive and specific methods for detection of mycotoxins.

Recent investigation in our laboratory has been expanded to include Fusarium trichothecenes such as nivalenol, fusarenon-X, T-2 toxin, neosolaniol, and others. This has been made possible by the following three steps: (i) biological screening of toxic Fusarium by a lethal toxicity test in mice and by examination of the inhibitory effect on protein synthesis in rabbit reticulocytes (5), (ii) histological detection of the “radiomimetic cellular injury” in poisoned mice (4), and (iii) chemical confirmation of toxic trichothecenes by thin-layer chromatography.

The fungal strains isolated from cereal grains, feeds, and vegetables were inoculated on peptone-supplemented Czapek-Dox medium, and, after cultivation at 25 to 27 C for 2 weeks, the “crude toxin” was prepared from the culture filtrate by the charcoal absorption method as previously reported (6). This fraction was employed both as starting materials for isolation of the mycotoxins and as test samples for the biological and chemical examinations for toxicity. In this way, the following mycotoxins have been isolated: nivalenol and fusarenon-X from Fusarium nivale and F. episphaeria (7-9); diacetylneosolaniol from F. oxysporum; T-2 toxin, neosolaniol (revised name of solaniol), and diacetoxyscirpenol from F. solani and other Fusarium species (2, 11, 12). Figure 1 illustrates chemical structures of the trichothecene mycotoxins. These toxins are divided into two groups according to the structural variation at C-8: (i) neosolaniol, T-2 toxin, HT-2 toxin, and diacetoxyscirpenol; and (ii) nivalenol, fusarenon-X, and diacetylneivalenol.

Among 20 mycotoxins tested, only the trichothecene compounds inhibited the uptake of radioactive amino acid in the reticulocyte biosay and showed parallel dose-response curves (5). This biochemical feature of trichothecenes gave a powerful tool for detection and semiquantitative determination of the toxic metabolites of Fusarium. The 50% inhibitory dose value of each toxin differed markedly depending upon the structure; T-2 toxin, the most potent inhibitor, was 100 times more active than nivalenol. Nevertheless, these toxins gave rather similar 50% lethal dose values in the acute toxicity test with mice (Table 1). A noticeable finding was that cellular degeneration and karyorrhexis of the actively dividing cells in the thymus, bone marrow, small intestine, testis, and ovary were marked in all the poisoned mice. This so-called “radiomimetic” injury of the tissues is considered to be a common pathological response of animals to the toxic trichothecenes.

From the above evidence, we have concluded that the trichothecene compounds are toxic principles of the crude toxins when they exhibit the inhibitory effect on 14C-leucine uptake in reticulocytes and cause the radiomimetic injury in poisoned mice.

Finally, chemical identification of the toxic principles was carried out by thin-layer chromatography with several solvent systems. Thin layers (0.25 mm) of kieselgel G were activated
FIG. 1. Trichothece mycotoxins of Fusaria.
at 100 °C for 30 min. Ten microliters of the standard solution of each toxin in methanol was spotted and developed with the following solvent systems: chloroform-methanol (95:5), ethyl acetate-n-hexane (3:1), benzene-acetone (3:2), chloroform-isopropanol-ethyl acetate (95:5:5), and chloroform-ethanol-ethyl acetate (90:5:5).

Mycotoxins were visualized under visible or ultraviolet light (360 nm) after spraying the plate with 20% H₂SO₄ and then heating at 100 °C for 10 to 20 min. Typical Rₜ values and colors observed are shown in Table 2. The B-type toxins gave a brown spot and several micrograms were required for quantification. The A-type toxins exhibited sky-blue fluorescence under longwave ultraviolet light, and the detection limit was in the order of 0.1 to 0.2 µg.

With the above-mentioned system of biological and chemical procedures, we carried out a screening of toxic strains of Fusarium for detection of trichothecenes. The fungal strains examined were isolated from cereal grains, feeds, and vegetables. The several standard strains generously supplied by C. W. Hesseltine (USA) and H. Kurata (Japan) were also examined.

From the data obtained during 1963 through 1971 (Table 3), the following observations were made. (i) The crude toxin fractions which were positive in both bioassay systems with reticulocytes (inhibition of ¹C-leucine uptake) and mice (radiomimetic cellular injury) invariably contained "toxic trichothecenes." (ii) Toxic strains of Fusarium were divided into two groups, A-type toxin producers (F. solani, F. tricinctum, F. sporotrichioides, etc.) and B-type toxin producers (F. nivale, F. episphearia, etc.). (iii) The former fungi were distributed in the northern districts of Japan and the latter fungi were in the southern districts. In both districts, "Akakabi (red-mold) poisoning" in men and farm animals was occasionally reported (7). (iv) Besides T-2 toxin and HT-2 toxin, F. tricinctum NRRL 3229, a fungus responsible for moldy corn poisoning in the United States (1), produced neosolaniol which was isolated first from F. solani M-1-1 (2, 11). (v) F. poae NRRL 3287 as well as F. sporotrichioides NRRL 3510, isolated from overwintered millet in connection with alimentary toxic aleukia (ATA) disease in the USSR (3), also produced trichothecenes such as T-2 toxin, HT-2 toxin, and neosolaniol (12).

In this respect, it is highly probable that "Akakabi poisoning" in Japan, moldy corn disease in the United States, and ATA disease in the USSR are presumably caused by intake of the common toxicant, 12-13 epoxy trichothecenes of Fusarium species.

| Mycotoxins | Rₚ solvent system* | Color after spray |
|------------|--------------------|-------------------|
|            | CM     | EH  | BA   | CPE | CEE | Visible light | UV* light |
| Type A     |        |      |      |     |     | Gray          | Skylight blue |
| T-2 toxin  | 0.55   | 0.61 | 0.56 | 0.68 | 0.78 | Gray          | Skylight blue |
| Diacetoxyscirpenol | 0.43 | 0.47 | 0.51 | 0.46 | 0.68 | Gray          | Skylight blue |
| Neosolaniol| 0.22   | 0.15 | 0.34 | 0.25 | 0.32 | Gray          | Skylight blue |
| HT-2 toxin | 0.20   | 0.10 | 0.30 | 0.17 | 0.23 | Gray          | Skylight blue |
| Type B     |        |      |      |     |     | Brown         | Skylight blue |
| Nivalenol  | 0.02   | 0.05 | 0.10 | 0.01 | 0.04 | Brown         | Skylight blue |
| Fusarenon-X| 0.17   | 0.31 | 0.41 | 0.21 | 0.32 | Brown         | Skylight blue |
| Diacetynivalenol | 0.43 | 0.47 | 0.51 | 0.43 | 0.54 | Brown         | Skylight blue |

* Abbreviations: CM, chloroform-methanol; EH, ethyl acetate-n-hexane; BA, benzene-acetone; CPE, chloroform-isopropanol-ethyl acetate; CEE, chloroform-ethanol-ethyl acetate.
* Ultraviolet.
Table 3. Screening data of toxic Fusaria and trichothecenes

| Fungus          | Strain         | Bioassay | Trichothecenes on thin-layer chromatography |
|-----------------|----------------|----------|---------------------------------------------|
| **Fusarium nivale** |                |          |                                             |
| Fn-2B           |                | +++      | Fusarenon-X, nivalenol                      |
| Fn-2LA          |                | +        | Fusarenon-X, nivalenol                      |
| Fn-2LB          |                | +        | Fusarenon-X, nivalenol                      |
| NRRL A-13-318   |                | +        |                                             |
| M               |                | -        |                                             |
| NRRL 3289       |                | -        |                                             |
| **F. episphaeria** |               |          |                                             |
| Fn M            |                | +++      | Fusarenon-X, nivalenol                      |
| Fn ML           |                | +        | Nivalenol                                   |
| M               |                | -        |                                             |
| **F. moniliforme** |               |          |                                             |
| Paddy 1         |                | -        |                                             |
| Paddy 6         |                | -        |                                             |
| Fn-HO           |                | +        |                                             |
| M-10-2          |                | -        |                                             |
| No. 9           |                | -        |                                             |
| USDA            |                | -        |                                             |
| NRRL 3197       |                | -        |                                             |
| **F. solani**   |                |          |                                             |
| M-1-1           |                | +++      | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol |
| M-1-2           |                | +++      | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
| M-13-2          |                | +        |                                             |
| M               |                | -        |                                             |
| No. 1 (clover)  |                | -        |                                             |
| No. 2 (alfalfa) |                | -        |                                             |
| **F. rigidiusculum** |              |          |                                             |
| M-1-3           |                | ++       | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
| M-10-1          |                | -        |                                             |
| M-13-3          |                | -        | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
| M               |                | +        |                                             |
| **F. roseum**   |                |          |                                             |
| "culmorum"      |                |          |                                             |
| M-2-5           |                | +        | Diacetoxyscirpenol                           |
| M-3-2           |                | +        |                                             |
| M-7-1           |                | +        |                                             |
| M-7-2           |                | -        |                                             |
| "scirp"         |                |          |                                             |
| M-8-1           |                | ++       | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
| "avenaceum"     |                |          |                                             |
| M-11-1          |                | ++       | Neosolaniol, T-2 toxin, diacetoxyscirpenol |
| "culmorum"      |                |          |                                             |
| M-13-1          |                | -        | diacetoxyscirpenol                          |
| M-14-2          |                | +        |                                             |
| M-15-2          |                | -        |                                             |
| 70-K-11         |                | ++       | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxyscirpenol |
| M               |                | -        |                                             |
| Abashiri-1      |                | +        | Neosolaniol, T-2 toxin                      |
| Abashiri-5      |                | -        |                                             |
| Asahikawa-1     |                | +        | Neosolaniol, T-2 toxin                      |
| Asahikawa-4     |                | ++       | Neosolaniol, T-2 toxin                      |
| Asahikawa-6     |                | ++       | Neosolaniol, T-2 toxin                      |
| Asahikawa-7     |                | -        |                                             |
| R 2029          |                | +        |                                             |

Continued on next page
| Fungus          | Strain          | Bioassay Reticulocyte* | Bioassay Mice* | Trichothecenes on thin-layer chromatography                      |
|-----------------|-----------------|------------------------|----------------|------------------------------------------------------------------|
| *F. gibbosum*   | M:14-1          | –                      | –              | Fusarenon-X, nivalenol                                           |
|                 | Abashiri-6      | +                      | +              |                                                                  |
|                 | Asahikawa-2     | –                      | +              |                                                                  |
| *G. zeae*       | Ōhita-II        | +                      | +              |                                                                  |
|                 | Ishii           | –                      | +              |                                                                  |
|                 | NRRL 2830       | –                      | –              |                                                                  |
|                 |                 |                        |                |                                                                  |
| *F. tricinctum* | M               | –                      | –              |                                                                  |
|                 | NRRL 3249       | –                      | –              |                                                                  |
|                 | NRRL 3299       | + +                    | + +            | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxy-scirpenol          |
|                 | Abashiri-2      | + +                    | + +            | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxy-scirpenol          |
|                 | R 2031          | + +                    | + +            | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxy-scirpenol          |
|                 | R 2031          | + +                    | + +            | Neosolaniol, T-2 toxin, HT-2 toxin, diacetoxy-scirpenol          |
| *F. sporotrichioides* | M:1-4 | + +                    | + +            | Neosolaniol, T-2 toxin, diacetoxy-scirpenol                      |
|                 | M:1-5           | + +                    | + +            | Neosolaniol, T-2 toxin, diacetoxy-scirpenol                      |
|                 | NRRL 3510       | + +                    | + +            | Neosolaniol, T-2 toxin, diacetoxy-scirpenol                      |
| *F. oxysporum*  | M:4-1           | –                      | –              |                                                                  |
|                 | M:15-1          | +                      | +              |                                                                  |
|                 | M               | +                      | –              |                                                                  |
|                 | NRRL 1943       | –                      | +              |                                                                  |
| “niveum”       | Melon-1         | + +                    | + +            |                                                                  |
| “niveum”       | Melon-2         | + +                    | + +            |                                                                  |
|                 | Abashiri-3      | + +                    | + +            |                                                                  |
|                 | Pimento-1       | –                      | –              |                                                                  |
|                 | Pimento-3       | +                      | –              |                                                                  |
|                 | Pimento-5       | +                      | –              |                                                                  |
|                 | Pimento-7       | –                      | –              |                                                                  |
|                 | No. 3 (alfalfa) | –                      | –              |                                                                  |
|                 | No. 4 (clover)  | –                      | –              |                                                                  |
| *F. lateritium* | M               | –                      | –              |                                                                  |
| *F. splendens*  | M               | –                      | –              |                                                                  |
| *F. poae*       | NRRL 3287       | + +                    | + +            | Neosolaniol, T-2 toxin, HT-2 toxin                               |

* The inhibitory effect of each crude toxin on the uptake of \(^{14}\)C-leucine was assayed in concentrations of 10 and 100 \(\mu g/ml\).

* The lethal effect of each crude toxin to mice was assayed after intraperitoneal administration of 10 and 40 \(\mu g/10\) g. The relative toxicity in both systems was cited.

This investigation was supported by a grant for Agriculture and Fishery Sciences Research from the Ministry of Agriculture and Forestry (1971). We are greatly indebted to C. W. Hesseltine (NRRL strains), H. Kurata (M strains etc.), and Ru-dong Wei (HT-2 toxin) for fungi and toxin samples.

**LITERATURE CITED**

1. Bamburg J. R., N. V. Riggs, and F. M. Strong. 1968. The structure of toxins from two strains of *Fusarium tricinctum*. *Tetrahedron* 24:3329-3336.
Joffe, A. Z. 1966. Toxin production by cereal fungi causing toxic alimentary aleukia in man, p. 77-86. In G. N. Wogan (ed.), Mycotoxins in foodstuffs. The M.I.T. Press, Cambridge.

Saito, M., M. Enomoto, and T. Tatsuno. 1969. Radiomimetic biological properties of the new scirpene metabolites of *Fusarium nivale*. Gann. 66:590-603.

Ueno, Y., M. Hosoya, and Y. Ishikawa. 1969. Inhibitory effects of mycotoxin on the protein synthesis in rabbit reticulocytes. J. Biochem. (Tokyo) 66:419-422.

Ueno, Y., Y. Ishikawa, K. Saito-Amakai, and H. Tsunoda. 1970. Environmental factor influencing the production of fusarenon-X, a cytoxic mycotoxin of *Fusarium nivale* F2B. Chem. Pharm. Bull. (Tokyo) 18:304-312.

Ueno, Y., Y. Ishikawa, M. Nakajima, K. Sakai, K. Ishii, H. Tsunoda, M. Saito, M. Enomoto, K. Ohtsubo, and M. Umeda. 1971. Toxicological approaches to the metabolites of *Fusaria*. I. Screening of toxic strains. Jap. Exp. Med. 41:257-272.

Ueno, Y., I. Ueno, K. Amakai, Y. Ishikawa, H. Tsunoda, K. Okubo, M. Saito, and M. Enomoto. 1971. Toxicological approaches to the metabolites of *Fusaria*. II. Isolation of fusarenon-X from the culture filtrate of *Fusarium nivale* F2B. Jap. J. Exp. Med. 41:507-519.

Ueno, Y., I. Ueno, Y. Itoi, H. Tsunoda, M. Enomoto, and K. Ohtsubo. 1971. Toxicological approaches to the metabolites of *Fusaria*. III. Acute toxicity of fusarenon-X. Jap. J. Exp. Med. 41:521-539.

Ueno, Y., and I. Ueno. 1972. Isolation and acute toxicity of citreoviridin, a neurotoxic mycotoxin of *Penicillium citreo-viride* Biourge. Jap. J. Exp. Med. 42:91-105.

Ueno, Y., K. Ishii, K. Sakai, S. Kanaeda, H. Tsunoda, T. Tanaka, and M. Enomoto. 1972. Toxicological approaches to the metabolites of *Fusaria*. IV. Microbial survey on "bean-hulls poisoning of horses" with the isolation of toxic trichothecenes, neosolaniol and T-2 toxin of *Fusarium solani* M-1-1. Jap. J. Exp. Med. 42:187-203.

Ueno, Y., N. Sato, K. Ishii, K. Sakai, and M. Enomoto. 1972. Toxicological approaches to the metabolites of *Fusaria*. V. Neosolaniol, T-2 toxin and butenolid, toxic metabolites of *Fusarium sporotrichioides* NRRL 3510 and *Fusarium poae* NRRL 3287. Jap. J. Exp. Med. 42:461-472.

Uraguchi, K., T. Tatsuno, F. Sakai, M. Tsukioka, O. Yonemitsu, H. Ito, M. Miyake, M. Enomoto, T. Shikata, and T. Ishiko. 1961. Isolation of two toxic agents, luteoskyrin and chlorine-containing peptide, from the metabolites of *Penicillium islandicum* Sopp, with some properties thereof. Jap. J. Exp. Med. 31:19-46.

Uraguchi, K. 1967. Mycotoxic origin of cardiac beriberi. J. Stored Prod. Res. 5:227-236.

Uraguchi, K. 1971. Pharmacology of mycotoxins, p. 143-298. In H. Raskova (ed.), International encyclopedia of pharmacology and therapeutics, 71-II. Pergamon Press, Oxford.

Wogan, G. N. 1965. Experimental toxicity and carcinogenicity of aflatoxins, p. 163-173. In G. N. Wogan (ed.), Mycotoxins in foodstuffs. The M.I.T. Press, Cambridge.